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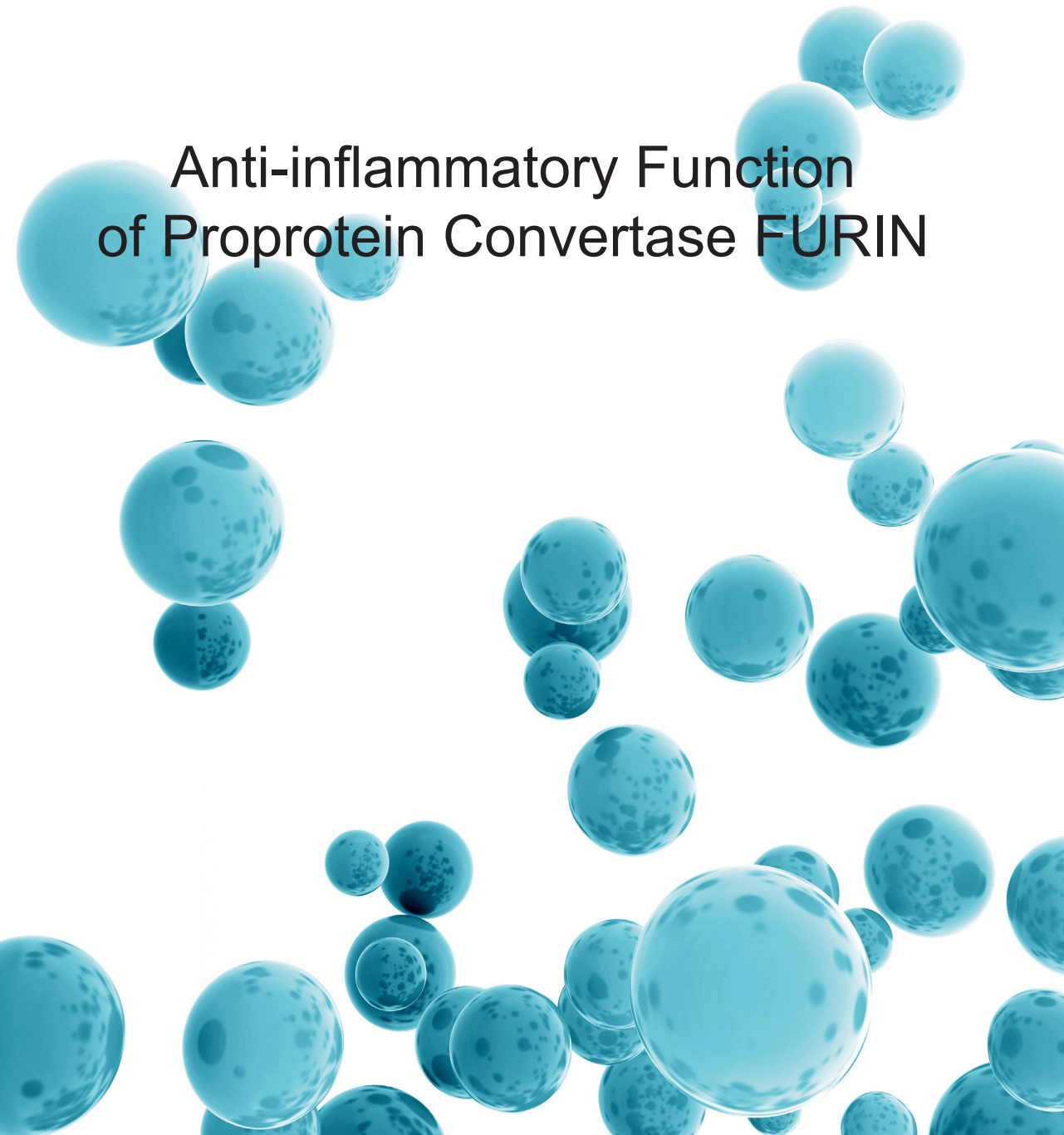
ZUZET MARTINEZ CORDOVA

Anti-inflammatory Function of Proprotein Convertase FURIN



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Anti-inflammatory Function of Proprotein Convertase FURIN

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ACADEMIC DISSERTATION
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“When life is good do not take it for granted as it will pass. Be mindful, be compassionate and nurture the circumstances that find you in this good time so it will last longer. When life falls apart always remember that this too will pass. Life will have its unexpected turns”.

Ajahn Brahm

“Thanks dear mother for your lessons of love and life, thank you dear son for the strength and love you give me, thank you dear Finland for the hope”

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is composed of the following original publications. They are designated with the Roman numerals (I-III):

I. **Cordova ZM**, Grönholm A, Kytölä V, Taverniti V, Hämäläinen S, Aittomäki S, Niininen W, Junttila I, Ylipää A, Nykter M, Pesu M. Myeloid cell expressed proprotein convertase FURIN attenuates inflammation. Oncotarget 2016; doi: 10.18632.

II. Ojanen MJ, Turpeinen H, **Cordova ZM**, Hammarén MM, Harjula SK, Parikka M, Rämet M, Pesu M. The proprotein convertase subtilisin/kexin furinA regulates zebrafish host response against Mycobacterium marinum. Infect Immun 2015; 83(4):1431-42.

III. Vähätupa M***,** Aittomäki S*****, **Cordova ZM***, May U, Prince S, Uusitalo-Järvinen H, Järvinen TA, Pesu M. T-cell-expressed proprotein convertase FURIN inhibits DMBA/TPA-induced skin cancer development. OncoImmunology 2016 (*In Press*)

*Equal contribution

ABBREVIATIONS

ADAM17	Metallopeptidase Domain 17 alpha M
ALRs	(AIM)-like receptors
APC	Antigen presenting Cell
Arg1	Arginase 1
Atf7	Activating Transcription Factor 7
BCL 6	B cell lymphoma 6
C5ar	Complement Component 5a Receptor 1
CBA	Cytometric Bead Array
CCL	Chemokine (C-C motif) Ligand
Ccnd1	Cyclophilin C
CD11b	Cluster of Differentiation molecule 11b
CD4cre-fur$^{(f/f)}$	T cell-specific FURIN conditional knockout
Ch25h	Cholesterol 25-Hydroxylase
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C motif Chemokine ligand
dLn	Draining lymph node
DMBA	7,12-Dimethylbenz[a]anthracene
Dusp6	Dual specificity phosphatase 6
cDC	Conventional or classic DC
pDC	Plasmacytoid dendritic cells
Egr1	Early Growth Response 1
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
Fcgr1	Fc Fragment of IgG, High Affinity Ia, Receptor
FOXP3	Forkhead box P3
furinAtd204e/+ fish	Furin A mutant zebrafish
GATA3	GATA-binding protein 3

Hcar2	Niacin Receptor 1
HIF1α	Hypoxia-inducible factor 1-alpha
ICOS	Inducible T cell co-stimulator
IFN-γ	Interferon gamma
IL	Interleukin
Il12rb1	Interleukin 12 Receptor, Beta 1
KO	Knockout
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6C
Ly6G	Lymphocyte antigen 6G
LysM	Lysozyme-M
LysMcre- $fur^{(f/f)}$	Myeloid cells-specific FURIN conditional
LDL-C	Low-density lipoprotein cholesterol
M.marinum	*Mycobacterium marinum*
M.tuberculosis	*Mycobacterium tuberculosis*
MCP-1	Monocyte Chemotactic Protein 1
MDSC	Myleoid-derived supressor cells
Nos2	Inducible Nitric Oxide Synthase 2
Olr1	Oxidized Low Density Lipoprotein (Lectin-Like)
OVA	Ovalbumin
PCSK	Proprotein Convertase Subtilisin/Kexin type
Ptgs2	Cyclooxygenase-2
pTreg	Peripherally derived regulatory T cell
qRT-PCR	Quantitative real time PCR
R848	Resiquimod Receptor 1
ROR	Retinoic acid receptor-related orphan receptor
RNAi	RNA interference

Serpinb1a	Serine (or cysteine) peptidase inhibitor, clade B
Serpinb2	Serpin Peptidase Inhibitor, Clade B (Ovalbumin)
STAT	Signal transducer and activator of transcription;
TACE	Tumor Necrosis Factor Alpha Converting Enzyme
TAMs	Tumor-associated macrophages
TAP	Transporter associated with antigen processing
TB	Tuberculosis
T-bet	T-box transcription factor
TCR	T-cell receptor
Teff	Effector T cell
TPA	12-O-Tetradecanoylphorbol-13-acetate
Tfh	T follicular helper
TGF-β1	Transforming growth factor-β1
TI	T cell independent-response antigen
TLR	Toll Like Receptor
TNF-α	Tumor Necrosis Factor alpha
TNG	Trans-Golgi network
Treg	Regulatory T cell
Trem1	Triggering receptor expressed on myeloid cells 1
tTreg	Thymus-derived regulatory T cell
VEGF	Vascular endothelial growth factor
VEC	Vascular endothelial cells
WT	Wild type

ABSTRACT

The ability of the immune system to generate an adequate immune response against potentially harmful pathogens and aberrant cells is critical in order to avoid disorders including autoimmunity, chronic inflammation and cancer. The appropriate immune response relies on an intricate network of several cell types and biomolecules, such as cytokines, growth factors, enzymes and receptors, which require proteolytic cleavage in order to become active. The proteolytic activity of proprotein convertase (PCSKs) enzymes determines the bioavailability of important molecules and PCSKs thereby play essential regulatory roles in cell biology. Our previous studies have convincingly demonstrated that the PCSK FURIN is upregulated in activated immune cells and it regulates T-cell dependent peripheral tolerance and Th polarization *in vivo*.

The present study investigated the role of FURIN in the regulation of the innate immunity using different methods and model organisms. Studies in FURIN-expressing myeloid cells demonstrated that FURIN played a regulatory role in maintaining the balance between M1 and M2-type macrophages and in the murine inflammatory response to LPS-induced endotoxemia. Furthermore, we discovered that FURIN was required for the normal production of the bioactive TGF-β1 cytokine in myeloid cells, and inhibited the maturation of the pro-inflammatory response-inducers TACE and Caspase-1. In addition, furinA was upregulated in adult zebrafish infected with mycobacteria, and similar to LysMcre-fur$^{(fl/fl)}$ mice, the infected furinAtd204e/+ mutants exhibited an enhancement of the early innate immune response characterized by elevated expression levels of genes encoding pro-inflammatory cytokines and a reduction in the number of copies of *M. marinum*. These studies convincingly unveiled, for the first time, a critical role for FURIN in the regulation of the activated state of macrophages in homeostasis and upon a harmful stimulus.

As the components of the innate immunity and the adaptive immunity have an essential role in several steps of carcinogenesis and tumor progression, aiming FURIN specifically in myeloid cells and/or T cells using could be a potent and well-tolerated targeted therapy strategy for the experimental treatment of malignancies. Therefore, we performed DMBA/TPA two-stage skin carcinogenesis in murine strains lacking FURIN in either T cells or macrophages and granulocytes, to address

whether the deletion of FURIN in the immune cells would regulate anti-cancer responses. Our results demonstrated that the absence of FURIN in T cells only led to an enhanced and accelerated development of tumors. In addition, we determined a regulatory role for FURIN in Th polarization at different stages of tumor development. In conclusion, our results show that the inhibition of FURIN specifically in T cells promotes carcinogenesis in a chemically induced squamous skin cancer model.

Collectively, our results demonstrated that the inhibition of FURIN in innate and adaptive immune cells strengthens host responses. Accordingly, the inhibition of FURIN in myeloid cells could be potentially applied as a therapeutic approach in the treatment and prevention of PCSK-dependent infections. In contrast, the inhibition of FURIN in T cells, but not in macrophages, appears to regulate the immune response in skin carcinogenesis. Consequently, the inhibition of FURIN at the systemic level or specifically in T cells may boost the development of certain cancer types caused by chronic immune insults.

These results provide new insights into the therapeutic use of FURIN inhibitors in infections and highlight the importance of evaluation when considering FURIN inhibitors in the treatment of human cancers.

TIIVISTELMÄ

Riittävä muttei ylimitoitettu immuunivaste mahdollisesti haitallisia patogeenejä ja epänormaaleja soluja vastaan on välttämätön, muuten seurauksena voi olla autoimmuunisairauksia, krooninen tulehdus tai syöpä. Puolustusjärjestelmä koostuu useiden solutyyppien ja biomolekyylien monimutkaisesta verkostosta. Monet näistä molekyyleistä ovat kasvutekijöitä, entsyymejä ja reseptoreja, jotka täytyy pilkkoa proteolyyttisesti, jotta niistä tulisi aktiivisia. Proproteiinikonvertaasit (PCSK:t) ovat proteolyyttisiä entsyymejä, jotka säätelevät monien soluille tärkeiden molekyylien aktiivisuutta. Aikaisemmat tutkimusryhmämme tulokset osoittavat, että PCSK-entsyymi FURINin ilmentyminen aktivoiduissa immuunijärjestelmän soluissa on lisääntynyt, ja se säätelee T-lymfosyyttivälitteistä perifeeristä toleranssia sekä Th-solujen polarisaatiota.

Väitöstyössä tutkittiin FURINin merkitystä synnynnäisessä immuniteetissa käyttäen erilaisia menetelmiä ja malliorganismeja. Tutkimus FURINia ilmentävissä myeloisissa soluissa osoitti, että FURIN säätelee M1- ja M2-tyyppisten makrofagien välistä tasapainoa ja tulehdusvastetta lipopolysakkaridilla (LPS) aikaansaadussa hiiren endotoksemiassa. Lisäksi havaittiin, että FURINia tarvitaan myeloisissa soluissa bioaktiivisen TGF-β1-sytokiinin normaaliin tuottamiseen, ja se estää tulehdusvastetta lisäävien entsyymien TACE:n ja kaspaasi-1:n kypsymistä. Aikuisissa, mykobakteereilla infektoiduissa seeprakaloissa FurinA:n ilmentyminen lisääntyi. Kuten LysMcre-fur(fl/fl)-hiirissä, myös infektoiduissa furinAtd204e/+-mutanttikaloissa tulehdusta kiihdyttävien sytokiinien määrä oli lisääntynyt ja Mycobacterium marinum -määrä vähentynyt, mikä viittaa varhaisen synnynnäisen immuunivasteen vahvistumiseen. Tutkimukset osoittivat näin ensimmäistä kertaa FURINin kriittisen merkityksen makrofagien aktiivisuuden säätelyssä sekä normaalitilanteessa että haitallisten ärsykkeiden läsnä ollessa.

Sekä synnynnäisellä että hankitulla immuniteetilla on keskeinen merkitys kasvainten synnyssä ja kehityksessä. Väitöskirjatutkimuksessa tutkittiin, miten FURINin puuttuminen joko T-lymfosyyteistä tai makrofageista ja granulosyyteistä vaikuttaa syövän syntyyn käyttämällä DMBA/TPA-indusoitua hiiren ihosyöpämallia. Tulokset osoittavat, että FURINin puuttuminen T-soluista mutta ei myeloisista

soluista johti voimistuneeseen ja nopeampaan kasvainten kehitykseen. Lisäksi havaitsimme, että FURIN säätelee T-auttajasolujen polarisaatiota kasvaimen kehityksen eri vaiheissa.

Väitöskirjatutkimus osoittaa, että FURIN-aktiivisuuden estäminen synnynnäisen ja hankitun immuniteetin soluissa voimistaa immuunivastetta. Myeloisissa soluissa FURINin inhibitiota voitaisiin käyttää PCSK-riippuvaisten infektioiden ennaltaehkäisyyn ja hoitoon. FURINin esto T-soluissa, muttei makrofageissa, näyttäisi säätelevän immuunivasteita ihosyövässä. Siten FURIN-aktiivisuuden esto koko elimistössä tai toisaalta spesifisesti T-soluissa saattaa lisätä kroonisen tulehduksen aiheuttamien syöpätyyppien kehitystä.

Tutkimuksessa saadut tulokset lisäävät tietämystä FURIN-inhibiittoreina toimivien lääkeaineiden vaikutuksista infektioissa ja syövän hoidossa.

1 INTRODUCTION

We are frequently exposed to microorganisms, whose ability to cause disease largely relies on their pathogenicity and the efficiency of our defense mechanisms. The immune system is an interactive web of soluble factors, cells, tissues and lymphoid organs, which are responsible for the effective host response against threats, such as viruses, bacteria, fungi, parasites and cancerous cells, that could disturb the organism's homeostasis. The immune system has been classified into two main branches according to the speed and specificity of the response they produce (Janeway, 1992). The first branch is called the innate immunity and it is characterized by an immediate detection and elimination of a wide spectrum of pathogens. The main drawbacks of this initial immune response are the limited range of common pathogenic molecules it can recognize and its inability to provide lasting immunity. This limitation is overcome by the second of branch the immune system called the adaptive immunity. The adaptive immunity has evolved to offer a broader spectrum of recognition of antigens and long lasting immunity. The innate and adaptive immunity are closely interconnected to together they provide an effective immune response (K. Murphy & Weaver, 2016).

An immune response is triggered in order to recognize pathogens or tissue damage and it involves soluble mediators, such as enzymes, cytokines, chemokines, growth factors, and receptors produced by the cells of the innate and adaptive immune systems. The aim of this effector function is the clearance of the foreign agent causing the imbalance in the organism's homeostasis. Under normal physiological conditions, the inflammation is resolved and the homeostasis reestablished. Nevertheless, an abnormally attenuated inflammatory response results in severe infections and tumor development, while an exaggerated response causes allergic reactions and autoimmune diseases. Therefore, the regulation of the immune system is essential in order to keep the organism protected against potential threats (Medzhitov & Janeway Jr, 2000).

One essential molecular mechanism involved in the regulation of soluble immune mediators is the post-translational proteolytic activation performed by different groups of enzymes, such as proprotein convertases (PCSK). Members of the proprotein convertase subtilisin/kexin (PCSK) enzyme family process and activate several proteins that mediate the activity of immune effectors.

Consequently, they are essential to the organism's homeostasis (Seidah, 2016). Among these enzymes, FURIN has been widely studied and several of its targets have been described, including cytokines, chemokines and growth factors, the components of several infectious agents, etc. Interestingly, the conditional deletion of FURIN in T cells causes the development of autoimmunity by impairing the peripheral tolerance (Pesu et al., 2008). However, the consequences of PCSK inhibition specifically in the cells of the innate immune system *in vivo* have remained ambiguous.

Modulating the activity of FURIN has been shown to be beneficial for the experimental treatment of infections, malignancies and autoimmune diseases(H. Lin et al., 2012; Seidah & Prat, 2012). Thus, the aim of this study is to investigate the role of FURIN in the regulation of the cells of the innate and adaptive immunity in the context of an infection and cancer. As FURIN inhibitors are considered therapeutic pharmaceuticals in several pathologies, it is important to investigate the consequences of inhibiting FURIN activity in the immune system.

2 REVIEW OF THE LITERATURE

2.1 The immune system

The immune system is an intricate network of cells, tissues and organs, which perform together to protect the organism from pernicious substances, pathogens, tissue damage and to prevent the development of diseases. The immune system has been classified into the innate and adaptive immunity according to differences in mounting immune responses to several threats. There is abundant evidence that the mammalian innate immune response has ancient roots and it is highly conserved in both plants, and animals (Medzhitov & Janeway Jr, 2000), whereas the adaptive immunity developed approximately 500 million years ago in jawed fish (Pancer et al., 2004).

2.1.1 General features of the innate Immunity

In vertebrates, the innate immune system is considered the first line of defense against pathogens, and is therefore essential for the initial detection of pathogens and the development of inflammation (Iwasaki & Medzhitov, 2004). Although the innate immune system has been traditionally described as relatively nonspecific, recent reports suggests that the components of the innate immunity can also generate an immunological memory (Levy & Netea, 2013; Netea, Quintin, & van der Meer, Jos WM, 2011).

The efficacious elimination of pathogens relies on the coordinated interaction of several cell types, such as innate immune cells and epithelial cells. Consequently, primary infections are controlled and the clearance of pathogens is facilitated through several mechanisms including epithelial barriers and the activation of complement and of specific cell types (K. Murphy & Weaver, 2016; Sonnenberg & Artis, 2015).

The surface epithelia comprises the skin, the gastrointestinal, respiratory, and urogenital tracts and the eyes (Elias, 2007). Epithelia form physical, chemical and

microbiological barriers for infections, consequently preventing pathogenic colonization.(Elias, 2007; K. Murphy & Weaver, 2016)

When the integrity of the epithelial barriers is lost, pathogens encounter a major component of the innate immunity called the complement system (Gasque, 2004). The complement system consist of a collection of interacting plasma proteins that produce a cascade of reactions leading to the elimination of the pathogen by phagocytes or the membrane attack complex. Moreover, the complement system provides a functional link between the innate and adaptive immunities in response to pathogens. (O'Neil et al., 1988).

Once the pathogen overcomes the first barriers of defense and begins to spread into the tissues of the host, it is promptly recognized by the cells of the innate immunity.

The innate immunity is composed of myeloid cells such as monocytes, macrophages, dendritic cells and granulocytes (eosinophils, basophils and neutrophils) and innate lymphocytes, including natural killer cells and the recently discovered innate lymphoid cells (Mebius, Rennert, & Weissman, 1997; Rivera, Siracusa, Yap, & Gause, 2016). Although traditionally macrophages and neutrophils have been associated with microbial infections and basophils, and mast cells and eosinophils with helminth infections, several reports have revealed that all these cells types frequently display their effector functions to a broader spectrum of pathogens (Rivera et al., 2016).

Macrophages, dendritic cells (DCs) and neutrophils remove dead cells and display robust endocytic, phagocytic, and secretory effector mechanisms to eradicate specific groups of pathogens. They can also display regulatory functions by affecting the properties of other cells of the immune system. (Egawa et al., 2013; Gordon, Plüddemann, & Martinez Estrada, 2014)

Basophils, eosinophils and mast cells have granules containing a variety of enzymes and toxic proteins, which are released when the cells are activated. They are important in allergic responses and basophils and eosinophils are involved in the response to various parasitic infections (Kang & Biswas, 2013).

Natural killer cells and the innate lymphoid cells are located in several tissues, where they can exert essential effector functions in the context of an infection, tissue damage and inflammation. These functions include cytotoxicity, the secretion of host protective factors and the production of cytokines and chemokines**.** Consequently, they facilitate the clearance or neutralization of pathogens, tumors,

allergens, etc (Feuerer, Shen, Littman, Benoist, & Mathis, 2009; Kirchberger et al., 2013).

The innate immune system uses a limited number of germ-line encoded receptors to detect conserved molecular patterns among invading microbes (Janeway, 1992).

Although the innate immune system does not display the specificity of the adaptive immunity, the cells of the innate immune system are able to discriminate self from non-self through the expression of a broad range of pattern recognition receptors (PRRs) that detect pathogen associated molecular patterns (PAMPs), specifically expressed by microbes (Coers, 2013). They also recognize endogenous stimuli known as “damage associated molecular patterns” (DAMPs) that are host molecules released after tissue damage (Matzinger, 1994; Matzinger, 1998).

The main members of PRRs family comprise Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-like receptors (RLRs), AIM2-like receptors (ALRs), Scavenger receptors and C-type lectin receptors (Matsunaga & Moody, 2009; Rathinam et al., 2010; Szabo et al., 2012). Some of the PRR are located on the cell surface (i.e. scavenger receptors and some TLRs) and they monitor the extracellular environment, whereas others, such as NLRs, RLRs and some TLRs, are localized intracellularly and become activated by foreign molecules such as foreign DNA or RNA (Kariko, Ni, Capodici, Lamphier, & Weissman, 2004; Martinon, Pétrilli, Mayor, Tardivel, & Tschopp, 2006; Tal et al., 2009)**.** Some examples of PAMPs recognized by PRRs are bacterial lipopolysaccharide (TLR4), RNA viruses (RLRs, TLR3), DNA viruses (ALRs, TLR9), bacterial MDP (NOD2) and fungal Dectin-1 (CLR)(Akira, Uematsu, & Takeuchi, 2006). The detection of PAMPS leads to the activation of PRRs and the production of several inflammatory mediators to help eliminate pathogens or reestablish tissue homeostasis. The aberrant activation of PRRs has been associated with autoimmune and inflammatory diseases, such as rheumatoid arthritis and asthma (Joosten, Abdollahi-Roodsaz, Dinarello, O'Neill, & Netea, 2016; Thorburn et al., 2016)**.** Although the innate immune system provides an immediate and efficient response against invading pathogens and other threats some microorganisms have developed mechanisms to escape the effector function of the innate immune response.

Among the components of the innate immunity, dendritic cells play an essential role in linking the innate immune system and the adaptive immune system. The majority of dendritic cells are strategically located in tissues all through the body ready to provide a rapid response to foreing threats (Coutant & Miossec, 2016).

Under healthy conditions, immature DCs travel through the blood stream and populate tissues to recognize and process antigens from apoptotic cells that die during physiological turnover (Somersan & Bhardwaj, 2001; Steinman, Inaba, Turley, Pierre, & Mellman, 1999). Dendritic cells ceaselessly present autoantigens to autoreactive T cells, but the production of immunosuppressive cytokines leads to the induction of tolerance through mechanisms, such as anergy, the deletion of potentially harmful T cells or the activation of regulatory T cells (Steinman et al., 2003).

In the presence of stressed or damaged tissues, immature DCs infiltrate the site of damage after the detection of inflammatory chemokines by a broad set of chemokine receptors (e.g; CCR1-6, CXCR1 and CX3CR1). Antigen capture by DCs in the presence of activation signals initiates a maturation program characterized by an optimization of the antigen presentation capacity and the ability to migrate to lymphoid tissues. So far, two types of DCs activation signals have been identified. One signal is derived from the recognition of exogenous molecules derived from pathogenes (PAMPs) by pattern recognition receptors such as TLRs, cell-surface C-type lectin receptors, NLR and RLR (Janeway Jr & Medzhitov, 2002). The second activation signal consists of endogenous molecules that are released by injured tissues (stressed, infection or cell death)(Gallucci, Lolkema, & Matzinger, 1999). These signals are denominated as damage associated patterns (DAMPs)(e.g; heat shock proteins, HMGB1, extracellular matrix proteins such as hyaluronic acid and metabolic waste products such as uric acid)(Gallo & Gallucci, 2013)

During maturation dendritic cells lose their endocytic functions and highly express cell-surface molecules including class I and class II major histocompatibility complex (MHC), and T-cell co-stimulatory molecules (such as CD40, CD80 and CD86), which are essential for the activation of adaptive immune responses. In addition, mature dendritic cells express the chemokine receptor CCR7 which, through the engagement of the chemokines CCL19 and CCL21, promotes the migration of DCs to the T-cell zones of secondary lymphoid organs. Once there, DCs communicate with lymphocytes to orchestrate adaptive immune responses (Coutant & Miossec, 2016).

Depending on the stimulus sensed in the periphery, mature dendritic cells secrete distintic cytokines (mainly IL-12, IL-23 and IL-10) that will induce the polarization of naïve T cells into specific subsets of Th1, Th17 or Tregs or Th2 through the expression of surface protein such as OX40 ligand. This suggests a remarkable functional plasticity based on the stimuli (Schlitzer et al., 2013; Segura et al., 2012; Yu et al., 2014).

2.1.1.1 Dendritic cells heterogeneity

Dendritic cells represent a heterogeneous population constituting of several subsets that can be defined based on their ontogeny, phenotype and transcriptional profile. Currently, DCs are subdivided into four subtypes widely distributed in mammals: the conventional or classic DC (cDC); plasmacytoid DC (pDC); inflammatory/monocyte derived DC; and Langerhans cells (LCs) (Coutant & Miossec, 2016). Except LCs, which self-renew mostly in situ, dendritic cells originate from progenitors in the bone marrow and populate several locations, including the thymus, blood, lymph, and most visceral organs (Alvarez, Vollmann, & von Andrian, 2008).

Under steady state conditions, human dendritic cell progenitors originating from the bone marrow generate four subsets of dendritic cells: plasmacytoid DCs (pDCs) and two types of classical myeloid dendritic cells (cDCs), which express CD141 (BDCA3) or CD1c (BDCA1). pDcs and cDC are found in the blood as well as in lymphoid and non-lymphoid tissues (León, López-Bravo, & Ardavín, 2007; Siegal et al., 1999). In addition, during inflammation, an additional subset of dendritic cells, known as inflammatory dendritic cells, arise (Hammad et al., 2010).

At the immature stage of development subsets of dendritic cells act as sentinels in peripheral tissues, where they sense environmental factors, take up proteins and dying cells. From the tissues DCs migrate to the T-zones in draining lymph nodes.

Functional heterogeneity characterizes each DC subset. For instance, pDCs express MHC class II and co-stimulatory molecules. Nevertheless, they are not as efficient as cDCs in priming T cells (Villadangos & Young, 2008). In addition, pDC not only perform immunogenic functions, but they can induce a tolerogenic immune response by promoting the differentiation of naive CD4+ and CD8+ T cells into induced Treg cells (Swiecki & Colonna, 2015)

CD141+ cDCs from blood are more efficient than CD1c+ cDCs at cross-presenting antigens derived from dead cells on MHC class I to CD8+ T cells, and at inducing Th2 polarization, due to the selective expression of the necrotic cell receptor C-type lectin domain family 9 member A and of the OX40 ligand (Jongbloed et al., 2010; Yu et al., 2014). In contrast, blood CD1c+ cDCs secrete high levels of IL-12, indicating an important role of this DC subset in Th1 responses (Nizzoli et al., 2013).

2.1.2 Monocytes and Macrophages

Monocytes and macrophages are mononuclear phagocytes that have different but essential roles in tissue homeostasis and immunity. Monocytes have crucial roles in the response to inflammation and the pathogen challenge, whereas the essential function of tissue-resident macrophages are related to development, tissue homeostasis and the resolution of inflammation. Furthermore, the various roles of monocytes and macrophages in the induction of protective immunity and homeostasis contribute to several pathologies (Ginhoux & Jung, 2014).

Until recently, it was universally accepted that monocytes and macrophages were two related cell types that arised from a continuum of differentiation (van Furth & Cohn, 1968). However, although monocytes display the ability to differentiate into macrophages in certain settings and during inflammation, recent studies have challenged the universality of this dogma. Firstly, monocytes are not a substantial source of most tissue macrophage compartments under steady state conditions or during certain types of inflammation. Second, adult tissue macrophages are derived from embryonic precursors that colonized the tissues before birth. And finally, tissue macrophages display a self-renewing capacity during adulthood (Wynn, Chawla, & Pollard, 2013).

2.1.2.1 Monocytes

The definition of monocytes refers to a population of cells called monocytes/macrophages or mononuclear phagocytes. Blood monocytes are bone marrow-derived leukocytes, which are functionally characterized by their ability to perform phagocytosis, produce cytokines, and act as antigen presenting cells (L. Ziegler-Heitbrock, 2015).

Monocytes are a population of leukocytes present in all vertebrates with some evidence indicating the existence of a monocyte-like population in fly haemolymph (Williams, 2007). These cells can be defined by their location, phenotype/morphology, characteristic gene expression and microRNA (miRNA) expression signatures (Cros et al., 2010; Etzrodt et al., 2012; Ingersoll et al., 2010; Mildner et al., 2013) . In organisms such as mice and humans, monocytes constitute 4% and 10%, respectively, of the nucleated cells in the blood. In addition, there is a pool of monocytes in the spleen and lungs that can be mobilized when necessary (Swirski et al., 2009; van Furth & Sluiter, 1986).

Monocytes originate from myeloid precursor cells in primary lymphoid organs, such as the fetal liver and bone marrow, during both embryonic and adult haematopoiesis, although in mice inflammation can also induce the production of monocytes in the spleen (Robbins et al., 2012) .

Peripheral blood monocytes are a heterogenous population. Based on surface markers, different monocyte subsets have been described in humans and mice (L. Ziegler-Heitbrock, 2015). In man, CD14 has been used as a marker (H. Ziegler-Heitbrock & Ulevitch, 1993), and in mice CD115 (CSF1) is often employed (Sunderkotter et al., 2004) .

In humans, a recent a nomenclature for monocyte subpopulations defines the major population of $CD14^{high}$ cells as classical monocytes, the minor population of cells with low CD14 and high CD16 as non-classical monocytes and the population in between these two subsets as intermediate monocytes (L. Ziegler-Heitbrock et al., 2010). In mice, the classical and non-classical monocyte subsets can be also identified, but different markers, such as CD115, Ly6C, and CD43, are used (Ingersoll et al., 2010; Sunderkotter et al., 2004).

The physiological function of the monocyte subsets *in vivo* is not clearly defined. Probably they have different roles during homeostasis, immune defense, and tissue repair, depending on their ability to become activated and depending on the pattern of inflammatory cytokines secreted in response to different stimuli. In addition, the subsets could display differences concerning antigen presentation and patrolling behavior (L. Ziegler-Heitbrock, 2015).

Bona fide monocytes are consider to be restricted to the blood compartment, bone marrow and spleen (Swirski et al., 2009). In bone marrow and spleen they are ready to be recruited to the blood first and subsequently to all organs and tissues.

The murine $Ly6C^{hi}$ monocyte subset gives rise to tissue-resident cells during inflammation (Ginhoux & Jung, 2014). $Ly6C^{hi}$ monocytes in mice and CD14+ monocytes in humans are considered “classical monocytes” because they are recruited to sites of inflammation and can act as the precursors for peripheral mononuclear phagocytes (L. Ziegler-Heitbrock & Hofer, 2013). The fast mobilization of $Ly6C^{hi}$ and CD14+ monocytes to the sites of inflamamation and damage makes them essential components of the host response to pathogens such as *Listeria monocytogenes*, *Mycobacterium tuberculosis* etc, (Serbina, Salazar-Mather, Biron, Kuziel, & Pamer, 2003). During inflammation and tissue damage, recruited $Ly6C^{hi}$ monocytes extravasate from inflammed tissues and differentiate into mononuclear phagocytes, including macrophages and DCs. Interestingly, in adults, monocytes

only seem to contribute to the maintenance of the majority of peripheral tissue macrophage populations under exceptional conditions (Hashimoto et al., 2013; Yona et al., 2013). Nevertheless, Ly6C^{hi} monocytes that have been recruited to injured tissues give rise to tissue-resident macrophages. Once inside the tissues monocyte-derived macrophages sense the microenvironement and define whether they contribute to the local inflammatory response or to its resolution (Ginhoux & Jung, 2014) .

The recruitment of monocytes is essential for an effective antimicrobial defence (viral, bacterial, fungal and protozoal infections) (Serbina, Jia, Hohl, & Pamer, 2008). On the other hand, monocytes also contribute to the pathogenesis of inflammatory and degenerative diseases such as atherosclerosis (Woollard & Geissmann, 2010) and they can inhibit tumour-specific immune defence mechanisms (Peranzoni et al., 2010).

In mice, infections with diverse pathogens promote the recruitment of Ly6C^{hi} monocytes to sites of infection, where they inhibit microbial growth and invasion (Shi & Pamer, 2011).

2.1.2.2 Tissue resident Macrophages

Macrophages are ancient cells in the metazoan phylogeny and were originally identified by Metchnikoff, who described their phagocytic nature. In adult mammals, macrophages are found in all tissues, where they are characterized by diverse anatomical and functional features (Wynn et al., 2013).

Recent studies have discarded the hypothesis that macrophages derive only from circulating monocytes (van Furth & Cohn, 1968) and have demonstrated that the majority of tissue resident macrophages develop during early embryogenesis and are maintained without the involvement of blood monocytes under steady state conditions (Carrero, Ferris, & Unanue, 2016). These studies have revealed three different sources for the production of macrophage precursors: the yolk sac, fetal liver and hematopoietic stem cells that colonize the bone marrow and produce bone marrow monocytes that seed the blood continuously throughout life (Christensen, Wright, Wagers, & Weissman, 2004; Ginhoux & Jung, 2014; Perdiguero et al., 2015)

The contribution of each of these sources of macrophage precursors to the establishment of a stable pool of resident macrophages in peripheral organs differs from one tissue to another. For example, the yolk sac has been identified as the

main source of macrophage precursors for brain microglia (Ginhoux et al., 2010), fetal liver for liver Kupffer cells and lung alveolar macrophages (Guilliams et al., 2013; Schneider et al., 2014), whereas bone marrow is the main source of intestinal, dermal, and cardiac macrophages (Bain & Mowat, 2012; Epelman et al., 2014; Tamoutounour et al., 2013)

In their basal state, resident tissue macrophages display significant diversity in their morphologies, transcriptome, locations and functions (Hume, 2012). The functional plasticity of resident tissue macrophages is probably caused by interactions between resident tissue macrophages and the cells they support (Wynn et al., 2013). Based on their tissue location, macrophages have different names, such as osteoclasts (bone), alveolar macrophages (lung), microglial cells (CNS), histiocytes (connective tissue) and Kupffer cells (liver). Differences in the transcriptional profiles of the macrophages make them unique populations, which is obvious from the specific functions they perform, for example alveolar macrophages promote the eradication of allergens from the lungs, whereas Kupffer cells in the liver contribute to the elimination of pathogens and toxins from the circulation (Murray & Wynn, 2011).

In tissues, macrophages shape the tissue architecture, generate and resolve inflammatory reactions, act as sentinel and effector cells, and maintain tissue homeostasis by eliminating apoptotic or senescent cells, and by remodeling and repairing tissues (Wynn et al., 2013).

2.1.2.3 Macrophage activation and plasticity

Macrophages are essential for the induction and resolution of immune responses as well as important regulators of tissue functions in health and disease (Wynn et al., 2013). Their remarkable plasticity and diversity allows them to perform a broad spectrum of functions. Numerous macrophage subsets have been identified according to the specific gene expression profiles they display after exposure to specific cytokines or microorganisms (Murray & Wynn, 2011)(Figure 1).

In order to facilitate the study of macrophage plasticity, these cells have been functionally grouped into two classes M1 and M2 macrophages (M1-M2 paradigm). Classically activated macrophages (M1) mediate the host response against bacteria, protozoa and viruses as well as display an important role in antitumor immunity, whereas the alternatively activated macrophages (M2) are described as anti-

inflammatory macrophages with a role in the regulation of wound healing. Other subsets include regulatory macrophages that secrete high levels of the anti-inflammatory cytokine IL-10 in the presence of immune complexes and TLR ligands (Sutterwala, Noel, Clynes, & Mosser, 1997; Sutterwala, Noel, Salgame, & Mosser, 1998).

After stimulation and depending on the context of the immune response, macrophages will adopt a phenotype that will promote or suppress the host's antimicrobial and inflammatory responses and antitumor immunity. Several reports have shown a flexibility in the activation of macrophages that allows the conversion from one functional phenotype to another in response to different signals from the microenvironment (Hagemann et al., 2008; Stout & Suttles, 2004; Stout et al., 2005). Consequently, macrophages exhibit a rainbow of activated phenotypes rather than one stable subpopulation.

Although the different phenotypes displayed by macrophages have important roles during various situations such as development, homeostasis, repair and the clearance of pathogens, in many cases those functions can produce harmful effects in the organism resulting in pathologies such as atherosclerosis, fibrosis, obesity, autoimmunity and cancer (Odegaard & Chawla, 2011; Pinderski et al., 2002; Sica & Bronte, 2007; A. M. Smith et al., 2009; Wynn & Barron, 2010).

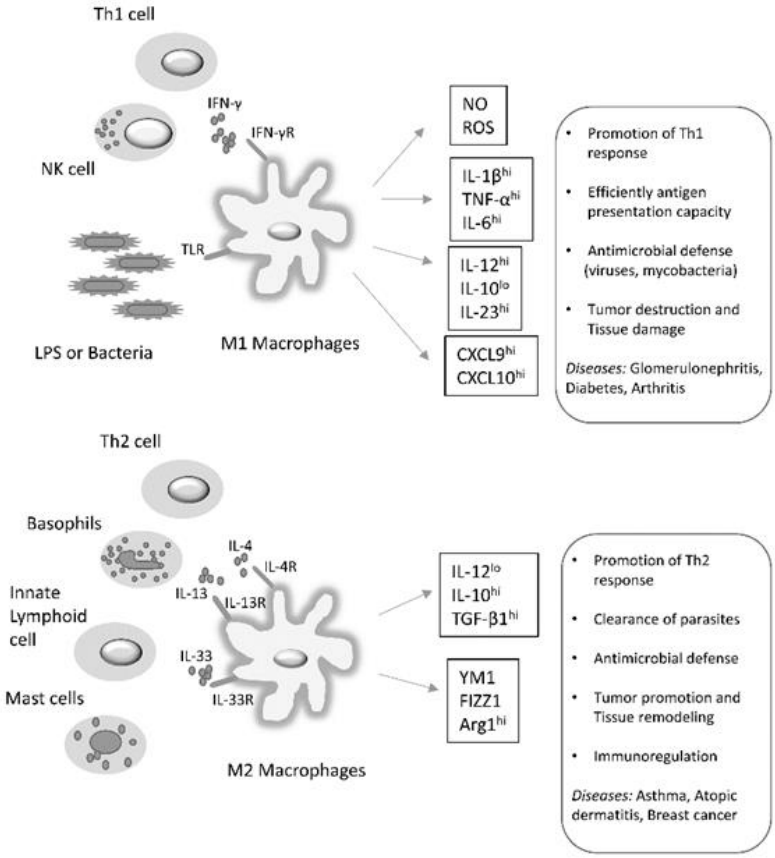

Figure 1. **Macrophage plasticity.** The figure represents a simplified approach to macrophage plasticity. The production of IFN-γ by Th1 cells and NK cells and/or the TLR engagement promote the activation of macrophages toward the M1-phenotype, which produce essential antimicrobial effectors. The uncontrolled inflammatory response caused by M1 type macrophages leads to substantial tissue damage and disease. In contrast, the cytokines produced by Th2 cells, and other cells types drive macrophages into M2 polarization and thus the production of several anti-inflammatory cytokines, such as IL-10 and TGF-β1, which promote tissue remodeling and promote tumors. A sustained M2 type response has been associated with several pathologies. The figure is based on Subhra K Biswas1 & Alberto Mantovani., 2010; Peter J. Murray & Thomas A. Wynn., 2011.

Based on a simplistic functional classification, M1/M2 tissue resident macrophages have been frequently classified as M2-like macrophages due to their dependence on CSF1R and the availability of M-CSF *in vivo* (Davies, Jenkins, Allen, & Taylor, 2013)

Tissue resident macrophages express several receptors that recognized PAMPs and DAMPs such as TLRs, NLRs, the RIG-I family, lectins and scavenger receptors (Akira, Takeda, & Kaisho, 2001; Inohara & Nunez, 2003; Taylor et al., 2005) . Nevertheless, there is great variation related to receptor usage among different tissues, and macrophages thus display an unique phenotype in distintic environments. This variability results in the implication of different subsets of macrophages in the activation of different classes of immune responses to pathogens (Davies et al., 2013).

After the initial encounter with a pathogen, resident macrophages and other tissue resident cells such as mast cells, dendritic cells and stromal cells produce inflammatory mediators that recruit inflammatory leukocyes, neutrophils and monocytes (source of inflammatory macrophages) to the site of infection. Monocyte derived macrophages rapidly colonize several inflammatory lesions and become the prevalent type of macrophage at the site of inflammation (Davies et al., 2013).

Tissue resident macrophages are essential for the initiation of the immune response. Several studies have demostrated that the depletion of tissue resident macrophages reduces the host's ability to protect againts an infection, promotes the loss of inflammatory mediators, and affects the recruitement of inflammatory cells (Ajuebor et al., 1999; Cailhier et al., 2005; Kolaczkowska et al., 2007; Kolaczkowska et al., 2009). Nevertheless, the specific role of resident macrophages in the initiation of the inflammatory response depends on the nature of the immune threat and its extent as well as on the distribution of the recognition receptors they express (Rosas et al., 2008).

After tissue damage or infection inflammatory monocytes ($Ly6C^+$ in mice) are recruited from the circulation into the affected tissues and they differentiate into macrophages (Geissmann et al., 2010).

Inflammatory monocyte-derived macrophages display a pro-inflammatory phenotype (M1) in the early stages of the immune response secreting proinflammatory cytokines such as TNF-α, IL-1β and nitric oxide that contribute to the elimination of invading pathogen (Murray & Wynn, 2011). Moreover, they produce IL-12 and IL-23, which promote the differentiation of T cells into the Th1

and Th17 subsets, respectively. Consequently, the antimicrobial response is promoted and the inflammatory response moves forward (Murray & Wynn, 2011).

Despite the essential role of inflammatory macrophages in the elimination of an invading organism, they also can cause collateral tissue damage due to the toxic activity of the molecular mediators they release during activation, such as reactive oxygen and nitrogen species. In addition, the exacerbated production of proinflammatory cytokines by Th1 and Th17 cells can contribute to tissue damage (Nathan & Ding, 2010).

Delays in the mechanisms that regulate the effector function of inflammatory macrophages, such as apoptosis or the switch into a suppressive/antiinflammatory phenotype, lead to several chronic inflammatory and autoimmune diseases (Krausgruber et al., 2011; Sindrilaru et al., 2011).

2.1.2.4 Resolution of inflammation

To prevent the progression from acute to persistent chronic inflammation, the inflammatory reaction must be resolved. It was generally believed that the resolution of inflammation was passive, however, several studies have demonstrated that it is a carefully regulated active process, where a deficiency in any of the main components leads to chronic inflammation (Headland & Norling, 2015).

Events that occur during the resolution of inflammation include**;** the reduction or termination of neutrophil infiltration into the tissue, the downregulation of chemokines and the production of cytokines and the induction of apoptosis in neutrophils and their efferocytosis by macrophages (Reville, Crean, Vivers, Dransfield, & Godson, 2006). Additional essential components in the resolution of inflammation are the change of the macrophage phenotype from classically activated to alternatively activated; the return of non-apoptotic cells to the vasculature or lymphatics and finally the initiation of tissue repair are (Headland & Norling, 2015).

Macrophages and neutrophils are essential cellular mediators of the resolution process associated with acute inflammation (Headland & Norling, 2015). During the resolution of inflammation, macrophages favour the return to homeostasis through the phagocytosis and elimination of apoptotic cells and cell debris. Additionally, they contribute to every stage of damage repair. Inflammatory bone marrow–derived

macrophages are often greater in number than tissue-resident cells during most of the resolution phase and have been ascribed active roles in the resolution of inflammation and wound repair (Gautier et al., 2012b).

The recruitment of monocytes and their differentiation into macrophages at the sites of injury is essential for the outcome of the inflammatory response and the initiation of tissue repair and homeostasis (Mantovani, Biswas, Galdiero, Sica, & Locati, 2013) . Tissue macrophages downregulate inflammatory signals and clear cytokines. Macrophages secrete proteases that cleave chemokines at motifs which impair binding to chemokine-receptors and thereby inactivate chemokines and prevent the recruitment of neutrophils. In addition, *in vitro* observations indicate that members of one macrophage subtype (M2b), derived from alternative macrophages, display immune-suppressive functions thus acting as regulators of inflammation. Moreover, several pro-resolving lipid mediatiors are able to upregulate microRNAs in macrophages that downregulate the translation of the mRNA of key inflammatory cytokines, chemokines and their receptors (Gautier et al., 2012a).

By releasing death receptor ligands, such as the Fas ligand (FasL), tumor necrosis factor (TNF)-α and TRAIL, macrophages can also control the lifespan of neutrophils, thus limitating the effector function of neutrophils during inflammation. In addition, macrophages have and essential role in the clearance of apoptotic neutrophils**.** This function is critical for the preservation of self-tolerance (Murray & Wynn, 2011)**.** The impairment of apoptotic cell clearance has been implicated in diseases such as systemic lupus erythematosus (Muñoz, Lauber, Schiller, Manfredi, & Herrmann, 2010).

Macrophages also have an essential role in tissue repair. Specifically M2 macrophages produce growth factors, such as TGFβ1, that contribute to tissue regeneration and wound repair by promoting the differentiation of fibroblasts into myofibroblasts, by blocking the the degradation of the extracellular matrix and by stimulating the synthesis of fibrillar collagens in myofibroblasts (Roberts et al., 1986).

2.1.3 General features of the adaptive Immunity

The adaptive immunity is mediated by B cells and T cells, and the immunological memory is one of its distinctive features. In addition, the adaptive immunity is highly specific and adaptable.

The lymphocyte antigen receptor is clonal, which means that each mature lymphocyte is specific for an antigen, and when lymphocytes proliferate they produce clones of identical daughter cells expressing identical antigen receptors. The diverse repertoire of antigen receptors is achieved by a specific genetic mechanism called clonal selection developed during lymphocyte production in the bone marrow and thymus (S. F. M. Burnet, 1959). During clonal selection, any lymphocytes expressing receptors reactive to self-antigens are eliminated before they fully develop into mature cells. Only the high affinity binding between the lymphocyte receptor and the foreign antigen leads to lymphocyte activation.

The mechanism of clonal selection determines which specific B lymphocyte or T lymphocyte clone will be selected to proliferate and eliminate a specific antigen.

The recognition of the antigens in B and T cells receptors occurs in different forms. B cell receptors (BCRs) are membrane bound immunoglobulins (IgM, IgD) that bind the intact antigen secreted by microorganisms as well as whole pathogens, such as virus particles and bacterial cells. There are different classes of immunoglobulins (IgG, IgM, IgA, IgD and IgE) and during B cell proliferation they experience a cellular process called affinity maturation somatic hypermutation, which enhances the ability of BCRs to recognize and bind a specific foreign antigen (Gearhart, Johnson, Douglas, & Hood, 1981; Griffiths, Berek, Kaartinen, & Milstein, 1984; Tarlinton, 2008).

The activation of naïve B cells occurs after the antigen is recognized by the BCRs and usually requires the participation of CD4+ T helper cells (Tfh). Nevertheless, T-cell independent antigens can also induce a strong BCRs stimulation leading to B cell proliferation and differentiation without the support of CD4+ T helper cells (Defrance, Taillardet, & Genestier, 2011; Seifert & Küppers, 2016). Antigens that elicit T cell independent-responses are generally divided in two classes: TI-1 or TI-2. TI-1 antigens include repetitive and ordered viral protein coats and microbial products containing ligands for both BCRs and TLRs such as lipopeptides, LPS, microbial CpG DNA, viral RNA and certain viral coat proteins (Bekeredjian-Ding & Jego, 2009). TI-1 antigens display an intrinsic B cells activating activity. TI-2 antigens

are typically multivalent antigens, examples of TI-2 antigens are the bacterial capsular repetitive polysaccharides found in *Streptococcus pneumoniae,* although TI-2 responses also occur in the presence of highly repetitive motifs found in viral capsids (De Vinuesa, O'Leary, Sze, Toellner, & MacLennan, 1999). TI-2 antigens lack an intrinsic B-cell activating capacity, and therefore only the cross-linking of a critical number of B cell receptors leads to B cell activation. In a TI-2 type response the repetitive antigens are able to activate B cells in the absence of help from T cells or a TLRs signal (Vinuesa & Chang, 2013).

Activated B cells proliferate and differentiate into effector plasma cells and resting memory cells. Plasma cells secrete soluble immunoglobulins that bind and neutralize extracellular pathogens and facilitate antigen uptake by phagocytes such as macrophages. Resting memory B cells provide protective immunity against recurring infectious agents (Treanor, 2012).

Unlike BCRs that directly recognize foreign proteins, polyssacharides, lipids, small chemicals etc, T cell receptors only recognize short peptide sequences derived from antigens that have been processed by antigen presenting cells (Swain, McKinstry, & Strutt, 2012). These peptides are bound to molecules expressed on the surface of APC that are called Major Histocompatibility Complex (MHC). Two different classes of MHC are recognized by TCRs, MHC class I and MHC class II. Consequently, TCRs are able to recognize peptides derived from internal proteins of certain pathogens. Pathogenic internal proteins display a lower rate of mutations compared to external proteins (Cole et al., 2007) . This provides protection against microorganisms with higher mutation rates in their surface proteins.

T cells are divided into two populations according to the expression of the cell-surface proteins CD4 (cluster of differentiation 4) and CD8 (cluster of differentiation 8). Both proteins mature in the thymus and migrate to secondary lymphoid organs, where they perform their effector function. CD8+ T cells are cytotoxic cells, whereas the main role of CD4+ T is the activation of other cells.

TCRs in naïve CD8+ T cells recognize peptide sequences bound to the MHC class I, whereas in naïve CD4+ T cells they detect peptides bound to MHC class II molecules expressed on the surface of APCs, such as B cells and dendritic cells. The affinity of a TCRs for the antigen along with co-stimulatory receptor–ligand interactions induce intracellular signals that activate transcription factors, which subsequently promote the proliferative expansion and differentiation of activated CD8+ and CD4+ T cells into effector T cells (van Panhuys, Klauschen, & Germain, 2014; Yamane & Paul, 2013). The T-cell response also produces memory T cells,

long-lived cells that protect the organism from further encounters with the same pathogen.

Effector CD8+ T cells eliminate virus-infected cells, cancer cells and damaged cells through the secretion of granzymes and perforins as well as cytokines such as interferon-γ (IFNγ) and tumor necrosis factor (TNF) (Cui & Kaech, 2010).

In contrast, activated CD4+ T cells proliferate and differentiate into specific subsets of effector CD4+ T cells that deliver distinctive protective immune responses such as T helper 1 (Th1) and T helper 2 (Th2), follicular helper T (Tfh) cells, Th9, Th17 subsets and peripherally induced (pTreg) and regulatory T cells from the thymus (tTreg). Each CD4+ T cell subset senses several cytokines and produces specific cytokines and chemokine receptors, which activate other effector cells such as CD8+ T cells and B cells to enhance the clearance of pathogens and prevent diseases (Figure1). Interestingly, recent reports have revealed that polarized T cells, specifically Th17 and pTreg cell subsets can modify their phenotype and repolarize. This new evidence could corroborate the hypothesis that CD4+ T cells are adaptable and can exhibit phenotypic plasticity in response to environmental changes (DuPage & Bluestone, 2016).

An infection in the skin or mucosa leads to the initiation of primary immune responses in the draining lymph nodes and on ocassion in the spleen. In these secondary lymphoid organs, mature DCs stimulate T cells to undergo clonal expansion and differentiation into short-lived effector and long-lived memory T cells (Di Rosa & Gebhardt, 2016).

Memory is the hallmark of the adaptive immunity. After the elimination of a pathogen as much as 90–95% of effector T cells die and a pool of memory cells is generated**.** Based on the expression of activation/memory markers circulating memory T cells are divided into central memory T cells (TCM), ($CCR7^+$ and $CD69^-$ $CD62L^+$), and effector memory T cells (TEM), ($CCR7^-$ and $CD69^-$ $CD62L^-$), which differ in their effector function, proliferative capacity, and migration potential (Sallusto, Lenig, Förster, Lipp, & Lanzavecchia, 1999)**.** TCM can secrete interleukin IL-2 and proliferate extensively, whereas TEM produce effector cytokines, such as interferon IFN-γ and display less proliferative capacity**.** TCM-Tcells are predominant in secondary lymphoid organs and TEM-T cells in peripheral compartments (Masopust, Vezys, Marzo, & Lefrancois, 2001; Reinhardt, Khoruts, Merica, Zell, & Jenkins, 2001)**.** Nevertheless, TEM can generate a heterogeneous population of cells with different migratory phenotypes and an extensive range of effector functions. Recently, the concept of tissue-resident memory T cells ($CCR7^-$ and $CD69^+CD62L^-$) emerged to

describe populations of memory T cells that permanently reside in peripheral non-lymphoid tissues tissues after an infection has been eliminated (Mueller, Gebhardt, Carbone, & Heath, 2013). New evidence revealed that mouse and human memory T cell populations are basically similar (Ahmed & Akondy, 2011).

T cells are constantly moving as they migrate around the body. Naïve T cells and TCM traffic from the blood into lymph nodes, scanning for antigens before returning to the circulation via the lymphatics (Grigorova, Panteleev, & Cyster, 2010; Tomura et al., 2008). The interactions between receptors expressed by T cells (e.g; CD62L and PSGL-1) and peripheral lymph node addressins (PNA and P-selectin, respectively) facilitate the traffic of T cells to the lymph nodes.

The recognition of antigens alters this pattern of migration favouring decreased exit from and increased input into lymph nodes (Cahill, Frost, & Trnka, 1976). Inflammatory stimuli that activate the lymph node vasculature or signals derived from dendritic cells can also promote the entry of effector and memory T cell into lymph nodes (Guarda et al., 2007; J. Smith, Cunningham, Lafferty, & Morris, 1970). The frafficking of T cells into secondary lymphoid organs primarily involves CCL21 expressed by endothelia that is bound by CCR7 on T cells (Stein et al., 2000).

During inflammation, predominantly effector cells and TEM enter nonlymphoid tissues, although some evidence indicate that a small proportion of naïve T cells may also enter tissues (Cose, Brammer, Khanna, Masopust, & Lefrançois, 2006). The downregulation of CD62L and CCR7 and the upregulation of other chemokine receptors and adhesion molecules promotes the migration of effector T cells into inflamed tissues. For example, the migration of T cells to the skin involves the upregulation of E-selectin ligands such as CD44 and CD43 that facilitate binding to E-selectin on the skin endothelium (Baaten, Tinoco, Chen, & Bradley, 2012; Matsumoto et al., 2007) . Receptors including CCR4, CCR10, CCR6, and CCR8, may also contribute to the recruitment of T cells into the skin (Mueller et al., 2013) . The recruitment of memory CD4+ and CD8+ T cell subsets to different regions of the skin is characterized by different chemokine requirements, with memory CD8+ T cells being more dependent on CCR10 and epidermal entry (Homey et al., 2002), whereas CD4+ T cells primarily involve CCR4 and access the dermis (Campbell, O'Connell, & Wurbel, 2007; Tubo, McLachlan, & Campbell, 2011). A cutaneous infection with HSV induces long-lasting CD8+ tissue-resident memory T cell populations at the sites of infection, the skin, and the dorsal root ganglia (Gebhardt et al., 2009).

2.1.3.1 Mechanisms of immune tolerance

One essential feature of the immune system is its ability to discriminate between a wide variety of microorganisms and self-antigens. This discrimination is based on self-non-self-discriminations (F. Burnet & Fenner, 1949; Jerne, 1971) or the detection of danger, modified self and discontinuity (Pradeu, Jaeger, & Vivier, 2013).

The recognition of self and non-self-antigens can be achieved through several mechanisms, such as the expression of PRRs that recognize pathogen-associated molecular patterns or danger-associated molecular patterns or thymic and bone marrow selection that eliminates developing T and B cells that recognize self-antigens (central tolerance).

Bone-marrow-derived progenitors of T lymphocytes migrate to the thymus**.** In the thymus, CD4 and CD8 double-positive (DP) thymocytes expressing TCRs that are unable to bind self-peptide–MHC complexes die by neglect. In addition, DP thymocytes with low affinity for self-peptide–MHC complexes differentiate into CD4 or CD8 single-positive thymocytes (positive selection). Nonetheless, DP with high-affinity TCRs for self-peptide–MHC complexes threaten the health of the organism, thus several mechanisms operate to ensure tolerance to self (central tolerance), including clonal deletion, clonal diversion, receptor editing, and anergy (Abramson, Giraud, Benoist, & Mathis, 2010). Nevertheless, the mechanisms of central tolerance are not sufficient to generate a peripheral T cell repertoire that shows broad specificity for pathogen-derived antigens and indifference to self-antigens. Consequently, the control of the intrinsic reactivity of mature T cells to self-antigens relies on the mechanisms of peripheral tolerance (Eberl, 2016).

One of the mechanisms of peripheral tolerance is the exclusion of naïve T cells and effector-memory T cells from sites where the cells express a high density of tissue restricted antigens. Restrictions in the trafficking patterns of naïve T cells and effector-memory T cells allows the ignorance of self-antigens (pMHC complexes). Other important mechanism of peripheral tolerance include tolerogenic dendritic cells, which present antigens to antigen-specific T cells, but fail to deliver adequate costimulatory signals. Finally, TCRs specific mechanisms such as the apoptosis of autoreactive T cells chronically engaged by self-pMHC in the periphery, and the promotion of anergy by costimulatory ligands (CTL4, PD-1) are also important for keeping peripheral tolerance (Eberl, 2016).

The suppression of autoreactive T cells mediated by regulatory T cells (Tregs) is another essential mechanism of peripheral tolerance. T regs can be generated in the thymus or induced from naïve T cells in the periphery**.** They can be divided into two major subsets: thymic-derived Tregs (tTregs) and peripheral-induced Tregs (pTregs)(FOXP3+). In addition, two other subsets have been defined (Tr1 and Th3) of FOXP3− iTregs. Examples of the mechanisms of suppression exerted by Foxp3+ Treg cell include a contact–dependent manner (eg killing of APCs or responder T cells by means of granzyme and perforin), the secretion of immunosuppressive cytokines (IL-10, TGF-β, IL-35 and galectin-1), the deprivation of cytokines necessary for the expansion and/or survival of responder T cells (eg;, IL-2) and CTLA-4–dependent suppression (Burchill et al., 2008; Shevach, 2009; Vignali, Collison, & Workman, 2008).

The failure of any of the mechanisms of tolerance listed above leads to the development of autoimmune diseases, cancer, etc. In addition, genetic factors and environmental factors, such as microbial infections also contribute to the development of autoimmune diseases.

2.1.4 CD4+ T cell plasticity

CD4+ T cells differentiate and exert distinct functions against specific pathogens, but can also adjust their functions according to enviromental variation. TCRs activation in a specific cytokine milieu induces the differentiation of naïve CD4 T cells into one of several lineages of T helper (Th) cells (J. Zhu, Yamane, & Paul, 2009).

The plasticity of CD4+ T cell is defined as the capacity of a single CD4+ T cell to develop the characteristics of different T cell subsets simultaneously or at different stages of its life cycle (Figure 2) (DuPage & Bluestone, 2016). This important feature of CD4+ T cells has been widely reported in immune diseases such as autoimmunity and cancer.

Specific cytokines are the driving force underlying the plasticity between CD4+ T cell subsets due to their capacity to provide a direct link between the environment and gene regulation (DuPage & Bluestone, 2016).

The majority of the polarizing cytokines function by interacting with their receptors, which leads to the activation of a phosphorylation cascade of receptor-associated Janus kinase (JAK) and signal transducer and activator of transcription

(STAT) proteins that result in the translocation of the STAT proteins to the nucleus, where they act as transcription factors (O'Shea et al., 2015).

Since Mosmann and Coffman published the hypothesis of T helper 1 (Th1) and T helper-2 (Th2) cells, the collection of CD4+ T cell subsets has expanded and includes Th17, Th9, T follicular helper (Tfh) cells, as well as thymically derived and peripherally induced regulatory T cells (tTreg cells and pTreg cells, respectively) (Burkett, zu Horste, & Kuchroo, 2015; Crotty, 2014; Schmitt, Klein, & Bopp, 2014; Wing & Sakaguchi, 2012).

T cell subsets display a distintic ability to sense different inductive cytokines, orchestrate specific transcriptional programmes and produce determined cytokines and chemokine receptor patterns in order to eliminate pathogens or halt immune pathology (Zhou, Chong, & Littman, 2009).

Recently, new studies have exposed the ability of polarized T cells, specifically Th17 and pTReg cell subsets, to modify their phenotypes and redirect their polarization towards different fates. These observations have boosted the hypothesis that CD4+ T cells are able to repolarize in response to changing circumstances (K. M. Murphy & Stockinger, 2010; O'Shea & Paul, 2010). Consequently, the concept of “plasticity” has resurfaced as opposed to “lineage stability” (Bluestone, Mackay, O'shea, & Stockinger, 2009)

The cytokines IL-2 and IL-4 were the first molecules described as promoters of the polarization of Th1 and Th2 subsets, respectively (Abbas, Murphy, & Sher, 1996).

Nevertheless, the addition of IL-4 to recently polarized Th1 cells in culture repolarizes the cells toward a Th2 phenotype by inducing the production of IL-4 and abolishing the production of IFN-γ. Similarly, Th2 cells can produce Th1-type cytokines when incubated with IL-12/IFN-γ and, essentialy, type I IFNs, which increase the expression of the IL-12 receptor β-chain (IL-12Rβ) in Th2-polarized cells (Hegazy et al., 2010; K. M. Murphy & Stockinger, 2010). Moreover, the treatment of Th2-polarized cells with transforming growth factor-β (TGFβ) induces the repolarization to the Th9 cell subset (Veldhoen et al., 2008). These observations revealed that the same cytokines, which drive the polarization of T helper cell subsets during initial priming, also mediate their functional plasticity (Figure 2).

Other examples that illustrate the plasticity of T cells are the ability of Th17 subsets to express IFNγ or repolarize completely to Th1 cells in the presence of the cytokines IL-12 or IL-23 (Bending et al., 2009) and the repolarization of murine Tfh to Th1, Th2 or Th17-type cytokines (cultured in the presence of IL21 and IL-12, IL-4

or IL-6 and TGFβ). Likewise, Th1, Th2 and Th17 express Tfh molecules (CXCR5, PD1) if they are cultured under Tfh conditions with IL-6 and IL-21 (Lu et al., 2011).

Plasticity has been shown to induce adverse effects in the host organism. For example, multiple forms of autoimmune disorders such as type 1 diabetes, multiple sclerosis or juvenile arthritis exhibit diminished stability of the transcription factor FOXP3 in Treg and/or increased numbers of IFN-γ producing FOXP3+ Treg (Bending et al., 2014; Long et al., 2010; McClymont et al., 2011). In addition, in patients with food allergies Treg display distinctive properties of Th2 cells such as IL-4 production (Rivas et al., 2015). Other examples are IL-17+FOXP3+ Treg in rheumatoid arthritis (Komatsu et al., 2014), RORγ+FOXP+ Treg that produce IL-17 in colorectal cancer (Blatner et al., 2012) and Th2 cells that produce IL-17 in atopic asthma (Becattini et al., 2015; Y. H. Wang et al., 2010). T cell plasticity is an essential factor in the development of several immunological diseases, therefore therapies targeting the regulation of T cell programming could ameliorate disease by promoting the beneficial functions of T cell effectors.

Despite of the adverse effects of plasticity, some new evidence has emerged that reinforce the benefits of plasticity and explain why it is tolerated by the organism. For example, plasticity allows a high degree of flexibility during a response to a different or the same pathogen in different environments (Becattini et al., 2015). Moreover, the conversion from one type of effector response to another could facilitate the effective elimination of pathogens that would otherwise avoid detection by populating new sites. For example, IL-17 producing T cells convert to IFN-γ producing T cells in order to clear pathogens that migrate from the extracellular to the intracellular space (DuPage & Bluestone, 2016).

Reprogramming memory T cells to display a different response in secondary infections could be important for keeping a strong immunity with age when the reserve of naïve T cells is diminishing (DuPage & Bluestone, 2016). In addition, T cell plasticity has proven to be essential for the production of IgA in the germinal centre of the small intestine, where FOXP3+Treg or Th17 convert to Tfh (Tsuji et al., 2009) and in controlling detrimental immunity (Lohning et al., 2008).

The increased knowledge of T cell plasticity will in the future allow the development of new therapeutics that regulate the transition between different T cell subsets in order to display their beneficial immune functions in patients.

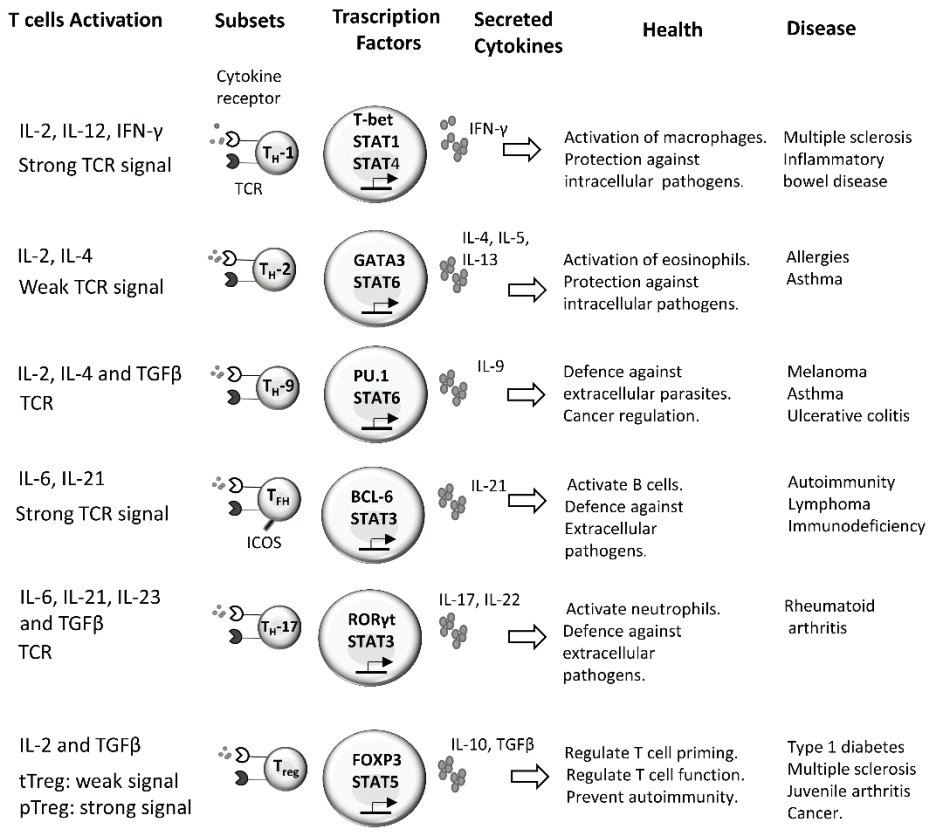

Figure 2. **CD4+ T cell plasticity.** The intensity of the TCRs signal together with polarizing cytokines drive the activation of specific transcription factors in CD4+ T cells, resulting in the production of typical cytokines that modulate immune responses in health and disease. The figure is based on Michel DuPage & Jeffrey A., 2016.

2.2 Proprotein Convertases enzymes (PCSK)

Proteins secreted by cells such as hormones, enzymes, receptors, antimicrobial peptides etc. are indispensable for cellular functions. One essential step in the maturation of several secretory proteins is the posttranslational processing that involves enzymatic modifications on a protein precursor after its translation by the ribosomes is completed. Consequently, the protein will mature functionally. The post-translational cleavage of an inactive precursor molecule was originally demonstrated by the discovery of proinsulin in 1967 (Steiner, Cunningham, Spigelman, & Aten, 1967). Since then, the study of human and mouse genomes has demonstrated approximately 600 distinct proteases, typically in five distinct classes; aspartic, metalloproteases, cysteine, serine, and threonine proteases (Lopez-Otin & Bond, 2008). Serine proteases can be further categorised based on their substrate specificity in trypsin-like, chymotrypisin-like, thrombin-like, elastase-like and subtilisin like proteases (Puente, Sánchez, Overall, & López-Otín, 2003).

Among these we find the chymotrypsin–trypsin like serine protease family that is implicated in a high spectrum of physiological processes such as embryogenesis, hemostasis, immune responses, tissue repair, cell activation, and apoptosis**.** In addition, the serine protease family includes proprotein convertases, which are related to the bacterial subtilisin (Siezen & Leunissen, 1997; Wright, Alden, & Kraut, 1969).

Since the discovery of the first proprotein convertase (van de Ven, Wim JM et al., 1990) numerous studies have demonstrated the functional importance of proprotein convertases in human homeostasis and in the development of disorders such as endocrinopathies, neoplastic diseases, infectious diseases, and atherosclerosis. Therefore, proprotein convertases constitute an attractive therapeutic target for the treatment of several human diseases (Seidah & Prat, 2012).

2.2.1 PCSK family

The family of proprotein convertases includes nine members that are synthesized as zymogens and undergo several post-translational modifications before becoming fully competent to convert immature proteins into end-functional products (Seidah, 2011).

The first seven members (PCSK1-2, FURIN, PCSK4-7) are biochemically similar to each other and are probably derived from the bacterial and yeast protein subtilisin (non-specific serine-protease found in prokaryotes) and kexin (prohormone processing protease found in the budding yeast) (Molloy, Anderson, Jean, & Thomas, 1999). They cleave precursor proteins at single or paired basic amino acids [R/K-(X)n-R/K-R↓] where R represents arginine, K lysine, X any amino acid other than cysteine and n the number 0, 2, 4 or 6 (Seidah & Chrétien, 1999).

The two remaining members of the proprotein convertase family membrane-bound transcription factor site 1 (MBTPS1/PCSK8) and PCSK9 are different from the seven core members displaying different domain structures. MBTPS1 cleaves non-basic residues at RX(L/V/I)X where L is leucine, V valine, I isoleucine and X represents any amino acid except for cysteine and proline, whereas PCSK9 has only an autocatalytic activity acting as a binding protein to specific cell surface receptors (Horton, Cohen, & Hobbs, 2007; Seidah et al., 2008).

Proprotein convertases are generally calcium-dependent proteases that perform their catalytic function within defined pH ranges at their site of action (Anderson et al., 2002). Theirs substrate selectivity often depends on their specific tissue expression, subcellular localization and secretory pathway (Thomas, 2002) (Figure 3). PCSK1 and PCSK2 are localized and active only in secretory granules, whereas the other members of PCSKs family process secreted proteins constitutively (Artenstein & Opal, 2011; Seidah & Prat, 2012).

PCSK1-2 are mainly found in neural and endocrine tissues (Day, Schafer, Watson, Chretien, & Seidah, 1992), although some reports suggest they may also be active in immune cells (Lansac et al., 2006; Mousa, Shakibaei, Sitte, Schäfer, & Stein, 2004). Another PCSK, PCSK4 is solely expressed in testicular germ cells and in the placenta and ovaries (Gyamera-Acheampong et al., 2006; Seidah et al., 1992). Although the PCSK5 gene is ubiquitously expressed, it undergoes alternative splicing thus producing a membrane bound (PCSK5b) and a secreted variant (PCSK5a) of the protease (De Bie et al., 1996). The small intestine and kidney are the main sources

of PCSK5B, while the other tissues express mainly PCSK5a. Furin, PCSK5b, PCSK6 and PCSK7 are ubiquitous and perform their function in several cellular compartments through a highly regulated process of trafficking and recycling between the trans-Golgi network, the cell membrane and endosomes (Seidah & Chrétien, 1999; Thomas, 2002). PCSK8 is ubiquitously expressed and it can be localized in the cis and medial Golgi, endosomes or lysosomes, but not at the cell surface (Seidah et al., 2008). In contrast, PCSK9 is a secreted protease that it is mainly expressed in the liver, small intestine and kidneys (Seidah et al., 2003; Zaid et al., 2008).

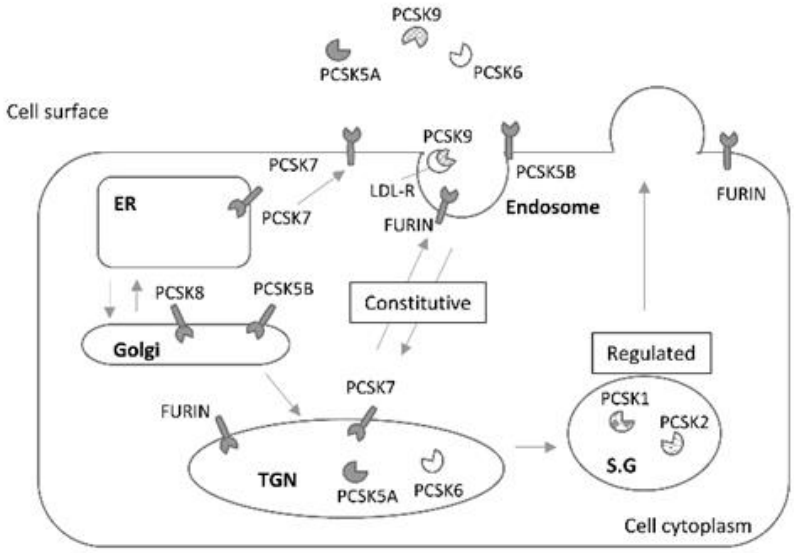

Figure 3. **Subcellular localization of proprotein convertases**. After leaving the endoplasmic reticulum (ER), most proprotein convertases travel across the Golgi apparatus towards the trans Golgi network (TGN). PCSK1 and PCSK2 are sorted into secretory granules where they are activated and awaiting signals for regulated secretion. In contrast, the constitutively secreted FURIN, PCSK5, PCSK6 and PCSK7 translocate to the cell surface from the TGN. In addition, PCSK7 can reach the cell surface directly from the ER. PCSK5A and PCSK8 remain on the cell surface, whereas FURIN, PCSK5B and PCSK7 are recycled via endosomes back to the TGN. The activated PCSK8 is localized in the Golgi apparatus and does not translocate to the cell surface. PCSK9 is released as an inactive molecule from the TGN into the extracelullar fluid. The complex PCSK9-LDLR (low density lipoprotein receptor) is embedded into endosomes and sent to lysosomes for degradation. The information in the figure is based on Nabil G. Seidah & Annik Prat., 2012.

2.2.2 PCSK knockout models and their phenotypes

Mammalian models have been widely used in modelling human diseases mainly because of the high level of homology between mammalian genomes as well as their similarities concerning anatomy, cell biology and physiology. However, the high degree of conservation in cell biological mechanisms between mammals and invertebrates advocates for the possibility of accurately studying diseases that affect these common cell mechanisms in models such as flies and worms (Y. Wang et al., 2013).

Genetic modification in animal models such as mice, zebrafish and Drosophila melanogaster has allowed the modelling of several human pathologies in order to decipher their mechanisms at the cellular and molecular level. In addition, genetic modifications such as gene deletions or insertions have provided information about the function and regulation of specific genes as well as their products in the organism.

Gene editing techniques such as Cre/loxP, transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs) and more recently CRISPR-Cas9 systems have allowed genetic modifications in animal models, such as zebrafish and mouse (Hwang et al., 2013; D. Li et al., 2013), monkeys and silkworms (Niu et al., 2014; Y. Wang et al., 2013).

The use of animal models, where proprotein convertase genes have been knocked out in the germline knockout or deleted in specific cell types, have provided invaluable information concerning their targets and function in health and disease.

PCSK1 and PCSK2:

Mouse models lacking PCSK1 and PCSK2 have helped to decipher their substrate specificities (Furuta et al., 2001; X. Zhu et al., 2002). Although individual knockout mice for PCSK1 and PCSK2 are viable, they show endocrinologic alterations (Furuta et al., 1997; X. Zhu, Zhou et al., 2002). In addition, mice lacking both proprotein convertases die during embryonic development suggesting certain redundancies in the functions of these enzymes (Seidah & Prat, 2012). The expression patterns and functions of PCSK1 and PCSK2 frequently overlap (Cyr, Stuart, Zhu, Steiner, & Nillni, 2012; Seidah, 2011b).

The growth rate PCSK1 knockout is severely reduced and they are characterized by dwarfism, possibly due to a deficit in growth hormone-releasing hormone (X. Zhu et al., 2002). Consequently, the mice display increased levels of precursor and

intermediate processing forms of hypothalamic growth hormone-releasing hormone, together with pro-opiomelanocortin, pro-insulin and intestinal proglucagon (Wardman et al., 2010; X. Zhu et al., 2002).

PCSK2 knockout mice also display retarded growth but to a lesser extent when compared with PCSK1 knockout mice, and they show chronic fasting hypoglycemia as well as altered levels of circulating glucagon (Dey et al., 2004; Furuta et al., 1997; Posner et al., 2004; X. Zhu et al., 2002). In addition, these mice are characterized by abnormalities in the processing of precursors such as: somatostatin, neuronal cholecystokinin, neurotensin, neuromedin N, dynorphin, nociceptin and pro-opiomelanocortin derived peptides. (Furuta et al., 1998; Zhang et al., 2010)

PCSK1 and PCSK2 deficiencies correlate with impaired processing of proinsulin and proglucagon into their active forms, resulting in defects in glucose homeostasis (Furuta et al., 1997; X. Zhu et al., 2002). In addition, PCSK1 and PCSK2 share specificity for substrates such as proopiomelanocortin, prorenin, proenkephalin, prosomatostatin, progastrin and proghrelin. (Turpeinen, Ortutay, & Pesu, 2013)

FURIN:

The embryos of FURIN knockout mice die at embryonic day 11 due to a defective ventral closure and axial rotation (Roebroek et al., 1998). These results suggest the existence of a specific substrate for FURIN or the lack of PCSK redundancy during mammalian embryogenesis. In addition, FURIN targets such as TGFβ like growth factors and bone morphogenetic protein 10 have been suggested to be among the substrates implicated in the phenotypes observed during embryogenesis in the absence of FURIN (H. Chen et al., 2004; Susan-Resiga et al., 2011).

The embryonic lethality of FURIN knockout mice has imposed limitations to studying the function of FURIN *in vivo*. Nevertheless, the deletion of FURIN in specific rodent tissues has revealed the absence of PCSKs compensatory effect on FURIN activity in endothelial cells or in the liver. Consequently, these studies have helped to decipher the substrate specificity for FURIN in different cell types (W. Kim et al., 2012; Roebroek et al., 2004). In addition, the inactivation of FURIN in salivary glands has revealed an important role for FURIN in the activation of the proto-oncogene pleomorphic adenoma gene 1, which induces the formation of salivary gland tumors when overexpressed in mice (De Vos et al., 2008).

In vitro studies have demonstrated that FURIN possesses a plethora of target proteins with important roles in the organism's biology such as growth factors (TGFβ), receptors (insulin receptor), adhesion molecules (alpha5 integrin),

metalloproteinases (MMP14), viral glycoproteins (HIV gp160), and bacterial toxins (anthrax pa83) (Seidah & Prat, 2012).

Notably, *in vitro* analyses have also revealed a high degree of functional redundancy between FURIN and other PCSKs, especially with PCSK5 and PCSK7. (Remacle et al., 2008).

Table 1 summarizes the main features of FURIN addressed in this thesis.

PCSK4:

PCSK4 knockout mice and *in vitro* experiments, where PCSK4 was deleted from sperm, revealed a severe impairment in fertility (Basak, Chretien, Mbikay, & Basak, 2004; Mbikay et al., 1997). These results were partially explained by an impairment in the processing and activation of metalloproteinases of the ADAM family (ADAM 2 and 3).

PCSK4 has been considered as an essential convertase in fertility and pregnancy due to its role in processing pituitary adenylyl cyclase-activating polypeptide in rodent gonads (M. Li, Mbikay, Nakayama, Miyata, & Arimura, 2000), and likely of insulin-like growth factor 2 in human placenta (Qiu, Basak, Mbikay, Tsang, & Gruslin, 2005).

PCSK5:

PCSK5 knockout mice develop several craniofacial and patterning defects that lead to death at birth (Essalmani et al., 2008). The explanation for this phenotype might be in part due to a defective processing and activation of the PCSK5 substrate GDF11 (McPherron, Lawler, & Lee, 1999). Other examples of PCSK5 substrates are the receptors (PTPRM) and the adhesion molecules (neuronal L1CAM, α4 integrin) (Seidah & Prat, 2012).

Strong redundancy between PCSK5 and FURIN and PCSK6-7 has been demonstrated *in vitro* and ex vivo (Seidah & Prat, 2012).

PCSK6:

The PCSK6 knockout mouse phenotype is characterized by severe cardiac malformations, left-right axis abnormalities and bone morphogenetic defects (Constam & Robertson, 2000). The abnormal mRNA levels of the PCSK6 substrates Nodal and Lefty at an early stage of embryonic development might contribute to the defects observed in the left-right axis (Blanchet et al., 2008).

Other examples of PCSK6 substrates are metalloproteinases (ADAM-TS4) and viral glycoproteins (HIV protein Vpr) (Seidah & Prat, 2012).

Strong redundancy between PCSK6 and FURIN, PCSK5 and PCSK7 has been shown *in vitro* and ex vivo (Seidah & Prat, 2012).

PCSK7:

PCSK7 knockout mice develop normally although they display an anxiolytic phenotype and exhibit deficiencies in cognitive factors such as learning and memory (Besnard et al., 2012; Constam, Calfon, & Robertson, 1996; Wetsel et al., 2013). PCSK7 KO mice exhibit reduced levels of brain-derived neurotrophic factor (BDNF), which seems to be the cause of the defects observed. Consequently, these results imply a specific role for PCSK7 in the central nervous system, possibly through the regulation of BDNF levels (Wetsel et al., 2013).

In contrast, the deletion of PCSK7 in Xenopus is lethal; the embryos lack eyes and a brain and show an abnormal anterior neural development (Senturker, Thomas, Mateshaytis, & Moos Jr, 2012). This observation argues for an essential role for PCSK7 in the embryogenesis of lower vertebrates.

The substrates for PSCK7, include proepidermal growth factor (Rousselet et al., 2011) and transferrin-1 receptor (Guillemot, Canuel, Essalmani, Prat, & Seidah, 2013).

PCSK7 activity overlaps with FURIN and PCSK5-6 (Seidah & Prat, 2012).

PCSK8:

PCSK8 knockout mice and zebrafish display a lethal phenotype due to the essential role of PCSK8 in the formation of cartilage (Mitchell et al., 2001; Schlombs, Wagner, & Scheel, 2003). In addition, a murine knockout model, where the expression of PCSK8 was deleted in 80% of the cells in the liver exhibits a decrease in the levels of circulating cholesterol and triglycerides (Yang et al., 2001). Another observation in line with this decrease was the low levels of nuclear sterol regulatory element-binding proteins (SREBPs) and the diminished expression of their target genes. These results confirmed the role of PCSK8 in the activation of the transcription factor for SREBPs. Furthermore, PCSK8 is required for the transcription of certain genes important for the bone matrix and mineralization in bone osteoblasts and osteocytes (Gorski et al., 2016).

Other targets of PCSK8 are AMP-response element-binding protein (Yang et al., 2001), transmembrane spanning b-ZIP transcription factors (Brown & Goldstein, 1999; Seidah et al., 2008) and viral glycoproteins, e.g., those of the Lassa virus (Lenz, ter Meulen, Klenk, Seidah, & Garten, 2001; Pasquato et al., 2006).

PCSK9:

The PCSK9 knockout mouse phenotype is characterized by higher levels of the LDL receptor (LDLR) protein in the liver and reduced levels of blood cholesterol (Rashid et al., 2005). Interestingly, although PCSK9 efficiently promotes the degradation of the hepatic LDLR via the acidic milieu of endosomes/lysosomes by resident hydrolases it does not perform this function through an intrinsic catalytic activity (Seidah, Awan, Chretien, & Mbikay, 2014; Seidah, 2016). Instead, it binds to the LDLR and it is endocytosed together with the receptor (Cariou, Le May, & Costet, 2011; Tibolla, Norata, Artali, Meneghetti, & Catapano, 2011) and modulates the recycling of LDLR in organs such as the liver, intestines, kidneys, lungs, pancreas and adipose tissue (Dadu & Ballantyne, 2014). The degradation of LDLR promoted by PCSK 9 leads to a diminished number of LDLR on the surface of cells (Dadu & Ballantyne, 2014).

Transgenic mice overexpressing PCSK9 display higher levels of LDL-cholesterol and mice with PCSK9 deleted in their hepatocytes showed lower levels of total cholesterol circulating in the blood when compared with normal mice (Seidah, 2016). These models support an inverse relationship between PCSK9 in the blood and the number of LDLR .

PCSK9 also promotes the degradation of other receptors such as VLDLR, ApoER2, low-density lipoprotein receptor-related protein 1, CD36, and CD81 (Seidah et al., 2014).

Table 1. **Furin at a glance**. Sources of Information: Oksanen et al., 2014; Turpeinen et al., 2012 and 2013; Seidah et al., 2012; Pesu et al., 2008; Silvestri et al., 2008.

TISSUE DISTRIBUTION	Ubiquitous (all vertebrates and many invertebrates)
TYPE OF MOLECULE	794-amino-acid type-I transmembrane protein
FAMILY	Proprotein convertase from the subtilisin/kexin family
LOCALIZATION	Trans Golgi network, cell surface, endosomes
ENZYMATIC FUNCTION	Processing and/or modulation of a large number of proprotein substrates in the secretory pathway leading to their activation and sometimes inactivation.
CLEAVAGE SITE	Single or paired basic amino acids within the motif (R/K)Xn(R/K)↓
ACTIVATION	Primarily activated in the *trans*-Golgi network
LOCALIZATION OF PROTEOLYTIC ACTIVITY	In immature secretory granules, in the Golgi, at the cell surface, in endosomes or in the extracellular matrix
MOUSE KNOCKOUT PHENOTYPE	Embryonic dismorphism: no axial rotation, no heart looping. Embryonic death at day 11
ORTHOLOGS	furina,furinb (ZEBRAFISH)
SUBSTRATES	Hormones (proinsuline); Growth factors (TGFβ); Tumor necrosis factor (TNF) ligand family (BAFF, APRIL); Chemokines (CXCL10); Receptors (Notch-1 receptor, integrins); Adhesion molecules (α5 integrin); Metalloproteinases (MMP14, ADAMs); Viral glycoproteins (HIV gp160, EBOV GP); Bacterial toxins (anthrax pa83, Pseudomonas exotoxin A (PE)), etc
INDUCTION	Several cancers and metastasis; Viral and bacterial Infections; Atherosclerosis; Autoimmune diseases; Immune cells (LPS-activated monocytes and activated lymphocytes); Shear stress in VEC; Iron deficiency and Hypoxia/HIF-1, etc
HUMAN POLYMORPHISMS	-rs2071410 (moderately associates with the hypertension phenotype) -rs2521501, rs2071410, rs6227, rs4702 (associated with an elevation in both the systolic and diastolic blood pressure)
FUNCTIONS	-Involved in several viral and bacterial infections by activating bacterial toxins and viral envelope glycoproteins -Contributes to the maturation of many proteins involved in tumorigenesis, neurodegenerative disorders, autoimmune diseases, colesterol metabolism, iron metabolism, atherosclerosis, etc -Regulator of peripheral tolerance and Th1/2 cell balance -Proteolytic processing of hTLR7 -Generation of MHC class I peptide antigens
INHIBITORS	Furin mRNA silencing FANG (Vigil) cancer vaccine (clinical trial); Small-molecule inhibitors (e.g, 2,5-ideoxystreptamine); Macromolecular compounds (α1-antitrypsin Portland (α1-PDX)); *Peptide inhibitors*; Polyarginines (nona-d-arginine); Serpins (furin natural inhibitors, e.g, serpinB8)

2.2.3 PCSK1-7 mutations in humans

Although biochemical studies of the PCSKs have substantially contributed to the deciphering of the physiological functions of these enzymes, the high degree of PCSK redundancy reduces the amount of information biochemical studies can provide. Consequently, genetic approaches have been undertaken to extensively understand the biological importance of PCSK enzymes.

The application of genome wide association study arrays to large sample collections has greatly contributed to the study of the genetics of PCSKs and their association with several human phenotypes (Table 2).

Table 2. **Examples of PCSKs gene polymorphisms and human traits/disease association.** The information is adapted from Turpeinen et al., 2013

PCSKs	Polymorphism	Association
PCSK1	rs6232 (N221D) in exon 6, rs6235 (S690T) and rs6234 (Q665E) in exon 14	Polygenic obesity
PCSK2	SNP rs6044834	Prevalence of myocardial infarction
FURIN	SNP rs4932178 in FURIN promoter	Course of an HBV infection.
PCSK5	SNP rs10512049	Alzheimer's disease related total ventricular volume
PCSK6	SNP rs1871977	High diastolic blood pressure
PCSK7	SNP rs508487	Levels of triglycerides and low-density lipoproteins LDL

Detailed information concerning PCSK1-7 genetics and their association with human diseases is discussed below.

PCSK1:

PCSK1 is responsible for processing several hormones and neuropeptides, thus it has been associated with numerous human diseases and endocrinal phenotypes such as hypogonadotrophic hypogonadism (Cadman, Kim, Hu, Gonzalez-Martinez, & Bouloux, 2007) and hypocortisolaemia/ hypoinsulinaemia (Jackson, Creemers, Ohagi, Raffin-Sanson, & Sanders, 1997). In addition, PCSK1 has also been linked to disorders such as hypo- and hyperthyroidism (Shen, Li, Brent, & Friedman, 2004), neoplastic human pituitaries (Lloyd et al., 1995) and breast cancer (Cheng et al., 1997).

Interestingly, PCSK1 cleaves hormones and neuropeptides essential for the regulation of thermogenesis and feeding behavior (Nilaweera, Barrett, Mercer, & Morgan, 2003; Suzuki, Jayasena, & Bloom, 2012) and it was one of the first genes to be associated with the severe early-onset of obesity (Montague et al., 1997).

The congenital PCSK1 deficiency in human and its association with severe obesity was reported for the first time in 1997 (Montague et al., 1997). Patients with PCSK1 deficiency are characterized by monogenic obesity, growth hormone deficiency, postprandial hypoglycemia, hypogonadism, and reduced thyroid function (Jackson et al., 1997; Jackson et al., 2003). Additionally to the monogenic obesity caused by a deficiency in the PCSK1 protein, studies on polygenic obesity and its association to polymorphisms in the PCSK1 gene have uncovered three nonsynonymous single nucleotide polymorphism variants (SNPs) of PCSK1 linked to common obesity in an age- and ethnicity-dependent fashion (Benzinou et al., 2008; Stijnen et al., 2014).

Some genetic variants of PCSK1 have also been associated with the age of onset of natural menopause (He et al., 2010).

PCSK2:

The proprotein convertases PCSK1/PCSK2 overlap in their expression patterns and frequently act jointly to functionally activate common substrates. However, differences in their enzymatic functions can generate distinct products from the same precursor.

Several PCSK2 SNPs in humans have been shown to be linked to type-2 diabetes in different ethnic populations (Leak et al., 2007; H. Yoshida et al., 1995; Zheng et al., 2012), total antioxidant levels in obese Hispanic children (Comuzzie et al., 2012), prevalence of myocardial infarction and chronic kidney disease in Japanese individuals (Fujimaki et al., 2010; T. Yoshida et al., 2009) as well as the dialysis

survival of African-Americans patients with type 2 diabetes (Murea et al., 2011), age of onset of menarche (Elks et al., 2010) and amyotrophic lateral sclerosis (Kwee et al., 2012).

FURIN:

The expression of FURIN has been investigated in many physiological and pathological processes. For example, *furin* mRNA levels are elevated in metastasizing human head and neck cancer (Bassi et al., 2001), and it is upregulated in the immune cells of advanced human atherosclerotic plaques (Turpeinen, Raitoharju et al., 2011).

The levels of FURIN are elevated in respiratory epithelial cells in cystic fibrosis enhanced toxin-induced cell death, fibrosis, and immunosuppression during the course of a Pseudomonas aeruginosa infection (Ornatowski, Poschet, Perkett, Taylor-Cousar, & Deretic, 2007). More recently, *furin* was described as one of the genes relevant for the development of schizophrenia (Fromer et al., 2016).

A polymorphism in the *furin* promoter (rs4932178) has been associated with the outcome of a HBV infection (Lei et al., 2009) and a poor prognosis in colorectal cancer (Försti et al., 2010).

Another important role for *furin* is the regulation of the renin-angiotensin system (Cousin et al., 2009) and SNPs in the *furin* gene have been suggested as candidates for an increased risk of developing hypertension in a Kazakh ethnic group (N. Li et al., 2010) and in three recent genetic association studies (Ganesh et al., 2013; International Consortium for Blood Pressure Genome-Wide Association Studies, 2011; Turpeinen et al., 2015).

PCSK5:

Polymorphisms in the PCSK5 gene region have been associated with neurobehavioural skills (D. S. Kim et al., 2012), Alzheimer's disease (Furney et al., 2011) and the age of onset amyotrophic lateral sclerosis (Alsgen Consortium, 2013). In addition, PCSK5 polymorphisms have been related to the development of anorectal atresia (Carter et al., 2013) and to the levels of high-density lipoprotein cholesterol (Iatan et al., 2009). Interestingly, one PCSK5 SNP showed a strong association with human height (Allen et al., 2010; Iatan et al., 2009).

PCSK6:

The PCSK6 SNP (rs11855415) has been associated with the direction of handedness in dyslexia, probably through the regulation of mechanisms involved in brain lateralization (Arning et al., 2013; Scerri et al., 2011). Furthermore, other SNPs in the PCSK6 gene region have shown sustained association with the severity of pain

in osteoarthritis (Malfait et al., 2012) and with high blood pressure (J. P. Li et al., 2004; Xu et al., 1999a; Xu et al., 1999b).

PCSK7:

GWA studies have unveiled an association between a human PCSK7 gene polymorphism and the level of the soluble transferrin receptor, thus PCSK7 could have an important role in the regulation of iron homeostasis (Oexle et al., 2011).

Another study found an association between the PCSK7 SNP rs508487 and the levels of triglycerides (Middelberg et al., 2011).

2.3 Proprotein convertases in immunity

The discovery that proprotein convertases such as PCSK1, PCSK2, PCSK5, FURIN and PCSK7 were expressed in primary and secondary lymphoid organs such as lymph nodes, the thymus and spleen, and their expression in immune cells under lipopolysaccharide stimulation suggested a role for these enzymes in immunity (Lansac et al., 2006).

Earlier studies detected the expression of PCSK1 in macrophages and PCSK2 in polymorphonuclear leukocytes (LaMendola, Martin, & Steiner, 1997; Vindrola, Mayer, Citera, Spitzer, & Espinoza, 1994). Recently, Refaie et al, studied a PCSK1 knockout mouse demonstrating a pro-inflammatory response after stimulation with LPS as well as an upregulation of the Th1 pathway (Refaie et al., 2012). In addition, they showed that PCSK1 modulated macrophage activation by regulating vesicle trafficking and secretion (Gagnon et al., 2013; Refaie et al., 2012). This evidence suggests a role for PCSK1 in the regulation of the innate immune response.

It has been also suggested that FURIN and PCSK5 regulate the inflammatory response in atherosclerosis through the cleavage of matrix metalloproteinases and integrins. Moreover, the expression levels of matrix metalloproteinases and integrins increase during macrophage differentiation suggesting a role for these proprotein convertases in the polarization of macrophages (Stawowy et al., 2005). FURIN, PCSK5 and PCSK7 are also expressed in human CD4+ T cells (Elhage et al., 2015).

The investigation concerning the function of the proprotein convertases in T cell biology has shown that the mRNA expression of FURIN, PCSK1, and PCSK7 was

elevated upon the activation of murine natural regulatory T cells, although the highest mRNA level was detected for PCSK7. An *in vitro* cleavage analysis indicated that PCSK1 and PCSK7 were responsible for the cleavage of Foxp3 at its C-terminus and that IL-10 was induced in cells where Foxp3 was cleaved at the C-terminus (de Zoeten et al., 2009). This study suggests that the proteolytic activity of proprotein convertases at the C-terminus of Foxp3 enhances the suppressive function of Tregs *in vitro* and *in vivo* thus preventing the development of inflammatory bowel disease in the adoptive transfer model of colitis in mice. Contrary to mice, another study performed in humans showed that the suppressive function of Tregs *in vitro* was almost absent when Foxp3 was cleaved at its C-terminus and the role of PCSKs in the cleavage of Foxp3 was unclear (Elhage et al., 2015). More research is required to determine the precise role of PCSK in the proteolytic cleavage of Foxp3+ as well as the physiological importance of Foxp3 isoforms.

PCSKs also display immunomodulatory functions in autoimmune diseases such as rheumatoid arthritis (RA), where the systemic administration of FURIN in a collagen induced arthritis murine model showed a protective effect characterized by a reduction in joint damage and inflammation as well as bone loss. The reduced level of inflammation observed after the systemic administration of FURIN was probably caused by a rebalance in the Th1/Th2 ratio and a restoration of the number of highly suppressive Tregs in the spleen (H. Lin et al., 2012). These results argue for a role of FURIN in immune tolerance.

In addition, Shiryaev et al; suggested the existence of an inflammatory proteolytic pathway that leads to the development of the autoimmune disease multiple sclerosis. This pathway involves the regulation of MMPs by the proteolytic activity of FURIN and PCSK2, and the cascade of activation results in the stimulation of specific autoimmune T cell clones. Consequently, the autoreactive T cells that crossed the brain barrier caused multiple sclerosis (Shiryaev et al., 2009).

2.3.1 Furin in T cell biology

One proprotein convertase described as important in T cell biology is FURIN. FURIN is induced by TGF-β1, but also in an IL-12/Stat4 dependent manner in both mice and humans (M. O. Li, Wan, & Flavell, 2007; Pesu, Muul, Kanno, & O'Shea, 2006) . Upon IL-12 stimulation FURIN is preferentially expressed in T helper 1 cells and its inhibition decreases the production of IFN-γ (Pesu et al., 2006). Interestingly,

a study of the genomic locus of *furin* has revealed a strong superenhancer region specifically in Th1 cells, suggesting an important role for FURIN in the definition of T cell identity (Vahedi et al., 2015) .

The important role of FURIN in the T cell effector function was demonstrated in a mouse model with a conditional deletion of *furin* in T cells (Pesu et al., 2008). This study showed that the deletion of *furin* does not interfere in the development of T cells, but affects the effector function of regulatory and effector T cells. In this model, regulatory T cells displayed a reduced ability to suppress inflammation in a model of colitis.

Furthermore, at approximately six month of age the mice developed a systemic autoimmune disease characterized by the hyperactivation of effector T CD4+ and T CD8+ cells, high levels of pro-inflammatory cytokines, the production of autoantibodies and a breakdown in the peripheral tolerance mediated by Tregs. These observations were explained at least in part by a deficiency in the production of the anti-inflammatory cytokine TGF-β1, one of the substrates cleaved by FURIN in T cells (Dubois et al., 2001; Pesu et al., 2006; Thomas, 2002) . However, when FURIN knockout T cells were compared to TGF-β1 deficient T cells and wild type controls it was apparent that the regulation of the bioavailability of TGF-β1 it is one of the functions of FURIN in T cells (M. O. Li et al., 2007; Pesu et al., 2008). In addition, FURIN has been shown to be an essential factor in the regulation of the T helper cell balance in mice that were chronically infected with an intracellular *Toxoplasma gondii* parasite (A. Oksanen et al., 2014)**.**

2.3.2 Proprotein convertase function in antigen presentation and the processing of TLRs

FURIN and others PCSKs not only activate several bacterial toxins and viral glycoproteins but also have a role in the process of antigen presentation. Consequently, they contribute to the pathogenesis of certain infectious diseases (Artenstein & Opal, 2011; Gil-Torregrosa, Raul Castano, & Del Val, 1998; Thomas, 2002).

As discussed earlier, antigen presentation in the context of MHC class I is essential for the effector function of $CD8^+$ T lymphocytes in response to viruses. Interestingly, some studies have demonstrated TAP- independent pathways that

involve FURIN in the trans-Golgi network (Medina et al., 2009). Notably, this alternative pathway mediated by FURIN provides intracellular viral peptides that bind to MHC class I molecules for presentation to CD8+ T cells and contribute to 30% of all the surface MHC class I complexes (Gil-Torregrosa et al., 1998).

One essential aspect in antigen presentation is to select and load stable and high affinity antigenic peptides onto MHC class I molecules. The optimization of this process takes place within a complex called peptide-loading complex (PLC). In virus infected and tumor cells where PLC is defective, several structurally fragile MHC class I/peptides complexes are produced that display a high level of disintegration thus affecting the presentation of the antigens to CD8+ T cells.

Leonhardt et al, showed that PCSK7, but not FURIN, is able to rescue the post-endoplasmic reticulum unstable MHC class I when PLC is defective. Cells with a functionally impaired PLC and which lack PCSK7, have reduced surface levels of MHC class I due to their fragility and delayed expression on the cell surface (Leonhardt et al., 2010). On the other hand, a genome-wide expression correlation analysis in humans has demonstrated a strong correlation between PCSK7 and the MHC-class genes (Turpeinen, Kukkurainen et al., 2011). These results highlight an important role for PCSK7 in the regulation of antigen presentation.

Important receptors for the antiviral immune response such as TLR7 and TLR8 have shown to be processed and activated by PCSKs. The human TLR7 recognizes single-stranded viral RNA in endosomes and FURIN, PCSK5 and PCSK7 have been reported as the enzymes responsible for the regulation of TLR7 activity and its localization in human monocytes (Hipp et al., 2013). Consequently, TLR7 processing by PCSKs guarantees an effective antiviral response. Likewise, another study suggested a role for furin-like proprotein convertases in TLR8 processing in the early/late endosomes of human monocytes and monocyte derived macrophages (Ishii, Funami, Tatematsu, Seya, & Matsumoto, 2014).

The regulation of the activity and localization of endogenous TLR7 and TLR8 by PCSKs prevent the response against self-nucleic acids and the development of autoimmune diseases.

2.4 Proprotein convertase in Therapeutics

The evidence of the regulatory role of proprotein convertases in the immune system has prompted the investigation of PCSKs as new therapeutic drugs in the treatment of cancer, arthritis, and infections (Seidah & Prat, 2012). Targeting of PCSKs is cumbersome since requires the generation of highly specific drugs that combat the disease without affecting the function of PCSKs in maintaining the homeostasis of the organism.

The upregulation of FURIN and PCSK6 in cancers and metastases has generated interest in the use of these enzymes as therapeutic targets (Bassi, Fu, Lopez de Cicco, & Klein-Szanto, 2005; Bassi et al., 2010; Couture, D'Anjou, & Day, 2011; D'Anjou et al., 2011). Consequently, a myriad of inhibitors has been produced such as small-molecule inhibitors, neutralizing antibodies, chemically generated prodomains and antisense RNAs (Seidah & Prat, 2012). Furthermore, the inhibition of FURIN can occur intracellularly and it is mediated by the FURIN-binding serpin proteinase inhibitor 8 (Kappert et al., 2013; Leblond et al., 2006).

In vitro studies have shown that small-molecule inhibitors of Furin are able to have an antimetastatic effect in human fibrosarcoma and a protective effect in skin cancer (J. M. Coppola, Bhojani, Ross, & Rehemtulla, 2008). On other hand, numerous *in vivo* studies support the use of protein inhibitors of furin-like-PCSKs in preventing tumor growth and spreading (J. M. Coppola et al., 2008).

A different approach to FURIN inhibitors is silencing the FURIN mRNA , which has been used as a cancer vaccine called bi-shRNAi(furin)/GMCSF DNA/autologous tumor cell (FANG) in patients with advanced cancer (Senzer et al., 2012). To create this inhibitor autologous harvested tumor cells have been transfected with a plasmid that expresses a recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) and a bifunctional short hairpin RNA (bi-shRNA) against the *furin* gene, with potential immunostimulatory and antineoplastic functions. After the intradermal aplication of the bi-shRNA-furin/GM-CSF-expressing autologous tumor cell vaccine**,** GMCSF promotes antigen presentation and the recruitment of immune effector cells to the site of injection, while the bifunctional short hairpin RNAi (bi-shRNAi) targeting *furin* gene leads to the dowregulation of the endogenous immunosuppressive transforming growth factors TGF-β1 and TGF-β2, which are Furin substrates (Senzer et al., 2012). Transforming

growth factors are strong immunosuppressive cytokines, thereby the reduction in their levels activates the antitumoral immune response (Senzer et al., 2012) .

One recent study of a three year follow-up of applying FANG to treating patients with metastatic advanced Ewing's Sarcoma revealed better survival estimates and non-significant toxicity (Ghisoli et al., 2016). In addition, the same vaccine has been reported as potentially effective in the treatment of advanced ovarian cancer and its safety, the strong induction of the immune system and survival improvement were demonstrated in advanced hepatocellular carcinoma (Nemunaitis et al., 2014; Oh et al., 2016).

Furin and other PCSKs process and activate several toxins from both bacteria and viruses such as anthrax, influenza A H5N1 flaviviruses, Marburg and Ebola viruses (Shiryaev et al., 2007). Consequently, the development of therapeutic FURIN/PCSK inhibitors could protect the host against all of these pathogens dependent on FURIN/PCSK activation and prevent the development of acute diseases.

Notably, the clinical use of two monoclonal antibodies (alirocumab and evolocumab) that inhibit PCSK9 has been recently approved in the USA and Europe based on their efficacy at lowering LDL-C levels and on their safety (Farnier, 2016).

Another significant reason for studying PCSKs is the possibility to use them as diagnostic markers. For example, FURIN and PCSK5 are secreted extracellularly (Essalmani et al., 2006; Meissner, Scheltema, Mollenkopf, & Mann, 2013). Consequently, the detection of PCSKs in serum samples and the knowledge that the levels of PCSKs change during the course of many acute and chronic pathologies could promote the development of new diagnostic methods (Kumar, Singh, Ahirwar, & Nath, 2014).

2.5 Zebrafish model

Although forward genetic mutagenic strategies have shown to be successful in invertebrate models for studying human diseases, these models face limitations, such as the absence of several structures, organ systems and a lack of an adaptive immune system with a role in human disease pathogenesis (Langenau & Zon, 2005).

In the case of mouse models, although forward and reverse mutagenic strategies are feasible and extremely reliable in modeling human diseases, it is challenging to carry out large scale studies, because of a necessity of considerable investment in

infrastructure support and staff. These limitations in the use of invertebrate and mammallian models can be overcome by using zebrafish (*Danio rerio*) models, mainly because forward and reverse genetics strategies are more manageable in fish than in mice. Also there are similarities in the molecular mechanisms, development and cellular physiology of zebrafish and mammals (Appleby & Ramsdell, 2003; Kari, Rodeck, & Dicker, 2007).

The zebrafish is a small tropical aquarium fish that has been recently introduced as a model organism (Grunwald & Eisen, 2002). Originally, it was used to study developmental biology, hematopoiesis and embryogenesis (Kari et al., 2007). Recently this model has also proved to be a powerful model in the study of the pathogenesis of several conditions such as infections and cancer as well as in the evaluation of drug efficacy and toxicity (Kari et al., 2007).

The advantages of using zebrafish as a model include external fertilization and development that allow the direct observation and manipulation of embryos under different laboratory conditions, the high number of small sized embryos produced that can be easely distributed onto plates and the numerous animals that are available for experimentation at a minimal cost. In addition, the zebrafish embryos and early adults areoptically transparent and develop fast ex utero with most organ systems in place by 5 days post-fertilization (Kari et al., 2007; Meeker & Trede, 2008). Consequently, the use of fluorescent markers allows the detection of different cells and organs in the zebrafish facilitating the microscopic imaging of specific cells affected in the context of several conditions.

The zebrafish embryo's permeability to small molecules and drugs during organogenesis effectively provides access for drug administration and dye staining. Furthermore, another important advantage of this model relies on the possibility to knockdown the expression of specific proteins using antisense oligodeoxynucleotides called morpholinos. In addition, the embryos can be microinjected with mRNA or plasmids to express specific proteins. These techniques have allowed the study of the role of specific proteins during development, in respose to drugs, in immunity, etc (Kari et al., 2007).

Finally, a recent report from Howe et al (2013) showed that the zebrafish genome expressed the largest set of genes among the vertebrates previously sequenced with 26,000 protein-coding genes (Howe et al., 2013). This increased gene number can be explained at least in part as a consequence of a teleost-specific genome duplication (Meyer & Schartl, 1999). Furthermore, the comparison

between the human and zebrafish genomes demonstrated that 70% of human genes have at least one orthologue in the zebrafish genome (Howe et al., 2013).

Despite the aforementioned advantages of zebrafish as an animal model, there are some potential limitations. For example, invertebrates lack several structures and organ systems (e.g; lung, prostate, skin, and mammary gland) that are involved in disease pathogenesis in humans, thereby the role of zebrafish in modelling human disease will be limited (Lieschke & Currie, 2007). In addition, human pathogens cause diseases at 37°C, whereas zebrafish are maintained at 28°C; therefore some pathogens can not be studied at this lower temperature. Furthermore, the almost nonexistent source of monoclonal antibodies against surface antigens of zebrafish immune cells greatly affects cell-biological studies of these processes. Finally, there are important differences between human and zebrafish concerning the physiology of the immune system, which might affect the validity and usefulness of modelling human infections in zebrafish (Lieschke & Currie, 2007).

2.5.1 Role of PCSKs in the zebrafish model

Zebrafish is a powerful *in vivo* model for deciphering the role of orthologous proteins and genes in vertebrate biology. For example, PCSK1-2 (Morash, MacDonald, Croll, & Anini, 2009), FURIN (Walker, Miller, Talbot, Stock, & Kimmel, 2006), PCSK5 (Chitramuthu et al., 2010), PCSK7 (Turpeinen et al., 2013) , PCSK8 (Schlombs et al., 2003) and PCSK9 (Poirier et al., 2006) have been studied using zebrafish.

Two co-ortologs (*furinA* and *furinB*) for the mammalian *furin* gene have been identified in zebrafish and both genes seem to play a role in the activation of endothelin-1 signaling, thus regulating craniofacial patterning. The *furinA* mutants in zebrafish display jaw and fin defects and 5 days post-fertilization the phenotype is characterized by an open-mouth and inability to form a swim bladder (Walker et al., 2006). In addition, modelling “Fraser syndrome” (an autosomal recessive polygenic, multisystem congenital human disorder) in zebrafish has implicated *furinA* in the proteolytic shedding of FRAS1 or FREM2, which are extracellular matrix proteins associated wih the aetiology of Fraser Syndrome (Carney et al., 2010).

PC5.1 is a co-ortholog gene of PCSK5 cloned in zebrafish that has been shown to have an important role in the deposition of the neuromast within the lateral line

system. Moreover, when PC5.1 was knocked down the embryos showed a lack of touch response and an aberrant circular swimming pattern advocating for a role for PC5.1 in the adequate development of the lateral line system, a sensory system that is present in fish and amphibians (Chitramuthu et al., 2010). Likewise, the specific knockdown of zebrafish PCSK9 displays an impairment in the development of the nervous system characterized by the general derangement of cerebellar neurons and a lack of hindbrain–midbrain boundaries, which lead to embryonic death at 96 h postfertilization (Poirier et al., 2006).

Another PCSK that has been shown to have an important role in zebrafish development is PCSK7. The deficiency of PCSK7 in developing larvae generates defects in several organs including the brain, eye and otic vesicle that leads to death at 7 days postfertilization. Interestingly, a study of PCSK7-dependent gene expression demonstrated a role for PCSK7 in the regulation of immune related pathways as well as in the mRNA expression and proteolytic cleavage of TGF-β1 (Turpeinen et al., 2013).

A PCSK8 mutant in zebrafish plays a role in cartilage formation and in processing and activating sterol regulatory element-binding proteins (SREBPs), which are transcription factors that regulate the expression of enzymes involved in lipid biosynthesis and are essential for the steatosis associated with chronic alcohol consumption (Schlombs et al., 2003). Consequently, the PCSK8 mutant in zebrafish has proved useful in modelling alcoholic liver disease (Howarth, Passeri, & Sadler, 2011; Passeri, Cinaroglu, Gao, & Sadler, 2009)

2.5.2 *Mycobacterium tuberculosis*

Tuberculosis (TB) is an airborne infectious disease caused by the *Mycobacterium tuberculosis* bacterium and it is a concerning cause of morbidity and mortality in poor developing countries. In 2015 the WHO reported an estimate of 10 million new TB cases worldwide, 90% of the new cases were adults (56% men, 34% women) and 10% were children (Myllymäki, Bäuerlein, & Rämet, 2016).

Mycobacterium tuberculosis is preferentially a pulmonary pathogen, although it can also affect bones, the central nervous system and several different organs. Consequently, TB can evolve from an asymptomatic infection to a severe disease (Myllymäki et al., 2016).

Patients with TB are classified as carriers of a latent TB infection (LTBI), a state characterized by the absence of symptoms and transmissibility, or as carriers of an active TB disease, which can be transmitted in the case of active pulmonary TB and can be diagnosed using cultures and molecular methods.

Although there is little knowledge concerning the early phase of a *Mycobacterium tuberculosis* infection in humans the use of animal models has helped to decipher the mechanisms involved in the early phase of an infection (Orme, Robinson, & Cooper, 2015).

Mycobacterium tuberculosis invades the respiratory tract after inhalation, then it is phagocytosed by alveolar macrophages and after infecting alveolar macrophages, it accesses the lung tissue where the infection progresses (Frieden, Sterling, Munsiff, Watt, & Dye, 2003). Once *Mycobacterium tuberculosis* accesses the parenchyma numerous pro- and anti-inflammatory signaling cascades are activated leading to the production of IFN-γ by Th1 cells and the consequent recruitment of more macrophages and other leukocytes to the site of infection. This multicellular response is called a granuloma and its main role is probably to localize and control the bacteria as well as restrict the immune response to a defined site (Adams, 1974; Adams, 1976; Ramakrishnan, 2012).

Although *Mycobacterium tuberculosis* can remain asymptomatic in the granuloma for years, an imbalance in the immune system can reactivate the mycobacteria leading to disease progression (Frieden et al., 2003).

Multiple efforts have been made in order to eradicate TB, but there are several drawbacks, such as the limited specificity and sensitivity of the diagnostic methods available, the prolonged antibiotic treatment required for overcoming the disease, the bacterial resistance to drugs and the poor protection provided by the vaccine BCG. Therefore, the development of new drugs and vaccines for the treatment of TB represent an essential and active area of research nowadays and animal models constitute an indispensable tool.

Mice, guinea pigs and non-human primates are animal models commonly used in TB research. Unfortunately, there are many limitations to the use of these models, for instance high costs and ethical requirements (Myllymäki, Niskanen, Oksanen, & Rämet, 2015; Tobin & Ramakrishnan, 2008). Consequently, animal models such as the zebrafish–*M. marinum* have recently become a popular in the study of the pathogenesis of TB.

2.5.3 *M. marinum* infection in zebrafish

Mycobacterium marinum is a ubiquitous pathogen of fish and amphibians that causes mycobacteriosis and it is closely related to *M.* tuberculosis (Stinear et al., 2008). *Mycobacterium marinum* is transmitted via water and can infect humans usually causing "fish tank granuloma" (Linell & Norden, 1954).

The similarities in the pathogenesis of the infection caused by *M. marinum* and *M. tuberculosis* make *M. marinum* a suitable model for the indirect study of TB. Important similarities include that both bacteria have the ability to survive and replicate within macrophages (Barker, George, Falkow, & Small, 1997; Tobin & Ramakrishnan, 2008), they both promote the development of acute and chronic forms of the disease and they generate granulomas (Parikka et al., 2012; Prouty, Correa, Barker, Jagadeeswaran, & Klose, 2003; Swaim et al., 2006). In addition, zebrafish and humans share similar primary components of the innate and adaptive immune system (Renshaw & Trede, 2012; Traver, Herbomel et al., 2003; Traver, Paw et al., 2003). Notably, zebrafish embryos depend only on their innate immunity with functional macrophages and neutrophils generated after 1 and 2 days post-fertilization, respectively. In contrast, lymphocytes develop 4 days post-fertilization and the adaptive immunity fully matures 4 weeks post-fertilization (Langenau et al., 2004). These particularities regarding the maturation of both the innate and adaptive immune systems in zebrafish enable the study of the biology of the components of the immune system (H Meijer & P Spaink, 2011; Lohi, Parikka, & Rämet, 2013).

One of the remarkable advantages of the zebrafish–*M. marinum* model is the formation of granulomas, which has been difficult to reproduce in other animal models (Myllymäki et al., 2015). Moreover, in the adult zebrafish the histology of mature granulomas is similar to those observed in human TB (Ramakrishnan, 2012; Volkman et al., 2010). In addition, the zebrafish–*M. marinum* model overcomes the limited experimental reproducibility observed in animal models in the study of the latent phase of TB (Parikka et al., 2012; Swaim et al., 2006) because akin to humans infected with TB, an *M. marinum* infection in fish shows a latent asymptomatic stagnant disease, where dormant bacteria remain within granulomas (Myllymäki et al., 2015).

Finally, an important aspect, which makes the zebrafish–*M. marinum* an extremely useful model for studying TB, is the observation that the reactivation of

the bacteria takes place spontaneously or can be induced as a result of the development of experimental immunosuppression caused by exposure to γ-irradiation. Both the spontaneous and the inducible reactivation cause the active proliferation of the bacteria leading to the appearance of symptoms and a high rate of lethality resembling a human TB infection (Parikka et al., 2012).

Collectively, the possibility to replicate several features of TB using the zebrafish–*M. marinum* model facilitates the study of the immunological mechanisms involved in the different stages of TB, as well as the search of new drugs, vaccines and potential biomarkers in the diagnosis of the different stages of TB.

Despite the aforementioned advantages of the *zebrafish–M. marinum* model in the study of TB, zebrafish and humans are evolutionarily distant, thereby the utility of the zebrafish system needs to be carefully validated in the context of TB drug discovery programs (Kari et al., 2007).

2.6 Squamous skin cancer

Commonly skin cancers are classified into two groups: melanoma and non-melanoma skin cancers. Non-melanoma skin cancers include cutaneous lymphomas, adnexal tumors, Merkel-cell carcinomas, etc; but the term has been generally used to refer to basal-cell carcinomas (BCC) and squamous carcinomas (SCC) (Madan, Lear, & Szeimies, 2010). Approximately 20% of non-melanoma skin cancers are squamous-cell carcinomas (SCC) (Kwa, Campana, & Moy, 1992).

SCC is the second most common skin cancer in Caucasoids and it is associated with a high risk of metastasis (Alam & Ratner, 2001). An annual incidence of 700000 cases of cutaneous SCC (cSCC) in the United States has been reported. (Karia, Han, & Schmults, 2013; Rogers et al., 2010). Furthermore, cSSC is more frequent in males than in females (Miller & Weinstock, 1994), with a elevated risk of incidence in the ageing population (Diffey & Langtry, 2005).

There are several genotypic, phenotypic, and environmental risk factors associated with the development of cSCC. The risk to develop cSSC increases in the presence of susceptibility markers to ultraviolet radiation such as fair skin, blue eyes, blonde or red hair, inability to tan, sun-associated skin disorders (actinic keratosis, and solar lentigines) (Neale, Davis, Pandeya, Whiteman, & Green, 2007). Exposure to the ultraviolet radiation in sunlight (UVB) is the main factor involved in the

development of this type of cancer and to a lesser extent ultraviolet A radiation (UVA). The direct mutagenic effect of ultraviolet radiation on DNA/RNA and the inability of the organism to repair these mutations may lead to the formation of tumors. In addition, the immunosuppressive effect of the UV radiation could also contribute to the development of cSSC (Ridley, Whiteside, McMillan, & Allinson, 2009; Rünger, 2007).

The clinical presentation of cSSC typically involves the presence of actinic keratosis described as small brownish or pink lesions; a type of carcinoma in situ involving only the epidermis. Some of these lesions can involute, while others can evolve into squamous-cell carcinoma. In addition, other precancerous conditions such as bowenoid papulosis and epidermodysplasia verruciformis may also evolve into cSCC (Alam & Ratner, 2001).

If not treated cSCC in situ can progress and become an invasive disease affecting principally the neck and the head (Alam & Ratner, 2001). Notably, in patients with primary cSSC up to 90% of local tumors can be eradicated with treatments such as electrodesiccation, cryosurgery, etc. However, the prognosis is poor (a 3-year disease-free survival rate of 56% in adult patients), for patients who develop metastases (Alam & Ratner, 2001).

The development of effective therapeutic strategies for the treatment of advanced cSSC relies on the study of the cellular and molecular mechanisms involved in the development of the disease. For this purpose, different mouse models have been used to investigate the mechanisms involved in the initiation and progression of cSSC. Two examples of these models are the induction of squamous cell carcinomas after the local application of UV light and multistage chemical carcinogenesis. In multistage chemical carcinogenesis a DNA-damaging agent 7,12 dimethylbenz[a]anthracene (DMBA) is combined with the phorbol ester 12 O tetradecanoylphorbol 13 acetate (TPA) to generate papillomas that may transform into cSCC, even in immunocompetent mice (Zitvogel, Pitt, Daillère, Smyth, & Kroemer, 2016)(discussed in detail below). This model has helped to study factors involved in skin cancer immunity. For instance, experiments in mice lacking CD4+ T-cells demonstrated a reduction in tumor incidence and number as well as delayed neoplastic progression, while in the CD8+ T-cells KO mice, tumorigenesis was enhanced (Daniel et al., 2003; Yusuf et al., 2008).

Another study using multistage chemical carcinogenesis revealed that elevated VEGF-D expression resulted in a reduction in the Th2 response characterized by the promotion of M1-like macrophages and CD4+T cell subsets (Th1 and Th17) in the

early stage of skin carcinogenesis, which led to an anti-tumoral immune environment and the regression of primary tumors (Honkanen et al., 2016).

After the topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA), mice accumulated granulocytic myeloid-derived suppressor cells (MDSC) in their skin resulting in a dramatic increase in the number of papillomas during epidermal carcinogenesis. This study suggests the accumulation of MDSC as an initial step in facilitating tumor formation, followed by the recruitment of IL-17 producing CD4+ T cells (Ortiz et al., 2015).

The exposure of mouse models to UV radiation has revealed that UV damages the Langerhans cells affecting their ability to present antigens, meaning that the proper induction of effector T cells is impaired. Consequently, a high number of immunosuppressive CTL4+ Tregs are generated causing a shift in the immunological state of the organism from T-cell-mediated immunity to immunosuppression facilitating the development of skin cancer (Rangwala & Tsai, 2011; Schwarz & Beissert, 2013) .

2.6.1 Multistage chemical carcinogenesis model in mouse skin

The multistage chemical carcinogenesis in mouse skin is one of the best *in vivo* models for studying the cascade of events during tumor development. Additionally, this model can be applied to assess strategies for preventing skin cancer as well as to evaluate the role of the genetic background in the initiation, promotion and progression of tumors (Abel, Angel, Kiguchi, & DiGiovanni, 2009).

The first stage of chemically-induced skin carcinogenesis is referred to as the “initiation” phase and it is characterized by the development of mutations in the genes of epidermal keratinocytes as a consequence of exposure to a chemical mutagen such as DMBA (J. M. Ward, Rehm, Devor, Hennings, & Wenk, 1986).

DMBA is usually topically applied and it primarily mutates the gene *Hras1* in keratinocyte stem cells (Ise et al., 2000). This gene belongs to the *Ras* oncogene family, which switches on/off important extracellular signaling cascades that regulate cell proliferation, differentiation and survival (Karnoub & Weinberg, 2008). The mutations in the *Hras1* gene can be observed after 3-4 weeks of a DMBA treatment and detected in the early papillomas that develop after the tumor promotion treatment (Balmain, Ramsden, Bowden, & Smith, 1984; Nelson, Futscher, Kinsella, Wymer, & Bowden, 1992).

The initiation stage is followed by a “promotion” phase where the repeated topical application of a chemical tumor promoting agent, such as TPA, enhances the clonal expansion of the mutated cells (Rundhaug, Fuscher, & Bowden, 1997). During the “promotion” stage the development of persistent epidermal hyperplasia is evident and characterized by thickness of the epidermis and a prevalent augmentation in the number of nucleated cells that have acquired mutations after the “initiation” stage (initiated cells) (Karen et al., 1999; Klein-Szanto, 1989). The result of the “promotion” stage is the development of papillomas (clonal outgrowth of the skin (Kemp, 2005). Promoting agents are structurally and mechanistically diverse and they can promote cell signaling, increase the production of growth factors as well as produce oxidative stress and tissue inflammation (DiGiovanni, 1992). Typical markers during the promotion stage are the augmentation of the epidermal thickness, proliferation of basal keratinocytes, dysregulation of DNA synthesis and the infiltration of inflammatory cells (Kemp, 2005). Notably, papillomas may evolve into invasive squamous cell carcinomas with a frequency that depends on the genetic background of the mice used (Woodworth et al., 2004). Additionally, the dose of the chemical initiator and promoter can determine the papilloma burden (Ewing, Conti, Kruszewski, Slaga, & DiGiovanni, 1988).

During the transformation of a papilloma into SCC, several chromosomal abnormalities are generated independently of the treatment with the promoting agents (Aldaz, Trono, Larcher, Slaga, & Conti, 1989; Ruggeri et al., 1991). In addition, major changes in gene expression occur. Roughly, 15-35% of mice with one or more tumors will show signs of metastasis in organs such as the lungs or lymph nodes (Hennings et al., 1986).

Importantly, the susceptibility to multistage chemical carcinogenesis in mice largely depends on the genetic background of the mice (DiGiovanni, 1992) and it is not caused by the initiating agent, but presumably due to differences in specific genes involved in the response to the treatment with the promoting agent (DiGiovanni & Colburn, 1989).

Although there is no direct human equivalent for papillomas in SCC, and *Hras* is not the primary gene mutated in human nonmelanoma skin cancer, the multistage chemical carcinogenesis model in mice shares several features with multistage carcinogenesis in humans (Benjamin & Ananthaswamy, 2007). For instance, the development of activating mutations in stem cells during the “initiation stage” is similar in mice and solid tumors in humans as well as in the SCC histology (Abel et al., 2009). Furthermore, there are numerous similarities between mouse and human

tumors such as mutations in *ras* family members, activation of the epidermal growth factor receptor stat3 and high expression of TGF-β1 (Kemp, 2005). Additionally, the multistage chemical carcinogenesis model in mice resembles the exposure of humans to several low doses of both carcinogens and promoting agents and the typical latency observed in human cancers also supports the role of a promotional agent in tumor development (Klein, 2005; Pitot & Dragan, 1991; Rundhaug et al., 1997). As a result of these similarities, the two-stage skin carcinogenesis model in mice constitutes an essential tool in the investigation of cancer risk genes, proto-oncogenes, tumor suppressor genes as well as signaling pathways involved in carcinogenesis. Moreover, the multistage chemical carcinogenesis model facilitates the investigation of the immunopathogenesis of cSCC and the evaluation of potential tumor inhibitors.

2.7 Inflammation and cancer

Inflammatory responses are essential at different phases of tumorigenesis including initiation, promotion, malignant convertion, invasion and metastasis. Inflammation also play an important role in immunesurveillance and response to cancer treatment (Grivennikov, Greten, & Karin, 2010). Although inflammation is usually resolved through apoptosis and the clearance of debris and immune cells, almost 20% of human cancers are triggered by chronic inflammation caused by infections (e.g; Helicobacter pylori infections are associated with gastric cancer and gastric mucosal lymphoma), exposure to irritants (e.g; tobacco) or autoimmune disease (e.g;, inflammatory bowel disease is associated with colon cancer) (Kundu & Surh, 2008).

Cancer and inflammation are connected by two pathways called intrinsic and extrinsic pathways (Mantovani, Allavena, Sica, & Balkwill, 2008). The intrinsic pathway is activated by genetic events including oncogene activation by mutation, chromosomal rearrangement or amplifications, as well as the inactivation of tumour-suppressor genes. These genetic events transform the cells into active producers of inflammatory mediators, thereby generating an inflammatory microenvironment in tumors lacking an underlying inflammatory condition (e.g; breast tumors) (Mantovani et al., 2008).

In contrast, the extrinsic pathway, which is activated by inflammatory or infectious conditions, increases the risk of developing cancer at specific anatomical sites such as the colon, liver, prostate and pancreas (for example, infections with hepatitis B or C viruses are major risk factors for hepatocellular carcinoma and infections with *Schistosoma* or *Bacteroides* species are associated with bladder and colon cancer, respectively) (Dolcet, Llobet, Pallares, & Matias-Guiu, 2005; Wu et al., 2009).

The intrinsic and extrinsic pathways converge leading to the expression of key proinflammatory transcription factors, such as NF-κB, STAT3 or HIF1α, within tumor cells,. The activation of the aforementioned trascription factors mediates the production of essential cytokines (e.g TNFα, IL6, IL-17) and chemokines (e.g CCL2) as well as inflammatory enzymes (such as COX-2), forming a rich and complex network of inflammatory responses within the tumor microenvironment (Mantovani et al., 2008).

Host immune cells including macrophages, dendritic, mast cells, NK cells and T cells are recruited by chemokines mediating the immune response within the tumor stroma. Tumor-infiltrating immune cells engage in a two-way molecular conversation (cross-talk) with cancer cells (Grivennikov et al., 2010) coordinating the production of mediators (e.g cytokines, chemokines, prostaglandins) actively involved in the proinflammatory responses (Mantovani et al., 2008). The secretion of cytokines within the tumor microenvironment contributes to sustaining the immune cells, and inflammatory enzymes involved in the synthesis of prostaglandins regulate several physiological processes involved in tumor immunity and inflammation**,** thereby initating the process of tumorigenesis (Crusz & Balkwill, 2015).

The infiltration of immune cells into the tumor can be either antitumoral or pro-tumoral and inhibit a protective immune response (Grivennikov & Karin, 2010). Consequently, the composition of the tumor inflammatory microenvironment is a critical determinant of the disease outcome.

In order to generate an antitumoral immune response some events must be initiated. In the first step, the cancer antigens from tumor cells are captured and processed by antigen presenting cells such as dendritic cells. Next, dendritic cells migrate to the lymph nodes where they present the antigenic peptides through a complex with MHC class I and II molecules for recognition by TCRs of CD8+ T cells and CD4+ T cells, respectively (R. Wang & Wang, 2017). Second, the activation of T cells also requires an interaction between the co-stimulatory surface molecules B7

on APCs and CD28 on T cells. In the absence of co-stimulation, T cells are not fully activated, thereby a T cell tolerance is induced.

Although stimulation with an antigenic peptide initiates T cell activation, the optimal anticancer T cell response is determined by the balance between co-stimulation (e.g CD28:B7.1) and co-inhibitory signaling (e.g CTLA4:B7.1). In addition, the optimal T cell activation also depends on the innate immune signaling-induced cytokines (for example, stimulatory cytokines: TNF-α, IL1, IFN-α; inhibitory cytokines: IL-10, IL-4, IL-13) (L. Chen & Flies, 2013; P. Sharma & Allison, 2015).

Apart from their intrisic regulation, the activation of T cells is also regulated by external factors (extrinsic regulation) including CD4+ regulatory T cells and myeloid-derived suppressor cells, which are potent inhibitors of effector T cells (Beyer & Schultze, 2006; Marvel & Gabrilovich, 2015; M. Wang, Yin, Wang, & Wang, 2014). The aforementioned immunosuppressive cells are usually present in high abundance in tumors and inhibit antitumor immunity, thereby their blockade might be vital for the development of successful immunotherapies.

2.7.1 Role of Macrophages in cancer

Macrophages are a major component of the cell infiltrate in tumors (Noy & Pollard, 2014). Despite the observation that activated macrophages show tumoricidal activity *in vitro* (Fidler, 1989), investigators found that they display a pro-tumoral phenotype *in vivo* at the primary and metastatic sites (Biswas, Allavena, & Mantovani, 2013). These studies suggested that macrophages could engage in a dual complex relationship with cancer (Mantovani, Marchesi, Malesci, Laghi, & Allavena, 2017).

Recent studies have provided evidence for abnormalities in the myeloid compartment in cancer, specifically the inhibition of the differentiation of DCs, the polarization of macrophages toward the M2-like phenotype, and the dramatic proliferation of myeloid-derived suppressor cells (MDSCs) (Gabrilovich, Ostrand-Rosenberg, & Bronte, 2012) . MDSCs constitute a heterogeneous population of pathologically activated myeloid cells (including precursors of neutrophils, macrophages, dendritic cells, and cells at earlier stages of myeloid cell differentiation). These cells are characterized by strong immune-suppressive properties and the capacity to promote tumor angiogenesis, tumor cell invasion, and metastases (Ortiz et al., 2015).

Evidence suggest that macrophages can contribute to the early elimination phase of immunoediting (immunosurveillance) orchestrated by T cells and interferons (Vesely, Kershaw, Schreiber, & Smyth, 2011), in nascent transformed cells. Depending on microenvironmental signals, macrophages polarize to either M1-like (anti-tumoral) or M2-like (pro-tumoral phenotypes), and CD4+ T cells differentiate into several T helper (Th) cell subsets with specific functions in tumor immunity (Sica et al., 2008). Briefly, Th1 subset which produce high levels of IFN-γ display tumor cell cytotoxicity and polarize macrophages towards the M1 phenotype, whereas IL-4 producing Th2 cells induce the alternative M2 activation program and limit antitumor responses (J. Zhu et al., 2009).

Classically activated (M1) macrophages can perform anti-tumoral effector functions through extracellular mechanisms which mediate tissue-destructive reactions including haemorrhagic necrosis (Biswas & Mantovani, 2010; Mantovani, Bottazzi, Colotta, Sozzani, & Ruco, 1992). However, once tumors have been established macrophages become pro-tumoral as a result of the production of certain cytokines and growth factors that promote a Th2 type immune environment (Noy & Pollard, 2014).

Unlike nascent tumors, in progressive tumors, including mouse and human breast or pancreatic cancers, IL-4 and IL-13 cytokines produced by Th2 cells, eosinophils, or basophils induce the alternative activation (M2) of Tumor-associated macrophages (TAMs) (Mantovani et al., 2017). In addition, signals produced by tumor cells (chemokines, CSF-1 and TGFB1), B cells (immune complexes), and stromal cells (IL-1) induce phenotypic shifts in macrophages (Mantovani & Allavena, 2015), which display functions different from those attributed to classic M1/M2 macrophage types. The polarization of TAM towards an immunosuppressive phenotype (M2-like) is a common feature of several cancers, and tumor progression is associated with the skewing and subversion of normal macrophage functions (Mantovani et al., 2017).

TAMs play an essential role as orchestrators of cancer-related inflammation. In addition, TAMs contribute to tumour progression by promoting genetic instability, supporting cancer stem cells and metastasis as well as regulating protective adaptive immunity (Mantovani et al., 2017).

TAMs influence the inherent properties of tumor cells and the tumour microenvironement. For instance, TAMs secrete growth factors such as epidermal growth factors, which induce the proliferation of breast cancer cells (Balkwill, Charles, & Mantovani, 2005; Qian & Pollard, 2010) . In addition, they produce

proteolytic enzymes that degrade components of the extracellular matrix, therby promoting tumor-cell spreading from the primary tumor site and contributing to metastasis. TAMS also promote angiogenesis, lymphoangiogenesis, and tissue remodelling (which support tumorigenesis), additionally they can be potent immunosuppressors of the cytotoxic activity of CD8+ T cells in progressing tumors (Leek et al., 1996; Murdoch, Muthana, Coffelt, & Lewis, 2008). Moreover, the reactive oxygen and nitrogen intermediates produced by TAMs are essential contributors to the genetic instability of cancer cells, thereby impairing the effective application of chemotherapy (Mantovani et al., 2017).

Several anticancer therapies aiming at targeting macrophages are under investigation. These include the inhibition of macrophage recruitment to tumors, the functional modification of the TAM phenotype to an "M1-like" antitumoral phenotype and monoclonal antibodies that promote macrophage-mediated extracelullar killing or phagocytosis, as well as the intracellular destruction of cancer cells (Mantovani et al., 2017).

2.7.2 CD4+ T-cell subsets and tumor immunity

CD4+ T cell have been suggested as targets for cancer immunotherapy due to their ability to orchestrate a variety of immune responses and to differentiate into multiple subsets that can promote and sustain destructive immune responses to self-antigens including tumor antigens. Animal experiments and clinical studies have revealed two effectors of CD4+T cells subsets ($CD4^+$-CTL and Tfh) that display potent antitumoral activity. In contrast, depending on the tumor microenvironment, Th17 and Tregs may function as protumoral effector cells (H. J. Kim & Cantor, 2014).

Previous studies have suggested that both Th1 and Th2 subsets mediate the antitumoral immunity, although Th1 may display a more potent effect due to their ability to produce high levels of IFN-γ and chemokines that stimulate and expand CD8+ T cells (Mattes et al., 2003; Nishimura et al., 1999; Schuler, Qin, Ibe, Noben-Trauth, & Blankenstein, 1999). Th1 cells can induce the eradication of a tumor by targeting the tumor stroma (in an IFN-γ–dependent manner) and by inhibiting angiogenesis (Qin & Blankenstein, 2000) . In addition, CD4+ T cells contribute to the recruiment of natural killer cells (NK) and M1-type of macrophages to tumor sites, which can act in unison toward tumor elimination (Jenner et al., 2009; Palucka & Banchereau, 2012).

In contrast, the contribution of Th2 to antitumoral immunity may depend on the context. Some evidence suggested that the IL-4 cytokine can mediate antitumor effects through the enhancement of eaosinophils, and macrophages infiltrating into the tumor (Tepper, Pattengale, & Leder, 1989). In contrast, the induction of Th2 may promote pancreatic tumor growth and the progressive growth of renal cell carcinoma and melanoma (Ochi et al., 2012; Tatsumi et al., 2002).

The intratumoral production of IL-17 by Th17 can promote angiogenesis and tumor growth through the production of a variety of proangiogenic factors, proinflammatory cytokines and the inhibition of CD8+ T cell infiltration (Langowski et al., 2006; Numasaki et al., 2005; Tartour et al., 1999). Conversely, *in vitro* studies have demonstrated that Th17 cells that are specific to a tumor antigen display superior antitumor activity compared with Th1 cells in a model of murine B16 melanoma (Martin-Orozco et al., 2009; Muranski et al., 2008).

CD4+ T cells that display cytolytic activity have an important antitumoral effector function. The tranference of tumor-reactive CD4+ T cells into lymphopenic hosts in concert with radiation and an anti-CTLA-4 antibody treatment increase the expression of IFN-γ and granzyme B resulting in the regression of tumors (Quezada et al., 2010)

Recent reports on colorectal and breast cancer have suggested that tumor-infiltrating Tfh play an important role in recruiting immune cells to the tumor as well as in the formation of intratumoral follicular structures, which are associated with a positive prognosis. Although the strict control of Tfh is vital in preventing the development of autoimmune disease, Tfh expansion promotes the production of effector cytokines and tumor-specific antibodies (D. Coppola et al., 2011; Gu-Trantien et al., 2013).

The frequency of Foxp3+ Treg in the tumor environment increases compared to peripheral lymphoid tissues due to their recruitment to the tumor bed by tumor-derived factors (Quezada, Peggs, Curran, & Allison, 2006).

Intratumoral Foxp3+ Tregs are highly proliferative and suppress the effective immunity against cancer. In addition, the limited availability of intratumor IL-2 facilitates the expansion of Treg over Teffs and the tumor microenvironment promotes Treg activation, thereby inhibiting the antitumoral response (Pandiyan, Zheng, Ishihara, Reed, & Lenardo, 2007; Predina et al., 2013; Preston et al., 2013; Sisirak et al., 2012)

2.7.3 CD8+ T cells in tumor immunity:

Several studies performed in patients with breast, lung, melanoma, colorectal, and brain cancer have revealed a positive correlation between the frequency of tumor-infiltrating CD8+ Tcells and cancer-free survival (Kmiecik et al., 2013; Mahmoud et al., 2011). Although it is challenging to define CD8+ T cells phenotypes due to the lack of specific biomarkers and differences between cancer types, six subsets of CD8+ T cell fates have been described in cancer patients and experimental models (effector T cells (Teff), exhausted T cells, anergic T cells, regulatory T cells and senescent T cells (Baitsch et al., 2011). This chapter will briefly introduce effector CD8+ T cells.

During antitumoral responses the potent activation of CD8+ T cell occurs in tumor-draining lymph nodes and directly in the tumor . Differentiated effector CD8+ T cells are IL-2 dependent and highly cytotoxic, expressing high levels of IFN-γ, TNF-α, perforin, and granzymes after activation (Pipkin et al., 2010). Tumor-antigen-specific effector CD8+ T cells that efficiently infiltrate primary tumors are called tumor-infiltrating CD8+ T cells. Tumor-infiltrating CD8+ T cells are mainly effector CD8+ T cells, which are able to recognize and sucessfully eliminate tumor cells *in vitro* and *in vivo* (this reponse varies depending on the tumor types) (Baitsch, Fuertes-Marraco, Legat, Meyer, & Speiser, 2012). However, the chronic activation (e.g uncontrolled tumor load) of CD8+T cells can induce phenotypic changes in these cells that promote functional deficiencies, ultimately leading to immune escape (Klebanoff, Gattinoni, & Restifo, 2006).

3 AIMS OF THE STUDY

One of the research lines followed in this study is the pharmacological inhibition of proprotein convertase enzymes that have shown to have a regulatory function in several pathologies. *In vitro* studies have suggested that FURIN can cleave several proteins known to be important for T cell biology and several other proteins important for the immune system and cancer development. Moreover, by proteolytically activating viral proteins and bacterial toxins FURIN/PCSK enzymes serve as virulence factors for many important pathogens. Therefore, not surprisingly, FURIN inhibitors have been suggested to be beneficial for the treatment of a wide variety of diseases. Our previous studies indicate that FURIN has an important role in regulating T cells, and thus inhibiting it may have consequences in the immune system, which may be either detrimental or beneficial when FURIN inhibitors are used as therapeutic drugs. Moreover, although recent findings demonstrate that FURIN is highly expressed in macrophages, the consequences of FURIN inhibition in the cells of the innate immune system *in vivo* have remained ambiguous. The objectives of this study are to assess the role of FURIN in the biology of the innate immune cells as well as to investigate the impact of FURIN activity in the regulation of the immune system during pathological conditions.

The main aims of the study are:

1- To characterize a novel tissue-specific knockout mouse model, in which FURIN is deleted in macrophages and granulocytes (LysMcre-*fur*$^{(fl/fl)}$).

2- To study the role of FURIN as an effector in the immune cells of *Mycobacterium marinum* –infected zebrafish.

3- To investigate the role of immune-cells expressed FURIN in a multistage skin tumor model using T cell specific FURIN KO and Myeloid cell specific FURIN KO mice strains.

4 MATERIALS AND METHODS

4.1 Experimental animals (I, II,III)

Table 3. General information about the animal models used in studies I, II, III

Animal model	Name of the strain	Type of knockout	Gene knockout
C57BL/6 mice	LysMcre-*fur*$^{(fl/fl)}$	Cell-specific (myeloid cells)	*furin*
C57BL/6 mice	CD4cre-fur$^{(f/f)}$	Cell-specific (T cells)	*furin*
Zebrafish in AB background	zebrafish *furin*A^{td204e}	whole-body	*furinA*

Mice were housed under pathogen-free standard conditions and fed with standard laboratory pellets and water ad libitum.

For further details please consult original publications I, II, III.

4.2 *In vivo* models (I, II, III)

Table 4. General information about the *in vivo* models used in studies I, II, III

Strain	*In vivo* model	Stimulus and route of application
LysMcre-*fur*$^{(fl/fl)}$	LPS-induced endotoxemia (I)	Intraperitoneal injection of LPS (25 mg/kg, *E.Coli* 0111:B4 serotype)
	Skin tumor induction (III)	DMBA (50ug/200 µl acetone) one topical application on the shaved area of the dorsal skin TPA (5 ug/200 µl acetone) topically applied to the back skin of the mice twice a week
CD4cre-fur$^{(f/f)}$	Skin tumor induction (III)	DMBA (50ug/200 ml acetone) one topical application on the shaved area of the dorsal skin TPA (5 ug/200 ml acetone) topically applied to the back skin of the mice twice a week
zebrafish	*M. marinum* infection (II)	1. Intraperitoneal injection of several concentrations of *M. marinum* (adult zebrafish *furin*A^{td204e})
		2. *furinA* and *furinB* MOs were simultaneously coinjected with M. marinum into the yolk sac (developing zebrafish embryos)

For further details please consult original publications I-III.

4.3 Histology (II, III)

In study II, the presence of *M. marinum* in infected adult zebrafish was detected using a histological analysis and Ziehl-Neelsen staining (K. E. Oksanen et al., 2013; Parikka et al., 2012). In order to exclude any background signal uninfected adult zebrafish were included as controls.

Sample sections were observed with an Olympus BX51 microscope and Olympus ColorView IIIu camera (×100 magnification) or with an Objective Imaging Surveyor virtual slide scanner (Objective Imaging, Cambridge, United Kingdom). The scanned sample sections were converted into digital format at a resolution of 0.4 µm per pixel using a 20× Plan Apochromatic microscope objective, and the images obtained were converted to JPEG2000 format (Tuominen & Isola, 2009).

In study III, shaved control mice or DMBA/TPA treated mice (43 hours or 17 weeks of treatment) were euthanized and skin samples from the back were collected, fixed with 4% paraformaldehyde and embedded in paraffin in accordance to standard protocols. Next, Hematoxylin/eosin and DAB were used to stain 6 µm thick paraffin sections as previously described (May et al., 2015). The primary antibodies used for immunohistochemical staining were: A0452 rabbit anti-CD3 (DakoCytomation, Glostrup, Denmark), MF48000 BM8 rat anti-F4/80 (Life Technologies Ltd., Paisley, UK) and 68672 rabbit anti-neutrophil elastase (AbCam, Cambridge, UK).

4.4 *In vitro* Experiments

4.4.1 Isolation, culture and ex vivo cell activation (I, II, III)

In study I, peritoneal cells from LysMcre-fur$^{(fl/fl)}$ and wild-type mice were extracted from the peritoneal cavity and cultured following a previously published protocol (Taverniti et al., 2013). After a 1 h incubation, WT and FURIN deficient peritoneal macrophages were stimulated with TLR ligands and/or cytokines as indicated in Table 5. After stimulation, the cells were collected for RNA extraction (RNAeasy, Qiagen, Düsseldorf, Germany). Neutrophils were isolated from the bone marrow of LysMcre-fur$^{(fl/fl)}$ and wild-type mice using the Anti-Ly-6G MicroBead Kit

(Miltenyi Biotec Norden AB, Lund, SE) and they were stimulated with 100 µM of fMLP for 1 hour at 37 °C. Unstimulated peritoneal macrophages and neutrophils were used as controls in the experiments.

In study II, for the flow cytometry analysis, zebrafish were sacrificed in a 0.04% 3-aminobenzoic acid ethyl ester anesthetic (pH 7.0; Sigma-Aldrich, Missouri, USA) then the kidneys were dissected and homogenized into a single-cell suspension of phosphate-buffered saline with 0.5% fetal bovine serum (Gibco/Invitrogen, California, USA). Bone marrows from LysMcre-fur$^{(fl/fl)}$ and wild-type mice were extracted and cultured until macrophage differentiation following a previously published protocol (Taverniti et al., 2013). After differentiation, WT and FURIN deficient bone marrow macrophages were stimulated for four hours with LPS as indicated in Table 5.

In study III skin draining lymph node cells from mice treated with DMBA/TPA for 17 weeks were stimulated with a plate-bound anti-CD3 antibody (10 µg/ml, clone 17A2, eBioscience) and soluble anti-CD28 Ab (2 µg/ml, clone 37.51, eBioscience) for 48 hours. After stimulation, the cells were used for *in vitro* analyses. In the case of the intracellular flow cytometry analysis, the cells were isolated from the skin draining lymph nodes of mice treated with DMBA/TPA for 48h and stimulated with PMA and Ca-ionomycin for 4 hours. Brefeldin A and Monensin were added for the last two hours of incubation.

Table 5. Stimuli applied to peritoneal macrophages and bone marrow macrophages in studies I, II

Stimulus	Concentration	Source and ligand type	Manufacturer
LPS	1 µg/ml	*E.coli* 0127:B8 serotype (TLR ligand)	Sigma-Aldrich, St Louis, MO, USA
Zymosan	10 µg/ml	*S.cerevisiae* (TLR ligand)	InvivoGen, San Diego, CA, USA
R848	1 µg/ml	Low molecular weight synthetic molecule (TLR ligand)	InvivoGen, San Diego, CA, USA
IFN-γ	20 ng/ml	*E.coli* (cytokine)	PeproTech, Rocky Hill, NJ, USA
IL-4	50 ng/ml	*E.coli* (cytokine)	PeproTech, Rocky Hill, NJ, USA

4.4.2 Flow cytometric analyses (I, II, III)

In study I peritoneal, splenic and bone marrow cells were stained for cell surface markers with antibodies against mouse F4/80, Gr-1, CD11b, B220 , CD3, CD4 , CD8, CD62L, Ly-6G, Ly-6C and CD11c (Table 6).

In manuscript II the relative percentages of hematopoietic stem cells, erythrocytes, granulocytes, and lymphocytes from kidney cell suspensions were analyzed using flow cytometry in uninfected *furinA*$^{td204e/+}$ mutants and WT controls. The hematopoietic cell populations were distinguished based on granularity (side scatter [SSC]) and particle size (forward scatter [FSC]) (Langenau et al., 2004). Flow cytometric sorting with a FACSAria I apparatus (Becton Dickinson) was used to purify granulocytes and lymphocytes from WT AB zebrafish kidneys in order to perform *furinA* expression analyses.

In study III, for surface staining, the skin draining lymph node (dLN) cells were stained for cell surface markers with antibodies against mouse CD4, CD8, CD44, CD62L and CD69 (Table 6). For intracellular staining cultured cells were stained with surface markers and subsequently fixed overnight with Fixation/Permeabilization

solution (from Foxp3/Transcription Factor Staining Buffer Set, eBioscience), permeabilized with Permeabilization Buffer (eBioscience) and stained with intracellular antibodies IL-10, IFNγ, IL-17A, Foxp3 (Table 6) according to the manufacturer's instructions .

In all of the studies the flow cytometry analyses were performed using a BD FACSCanto™ II II system (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) and the data were analyzed with the FlowJo software (Flowjo LLC, Ashland, Oregon, USA).

Table 6. Antibodies used for flow cytometry (I, III)

Name	Clone	Provider	Used in study
anti-CD16/CD32	93	eBioscience	I, III
anti- F4/80	BM8	eBioscience	I
anti-Gr-1	RB6-8C5	eBioscience	I
anti-Ly-6G	RB6-8C5	eBioscience	I
anti-Ly-6C	HK1.4	eBioscience	I
anti-CD11c	N418	eBioscience	I
anti-CD11b	M1/70	eBioscience	I
anti-B220	RA3-6B2	eBioscience	I
anti-CD3	145-C11	eBioscience	I
anti-CD4	RM4-5	eBioscience	I, III
anti-CD8	53-6.7	eBioscience	I, III
anti-CD62L	MEL-14	eBioscience	I, III
anti-CD44	IM7	eBioscience	III
anti-CD69	H1.2F3	eBioscience	III
anti-Foxp3	FJK-16s	eBioscience	III
anti-IL-10	JES5-16E3	eBioscience	III
anti-IFNγ	XMG1.2	eBioscience	III
anti-IL17A	eBio17B7	eBioscience	III

4.4.3 Cytokine measurements (I, III):

Table 7 : General information related to cytokine measurements in study I

Study I	
Strain	LysMcre-fur$^{(fl/fl)}$ and WT mice (6-8 weeks of age)
Stimulus	LPS (*E.Coli 111:B4 serotype*)
	Steady state (non stimulation)
Dose	100 µg/kg (LPS)
Route of inoculation	Intraperitoneal
Biological samples collected	Serum from steady state and LPS injected mice
	Peritoneal cells from steady state mice
Time of collection	0, 1h and 3 h (LPS injected mice)
Measurements	Quantification of serum cytokines TNF-α, IL-6, MCP-1 and IL-10 (Steady state and LPS injected mice)
	Levels of serum cytokine IL-1β in steady state mice
	Quantification of bioactive TGF-β1 from the supernatant of non-stimulated peritoneal macrophages
Method of measurement and manufacturer	BD™Cytometry Bead Array Mouse Inflammation Kit (BD Biosciences, Franklin Lakes, NJ, USA)
	Mouse IL-1 beta ELISA Ready-SET-Go!® (eBioscience, San Diego, CA, USA)
	Human/Mouse TGF beta 1 ELISA Ready-SET-Go! (2nd Generation) (eBioscience, San Diego, CA, USA).

For further details about cytokine measurements in publication I please consult the original publication.

For publication III, a Multiplex cytokine/chemokine measurement was performed in the culture supernatants of stimulated skin lymph node cells from mice treated with DMBA/TPA for 17 weeks (see subheading 4.4) using the ProCartaPlex assay (Mouse Cytokine & Chemokine 26-plex, eBioscience) according to the manufacturer's instructions, and with a Bio-Plex 200 instrument (Bio-Rad). In addition, IFN-γ levels were determined from the cell culture supernatants with a Ready-Set-Go! ELISA kit (eBioscience), according to the manufacturer's instructions.

4.4.4 Transcriptome analysis

4.4.4.1 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) (I, II, III):

qRT-PCR was performed in all of the studies to determine the expression of different genes. For further details concerning the cell types and methods used see Tables 8 and 9. The primer sequences for the three studies are described in Tables 10, 11 and 12.

Table 8. qRT-PCR method used in the studies I, II

Study I (mice)	
Cell types used	Stimulated and non-stimulated peritoneal macrophages
RNA Extraction	RNeasy kit (Qiagen, Düsseldorf, Germany)
RNA quantification	NanoDrop ND-2000 (Thermo Fisher Scientific, Massachusetts, USA)
Reverse Transcription	iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA)
qRT-PCR machine, software and mix	Bio-Rad CFX96 Real-Time System, Bio-Rad CFX Manager software v1.6 (Bio-Rad) and Sso Fast Eva Green Supermix (Bio-Rad, Hercules, CA, USA)
Housekeeping gene	*18s*
Calculations (I, II, III)	The normalized threshold cycle (Ct) values were subtracted from the target Ct values of each sample (deltaCt). Relative levels of target mRNA were calculated as 2-deltadeltaCt.
Study II (zebrafish)	
Cell types used	cells from kidneys, lymphocytes, granulocytes and homogenates of organs in the abdominal cavity
RNA and/or DNA extraction.	RNeasy kit (Qiagen, Düsseldorf, Germany) or RNA-DNA coextraction method for TRIreagent (Molecular Research Center, Ohio, USA. Total DNA was isolated simultaneously with the RNA to quantify the *M. marinum* load in the fish.
RNA quantification	NanoDrop ND-2000 (Thermo Fisher Scientific, Massachusetts, USA)
Reverse Transcription	iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA)
qRT-PCR machine and mix	Bio-Rad CFX96 Real-Time System, Bio-Rad CFX Manager software v1.6 (Bio-Rad, Hercules, CA, USA) and Maxima SYBR green qPCR master mix (Fermentas, Burlington,Canada)
Housekeeping gene	*eef1a1l1* (*ef1a*)

Table 9. qRT-PCR method used in study III

Study III (mouse)	
Cell types used	Samples form mice back skin were harvested into RNAlater RNA stabilization Reagent (Qiagen, Hilden,Germany)
RNA Extraction	Invitrogen Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA)
RNA quantification	NanoDrop ND-2000 (Thermo Fisher Scientific, Massachusetts, USA)
Reverse Transcription	Thermo Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific)
qRT-PCR machine, software and mix	7500 Real-Time PCR System, LinRegPCR program and Thermo Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Fisher Scientific)
Housekeeping genes	For *ifng* and *il17a* the reference genes used were *hprt* and *tbp* and for *furin 18S* was used as a reference gene.

Table 10. Primers used in qRT-PCR analyses (F: forward R: Reverse)(I)

Study I (mouse)	Sequences 5´-3´
il1b	F 5'-CGT GGA CCT TCC AGG ATG AG-3'
	R 5'-CAT CTC GGA GCC TGT AGT GC-3'
Tnfa	F 5'-CTT CTG TCT ACT GAA CTT CGG G-3'
	R 5'-CAG GCT TGT CAC TCG AAT TTT G-3'
il6	F 5'- TGT GCA ATG GCA ATT CTG AT- 3'
	R 5'- CTC TGA AGG ACT CTG GCT TTG- 3'
nos2	F 5'-GGG CAG TGG AGA GAT TTT GC-3'
	R 5'-CCA GAG GGG TAG GCT TGT CT-3'
il10	F 5'-GCC CAG AAA TCA AGG AGC AT-3'
	R 5'-TGT AGA CAC CTT GGT CTT GGA G-3'
arg1	F 5'-AAG AAT GGA AGA GTC AGT GTG G-3'
	R 5'-GGG AGT GTT GAT GTC AGT GTG-3'
pcsk1	F 5'-GTA CAC ATC CTA CAA TAC AGT CCA G-3'
	R 5'-TCC CTT CTA CCC TCC ACA TT-3'
pcsk2	F 5'-AGG TGT GCA GGA GAA GTT TC-3'
	R 5'-GTC TGT CAT AAA GGG CTG GTC-3'
Furin	F 5'-CAG AAG CAT GGC TTC CAC AAC-3'
	R 5'-TGT CAC TGC TCT GTG CCA GAA-3'
pcsk4	F 5'-TCT TGG ACG ATG GCA TTG AG-3'
	R 5'-TTC CAT GTC GGT TCT CAT CG-3'
pcsk5	F 5'-CGC TTT CAA CGC CAA GAT TG-3'
	R 5'-AGT CTT GCC ATC GTC ATC TG-3'
pcsk6	F 5'-CTA AAC AAG CTT TCG AGT ATG GC-3'
	R 5'-TGG TGT AGA TGC TGT TGG TG-3'
pcsk7	F 5'-TTC TGT GCA GTG GGT GTG-3'
	R 5'-CTG TCA GTA AGT GGT CCA TCC-3'
tgfb1	F 5'-CCTGAGTGGCTGTCTTTTGA-3'
	R 5'-CGTGGAGTTTGTTATCTTTGCTG-3'
18s	F 5'-GTG ATC CCT GAG AAG TTC CAG-3'
	R 5'-TCG ATG TCT GCT TTC CTC AAC-3'

Table 11. Primers used in qRT-PCR analyses (F: forward R: Reverse)(II)

Study II (zebrafish)	Sequences
furinA	F 5'- CCAAAGAGGCTTTCCAACGC-3'
	R 5'- CGTACTGCTGCTGATGGACAG-3'
pcsk1	F 5'-CGGGAAAAGGAGTGGTCAT-3'
	R 5'-GGTGGAGTCGTATCTGGG-3'
pcsk2	F 5'- CGGATCTGTATGGAAACTGC- 3'
	R 5'- GCCGGACTGTATTTTATGAAT- 3'
furinB	F 5'-CCAAGGCATCTACATCAACAC-3'
	R 5'-ACACCTCTGTGCTGGAAA-3'
pcsk5a	F 5'-GGAGTTTCAATGACCCCAA-3'
	R 5'-ACCACAACTCCTTTCCCA-3'
pcsk5b	F 5'-TGTTCCTCGACCCTTACCAC-3'
	R 5'-ATCTCGCCATGTCAGGAAAG-3'
pcsk7	F 5'-AGAGTGTTGGACGGG-3'
	R 5'-TGCCTAATGGATGCGGT-3'
cd247 (cd3zeta)	F 5'-CATCACCGGCTTCTTTGTGC-3'
	R 5'-CCCCAGTTTATCAATGGCCTGA-3'
tbx21(T-bet)	F 5'-GGCCTACCAGAATGCAGACA-3'
	R 5'-GGTGCGTACAGCGTGTCATA-3'
gata3	F 5'-GGATGGCACCGGTCACTATT-3'
	R 5'-CAGCAGACAGCCTCCGTTT-3'
foxp3a	F 5'-CAAAAGCAGAGTGCCAGTGG-3'
	R 5'-CGCATAAGCACCGATTCTGC-3'
rorca	F 5'-GAAGGCTGCAAGGGCTTCTT-3'
	R 5'-TGCAGTTCCTCTGCCTTGAG-3'
tnfa	F 5'-GGGCAATCAACAAGATGGAAG-3'
	R 5'-GCAGCTGATGTGCAAAGACAC-3'
il1b	F 5'-GTTCACTTCACGCTCTTGGATG3'
	R 5'-CGTGGAGTTTGTTATCTTTGCTG-3'
Lta	F 5'-CCACAGTTCAGCAGGACCTC-3'
	R 5'-TTTCCTGCGTGCTCTCATGTC-3'

Table 11 (cont). Primers used in qRT-PCR analyses (F: forward R: Reverse)(II)

Study II (zebrafish)	Sequences
ifng1-1	F 5'- AAATGGTGCTACTCTGTGGAC-3'
	R 5'- TTCCAACCCAATCCTTTG-3'
il22 (ifnphi6)	F 5'-TCAGACGAGCACACAGATATG-3'
ZDB	R 5'-GATGGCTGGAGTAGTCGTGG-3'
il17a/f3	F 5'- GGCTCTCACGGGTTTTCAG- 3'
	R 5'- ACACTTCTTCACACCAGAACATC- 3'
il10	F 5'-GCTCTGCTCACGCTTCTTC-3'
	R 5'-TGGTTCCAAGTCATCGTTG-3'
tgfb1a	F 5'-TCGTCTTCCAGCAAGCTCAG-3'
	R 5'-TTGGAGACAAAGCGAGTTCC-3'
eef1a1l1 (ef1a)	F 5'-CTGGAGGCCAGCTCAAACAT-3'
	R 5'-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3'
Mmits	F 5'-CACCACGAGAAACACTCCAA-3'
	R 5'-ACATCCCGAAACCAACAGAG-3'

Table 12. Primers used for qRT-PCR (F: forward R: Reverse) (III)

Study II (mouse)	Sequences 5´-3´
il17a	F 5'-GACTCTCCACCGCAATGAA-3'
	R 5'-GACCAGGATCTCTTGCTGGA-3'
Ifng	F 5'-CCAAGTTTGAGGTCAACAACC-3'
	R 5'-GCTTCCTGAGGCTGGATTC-3'
furin	F 5'-CAG AAG CAT GGC TTC CAC AAC-3'
	R 5'-TGT CAC TGC TCT GTG CCA GAA-3'
hprt	F 5'-AGGGATTTGAATCACGTTTGTGT-3'
	R 5'-GGCCACAGGACTAGAACACC-3'
Tbp	F 5'-CCCACCAGCAGTTCAGTAGC-3'
	R 5'-TCTGCTCTAACTTTAGCACCTGTT-3
18s	F 5'-GTG ATC CCT GAG AAG TTC CAG-3'
	R 5'-TCG ATG TCT GCT TTC CTC AAC-3'

4.4.4.2 Microarray data analysis (I)

FURIN KO and WT peritoneal macrophages were left unstimulated or were stimulated with LPS (1 µg/ml)(*E. Coli* 0127:B8 serotype; Sigma Aldrich, St Louis, MO, USA) different time points (1, 4 and 24 hours)(two biological replicates). (for details about RNA isolation and purification please consult publication I)

300ng of a Cy-3 and 300ng of a Cy-5 labeled sample were hybridized together on an Agilent Mouse Chip 8x60K (Design ID 028005) (Agilent Technologies, California, USA) overnight at 65°C using the Gene Expression Hybridization kit. The chips were washed with the Gene Expression Wash Pack according to the instructions. Afterwards the chips were scanned using an Agilent Technologies Scanner model G2565CA with the scan profile AgilentG3_GX_2Color. The scanned results were converted into numerical data by the Agilent Feature Extraction software version 10.7.3.

The analysis of the data was performed using the R software (Team, 2013). Next, the raw probe level intensity values were normalized with loess regression and

quantile normalization using R package “limma” to robustly generate sample-wise comparability (Ritchie et al., 2015).

Differential gene expression was assessed using the log2-transformed fold change difference and statistically analyzed applying the two-sample Student’s t-test. Genes with an average expression lower than the log2 transformed intensity value 6 were discarded as non-informative. In order to obtain a gene set involved in Toll-like receptor signaling we used The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa & Goto, 2000).

4.4.5 Western blot analysis (I)

To detect Caspase-1 bone marrow macrophages were cultured according to the standard protocol [63). Next, bone marrow macrophages were kept unstimulated or were stimulated for 6 hours with ultrapure LPS *(E.Coli* 0111:B4, 500 ng/ml; InvivoGen, San Diego, CA, USA). After the LPS stimulation, the macrophages were washed twice with PBS and incubated with ATP (1mM, Sigma Aldrich, St Louis, MO, USA). Finally, the cells were dislodged and collected in a lysis buffer specific for Caspase-1 (50 mM Tris, pH: 7. 4, 150 mM NaCl, 2 mM Ethylenediaminetetraacetic acid (EDTA) pH: 8.2; 2mM Ethyleneglycoltetraacetic acid (EGTA) pH:7.5, 10% Glycerol, 1% Triton X-100, 50 mM Sodium fluoride, 200 µM Sodium vanadate) (Netea et al., 2009).

For the TACE experiment, peritoneal macrophages were left at steady state or were stimulated with LPS for 5-30 minutes (1 µg/ml, *E.Coli* 0127:B8 serotype; Sigma Aldrich, St Louis, MO, USA). Afterwards, the cells were dislodged and collected in a lysis buffer specific for TACE (1% Triton X-100, 15 mM NaCl, 50 mM Tris HCl pH: 7.4, protease inhibitors cocktail (Roche), 10mM 1,10-phenanthroline) (McIlwain et al., 2012).

Equal amount of proteins were loaded onto 12% polyacrylamide gels. Next, the separated proteins were immobilized onto nitrocellulose membranes (Whatman, GE Healthcare, Pollards Wood, UK). The detection of the proteins was performed using primary antibodies, either anti-ADAM17-cytoplasmic domain (ab39162; abcam) or anti-caspase-1-t/ICE (AHZ0082; Invitrogen), followed by a HRP-conjugated secondary antibody (R&D system). The loading control used was Anti-actin (MAB1501R; Merck Millipore, Billerica, MA, USA) and the blots were visualized

with an ECL detection system (GE Healthcare, Pollards Woods, UK). The signal intensities of the samples were analyzed using the NIH ImageJ software.

4.5 Statistical analyses (I, II, III)

In publication I, III statistical analyses were performed using the two-tailed unpaired Student's t-test. P values of <0.05 indicate statistical significance (I, II, III). In publication I the variability was represented by the standard error of the mean (SEM) and the Kaplan Meier test was used to estimate survival in the *in vivo* model of endotoxemia.

In publication II, III a log-rank (Mantel-Cox) test was applied in the analysis of the survival experiments. A nonparametric Mann-Whitney analysis was used in flow cytometry and qRT-PCR zebrafish experiments (II).

In study III mean averages are shown with 95% confidence intervals except Figure 1B where SEM was used. The normal distribution of the immunohistochemistry data was determined using the D'Agostino & Pearson omnibus and Shapiro-Wilk normality tests (III). Non-normally distributed time course experimental data were analyzed by non-linear regression (III).

Statistical analyses were performed with the Prism v5.02 and 6 programs (GraphPad Software, Inc., California, USA). (I, II, III). In publication III the STATA 13.0 (StataCorp LP, College Station, Texas, USA) statistical analysis software was used for the non-linear negative binomial regression analysis.

4.6 Ethical aspects (I, II, III)

All of the experiments in animal models were performed in accordance with the guidelines of the National Animal Experiment Board, Finland.

Permits for experiment in mice (I, III): ESAVI/2837/04.10.07/2015

Permits for experiment in zebrafish (II): LSLH-2007-7254/Ym-23, ESAVI/6407/04.10.03/2012,ESAVI/733/04.10.07/2013, ESAVI/2267/04.10.03/2012, and ESAVI/8125/04.10.07/2013).

5 SUMMARY OF THE RESULTS

5.1 The proprotein convertase FURIN expressed in myeloid cells regulates inflammation (I)

Previous reports have described an increased expression of FURIN in human atherosclerotic plaques and its upregulation upon the LPS activation of human CD14+ monocytes (Turpeinen et al., 2011) . Furthermore, FURIN is expressed in human monocytes and granulocytes (Stawowy et al., 2005) and elevated levels of FURIN have been detected in the serum of patients infected with salmonella (Kumar et al., 2014). However, the consequences of FURIN inhibition in the components of the innate immunity remain unknown.

In order to assess the contribution of FURIN to immune responses mediated by myeloid cells we characterized a conditional knockout mouse model in which FURIN is deleted specifically in macrophages and granulocytes (LysMcre-*fur*$^{(fl/fl)}$).

5.1.1 Under steady state conditions the LysMcre-fur$^{(fl/fl)}$ mice phenotype is characterized by high levels of serum IL-1β and reduced numbers of splenocytes

Previous studies have demonstrated a regulatory role for PCSK1 and PCSK7 in innate cytokine secretion (Refaie et al., 2012) and antigen presentation (Leonhardt et al., 2010) , respectively. In addition, FURIN is upregulated in LPS activated CD14+ cells (Turpeinen et al., 2011), and in the plasma of chronic typhoid carriers (Kumar et al., 2014). To address whether FURIN, when expressed in myeloids, regulates the innate immunity *in vivo* we generated a conditional knockout mouse model in which *furin* is specifically deleted in Lysozyme M positive cells. LysMcre-fur$^{(fl/fl)}$ mice developed normally and unlike T cell FURIN KO mice (Pesu et al., 2008) LysMcre-fur $^{fl/f}$ mice did not manifest with age-related health problems such as autoimmunity and inflammatory bowel disease (I, supplementary Fig S2A)**.** Interestingly, unlike the large spleens observed in the PCSK1 KO model mice (Refaie et al., 2012) LysMcre-

fur$^{(fl/fl)}$ animals had significantly reduced numbers of splenocytes compared to WT littermates (I, Figure 1A).

Predictably, the expression of *furin* was practically abrogated (>95% less mRNA) from the myeloid cells (neutrophils/macrophages) of LysMcre-*fur*$^{(fl/fl)}$ mice (I, supplementary Figure S2B, C). A flow cytometric analysis of the peritoneal cavity (I, Figure 1B), spleen (I, supplementary Figure S3A) and bone marrow (I, supplementary Figure S4A) revealed that the FURIN deficiency did not significantly affect the proportion of macrophages (F4/80+CD11b+) and neutrophils (Gr1+CD11b+). Next, we investigated whether the FURIN deficiency in myeloid cells generated secondary effects in lymphoid cells, and we observed that the proportions of splenic CD3+ T and B220+ B cells were unaltered (I, supplementary Figure S3B). Nevertheless, there was a significant reduction in the percentages of splenic CD3+CD4+CD8- T helper cells, while the percentage of splenic CD3+CD4-CD8+ cytotoxic T cells was elevated in the LysMcre-*fur*$^{(fl/fl)}$ mice (I, supplementary Figure S3C).

Previously, we demonstrated that the lack of FURIN in T cells causes he upregulation of Th1 and Th2 cytokines in serum (Pesu et al., 2008). To study whether the expression of FURIN in myeloid cells regulates the production of innate cytokines under steady state conditions in LysMcre-*fur*$^{(fl/fl)}$ and WT mice, we determined the levels of pro-inflammatory IL-1β, TNF-α, IL-6 and MCP-1 cytokines as well as the anti-inflammatory IL-10 in serum (I, Figure 1C). The data obtained demonstrated that the lack of FURIN in myeloid cells increases the production of pro-inflammatory IL-1β, which suggests an autoinflammatory phenotype (Goldbach-Mansky et al., 2006; Masters, Simon, Aksentijevich, & Kastner, 2009).

5.1.2 The expression of pro-inflammatory genes is upregulated in FURIN deficient macrophages

The deletion of PCSK genes in *in vitro* and *in vivo* experiments has been shown to affect gene expression in specific cell types. These studies have contributed to the uncovering of some of the effector functions of PCSKs (Ortutay, Oksanen, Aittomaki, Ortutay, & Pesu, 2015; Pesu et al., 2006; Turpeinen et al., 2013). In order to assess the role of FURIN in the regulation of the genetic signature in macrophages we performed a genome-wide RNA microarray and a qRT-PCR analysis. Similarly to human CD14+ monocytes (Turpeinen et al., 2011) FURIN was upregulated following

the *in vitro* activation of mouse peritoneal macrophages with LPS+/-IFN-γ (I, supplementary Figure S2B). Unlike to the functional compensation that different PCSKs caused in FURIN deficient endothelial cells (Tie2Cre) (W. Kim et al., 2012) we observed that the deletion of FURIN in macrophages had no effect on the expression of other PCSK enzymes (I, supplementary Figure S2D).

In addition, we studied the anti-inflammatory role of FURIN in bone marrow-derived macrophages. Briefly, the bone marrows from WT and LysMcre-fur$^{(fl/fl)}$ mice were isolated (II, Figure 5) (Clausen, Burkhardt, Reith, Renkawitz, & Förster, 1999; Taverniti et al., 2013)**,** cultured and differentiated into macrophages. Next, bone marrow-derived macrophages were activated with LPS (1ug/ml). Finally, we performed qRT-PCR on FURIN deficient and WT bone marrow macrophages. Our results showed that the lack of FURIN in bone marrow macrophages correlated with significantly upregulated mRNA levels of the pro-inflammatory cytokine gene *Tnfa.*

The characterization of FURIN-dependent gene expression patterns in resting peritoneal macrophages from LysMcre-fur$^{(fl/fl)}$ and littermate WT mice was performed using a genome-wide microarray analysis. (I, Figure 2A, 2B). We observed the upregulation of several genes frequently expressed in activated macrophages such as *Serpinb1a, Serpinb2, Hcar2 , Egr1, Il6, Il1β, Ptgs2, Ccl2, Ccl7*, *Ccnd1, Dusp6, Fcgr1 and C5ar1* (Blanchette, Day, Dong, Laprise, & Dubois, 1997; Feingold, Moser, Shigenaga, & Grunfeld, 2014; Gong et al., 2011; Gundra et al., 2014; Murray et al., 2014; Piao et al., 2015; Udofa et al., 2013). Among the downregulated genes we found *Atf7,* which has been associated with the constitutive activation of macrophages (K. Yoshida et al., 2015).

5.1.3 Decreased survival of LysMcre-fur$^{(fl/fl)}$ mice during LPS-induced endotoxemia

To test whether the pro-inflammatory phenotype observed in resting FURIN-deficient peritoneal macrophages affects the inflammatory response in LysMcre-fur$^{(fl/fl)}$ mice we performed a model of endotoxemia. First, LysMcre-fur$^{(fl/fl)}$ and littermate WT mice were intraperitoneally injected with 25 mg/kg (LPS) and surveyed for 72 hours (I, Figure 3A). Notably, the rate of mortality was significantly increased in LysMcre-fur$^{(fl/fl)}$ mice 24 hours post-injection (62% mortality in KO vs. 38% mortality in WT) ($p = 0.0109$), which strongly suggests an increased susceptibility to pro-inflammatory stimuli. Next, a lower dose of LPS (100 µg/kg) was

intraperitoneally injected into LysMcre-fur$^{(fl/fl)}$ and WT littermate mice in order to assess how FURIN expression in myeloids regulates cytokine levels in serum as well as the number of macrophages (F4/80+CD11b+Ly6C+Ly6G-) thought to be involved in the inflammatory response (Grainger et al., 2013; Swirski et al., 2009). We found that injecting LPS into LysMcre-*fur*$^{(fl/fl)}$ mice induced the production of the pro-inflammatory cytokines TNF-α and IL-6 1 and 3 hours post-infection, respectively. In addition, 3 hours post-injection the production of anti-inflammatory IL-10 decreased (I, Figure 3B-3D) and there was a significant enrichment of splenic F4/80+CD11b+Ly6C+Ly6G- type of monocytes, which are said to express the C-C chemokine receptor type 2 (CCR2) and are frequently described as classical or inflammatory monocytes (Geissmann, Jung, & Littman, 2003). Although more specific surface markers (e.g CCR2) and functional studies need to be performed in order to definitely assigned the inflammatory phenotype to F4/80+CD11b+Ly6C+Ly6G- cells and clarify their origin (monocytes or monocytes-derived macrophages), this study considers these myeloid cells potential inflammatory cells and defines them as "inflammatory" based on previous reports as well as the FACS gating analysis applied (I, Figure 3E) (Jablonski et al., 2015; Rose, Misharin, & Perlman, 2012).

Previous reports have found an association between the phenotype displayed by macrophages (M1 or M2) and susceptibility to a septic shock (López-Bojórquez, Dehesa, & Reyes-Terán, 2004; Mehta et al., 2004). To address this conundrum, we performed qRT-PCR *ex vivo* in LPS/IFN-γ activated FURIN KO peritoneal macrophages and we detected an upregulation in the expression of *Nos2,* which has been reported as a marker gene for M1 macrophages (Murray & Wynn, 2011). In addition, when FURIN KO peritoneal macrophages were activated with IL-4 we observed a reduction in the expression of *Arg1* (M2 marker gene) (Murray & Wynn, 2011) (I, Figure 3F).

5.1.4 FURIN is dispensable for the immediate TLRs response

The direct proteolysis of TLR7 by PCSKs has proven to be essential for the activation of the human Toll-like receptor 7 (TLR7) (Hipp et al., 2013). In order to study the role of FURIN in the activation of TLR4 we determined whether the expression of the genes associated with LPS/TLR4 responses was affected by the

lack of FURIN in peritoneal macrophages. First, using qRT-PCR we investigated the time course of changes in the mRNA expression levels of the *Il1b, Tnfa, Il6 and Il10* genes in response to LPS. Our data indicated that LPS-stimulated FURIN-deficient peritoneal macrophages and control macrophages displayed roughly the same dynamics in the expression patterns of the aforementioned genes (I, Figure 4A). Interestingly, FURIN KO peritoneal macrophages showed an upregulated base line expression of the pro-inflammatory cytokine genes compared to controls. Similarly, microarray data obtained from the expression of TLR-associated mRNAs in WT and FURIN KO macrophages (I, Figure 4B) revealed scarcely any difference between their gene expression dynamics in response to LPS. Nevertheless, we found that the lack of FURIN in LPS activated macrophages upregulated the dynamic response of several pro-inflammatory genes including *Trem1, Nos2, Il15, Il33,and Il12rb1* (Bouchon, Facchetti, Weigand, & Colonna, 2001; C. Li et al., 2014; Mattiola et al., 2015; Russell et al., 2003) (I, Figure 4C) and suppressed genes such as *Ch25h, Olr1 and Arg1*, which are induced in alternatively activated macrophages (Gundra et al., 2014). Finally, we stimulated WT and KO peritoneal macrophages with different TLR ligands and IFN-γ (I, supplementary Figure S5) and investigated the expression of pro- and anti-inflammatory cytokines using qRT-PCR. The results showed the ability of FURIN to display an anti-inflammatory response in TLR2, TLR4 and TLR7/8 activated macrophage**s**. Thus, the biological function of FURIN as a regulator of the inflammatory response in activated macrophages is stimulus-independent.

5.1.5 The lack of FURIN in peritoneal macrophages affects the production of the bioactive TGFβ-1 cytokine activated TNF-α Converting Enzyme (TACE) and Caspase-1 p20.

Several of the cytokines secreted by macrophages require proteolytic processing in order to perform their effector functions. Among these cytokines we found TNF-α, IL-1β and TGF-β1 (Biswas & Mantovani, 2010) . Specifically, the conversion of TGF-β1 into an active molecule requires a post-translational enzymatic cascade of reactions, where FURIN catalyzes one of the cleavage steps in the functional maturation of pro-TGF-β1 (Dubois et al., 2001; Pesu et al., 2008). Another important molecule for macrophage biology is TNF-α Converting Enzyme (TACE). TACE is

converted into a functional molecule by a FURIN-like proprotein convertase (Endres et al., 2003) and has an essential role in releasing the pro-inflammatory cytokine TNF-α from its membrane-bound precursor.

The modulation of the Caspase-1 cascade, which directly processes IL-1β, seems to play an essential role in the transition between different macrophage phenotypes (Pelegrin & Surprenant, 2009). Previous reports have suggested a functional connection between the proteolytic activity of PCSK and IL-1β (H. Lin et al., 2012). In addition, the cleavage of the anthrax toxin by FURIN leads to the activation of Caspase-1 in macrophages (Bugge & Leppla, 2006).

To investigate the potential role of FURIN in the regulation of the maturation of the aforementioned molecules in macrophages, we studied the production of bioactive TGF-β1 as well as the proteolytic activation of TACE and Caspase-1 in LysMcre-fur$^{(fl/fl)}$ and WT mice. First, an ELISA was performed in resting FURIN KO and WT peritoneal macrophages to analyze the levels of the production of bioactive TGF-β1. We found a reduction in the production of bioactive TGF-β1 in FURIN KO macrophages, whereas the levels of *Tgfb1* mRNA remained unaltered (I, Figure 5A, supplementary Figure S6). In contrast, we observed that the lack of FURIN in LPS-stimulated peritoneal macrophages increased the levels of mature TACE (I, Figure 5B). The aforementioned result is in tune with the high production levels of TNF-α that we detected in the *in vivo* model of endotoxemia (I, Figure 3B). Finally, the study of Caspase-1 processing, using western blotting, revealed a higher production of Caspase-1p20 in FURIN KO macrophages compared to controls (I, Figure 5C). These data support the higher levels of serum IL-1β observed in resting FURIN KO peritoneal macrophages and suggest a role for FURIN as a novel proteolytic regulator of IL-1β.

5.2 Role of FURIN in the regulation of the host response against *Mycobacterium marinum* (II)

Previously, we have demonstrated that the lack of FURIN in T cells disrupts the peripheral immune tolerance. This observation supports a role for FURIN in the regulation of the adaptive immunity. Nevertheless, the function of FURIN in the innate immunity and in infections has remained elusive. Here, we used a model of

a *Mycobacterium marinum* infection in zebrafish (*Danio rerio*) in order to evaluate the role of FURIN in the regulation of the host immune response against *Mycobacterium marinum.*

The zebrafish has two co-orthologs of the mammalian *furin* gene: *furin*A and furinB. Likewise, mammalian *furin, furin*A has a crucial and nonredundant role in several biological events essential for the development of a organism (Walker et al., 2006). In this publication, we used a furinAtd204e/+ mutant zebrafish strain to investigate the role of *furin*A in the host response against mycobacteria.

5.2.1 FurinA+B regulates the survival of ? in the embryonic *M. marinum* infection model.

In this study, we silenced the expression of the *furin* genes in developing fish and used a furin$^{Atd204e/+}$ mutant zebrafish strain (44% decrease in *furinA* gene expression) to assess the role of *furin*A in the regulation of the immune response during a mycobacterial infection.

To assess the role of FurinA in the immune response of zebrafish against *M. marinum*, furinA$^{td204e/+}$ and WT zebrafish, were inoculated fish with a high-dose of *M. marinum* (8,300 ± 1,800 CFU) and monitored them for 11 weeks (II, Figure 3A). Both WT and *furinA*$^{td204e/+}$ mutants showed no significant statistical difference in survival (II, Figure 3A). Furthermore, the results from a histological analysis revealed similarities between WT and *furinA*$^{td204e/+}$ mutants concerning the number and organized granulomas at 3 and 11 wpi. (II, Figure 3B). Nevertheless, when a morpholino was used to silence the expression of *furinA and furinB* in the embryonic *M. marinum* infection model (Benard et al., 2012; Carvalho et al., 2011) we observed a significant reduction in the survival of infected *furinA+B* morphants compared to controls (*furin*A+B versus RC, $P < 0.01$).

5.2.2 Furin A is responsible for the inhibition of the early expression of pro-inflammatory cytokine genes in *M. marinum*-infected zebrafish

The restraint of the disease caused by mycobacteria is highly dependent on adequate adaptive and innate immune responses (Gupta, Kaul, Tsolaki, Kishore, & Bhakta, 2012; Philips & Ernst, 2012; Velez et al., 2010). Despite the role of FURIN in processing several molecules important for the innate immunity (Adrain, Zettl, Christova, Taylor, & Freeman, 2012; Hipp et al., 2013), the mechanism for how FURIN regulates the innate responses in infections *in vivo* remains unclear. To address this conundrum, furinAtd204e/+ and WT adult zebrafish were first infected with a high dose of *M. marinum* (10,300 ± 3,300 CFU). Then we performed a qRT-PCR expression analysis to decipher the early cytokine gene expression profile. The expression of pro-inflammatory (*tnfa, il1b, lta, ifng1-1, il22, and il17a/f3*) and anti-inflammatory *(il10 and tgfb1a*) cytokine genes was analyzed at 1, 6, and 12 dpi (II, Figure 4). Collectively we observed an efficient induction of pro- and anti-inflammatory cytokine genes already during the first 12 days after the infection.

Next, to decipher the contribution of FurinA to the early cytokine induction during an *M. marinum* infection we compared the expression of the previously studied cytokine genes in *furinA*$^{td204e/+}$ and WT infected zebrafish (II, Figure 4B). At 1 dpi we observed a significantly higher relative expression of the pro-inflammatory cytokine genes *tnfa* , *lta* and *il17a/f3* in *furinA*$^{td204e/+}$ compared to WT fish. Notably, the intrinsic upregulation of *tgfb1a* in *furinA*$^{td204e/+}$ mutants was abrogated by the 12th dpi day and co-occurred with a relative reduction in the expression of *il10*. Moreover, the attenuated expression of *furinA* correlated with a sustained upregulation of *il17a/f3*.

Overall, our results suggest a inherent role for FURIN in myeloid cells in the regulation of the innate cytokine responses in *M. marinum*-infected furinAtd204e/+ mutant fish.

5.3 T-cell-expressed proprotein convertase FURIN inhibits the development of DMBA/TPA-induced skin cancer (III)

5.3.1 Lack of FURIN in T-cells and not in myeloid cells promotes skin tumorigenesis

The pharmacological inhibition of proprotein convertase (PCSK) enzymes in various cancer types (J. M. Coppola et al., 2008; Fu et al., 2012) constitutes a promising therapeutic approach to the treatment of cancer. FURIN expression has been shown to be elevated in non-small cell lung carcinoma as well as in human head and neck squamous cell carcinomas, and the upregulation of FURIN activity correlates positively with tumor progression. In addition, Fu J et al. demonstrated the pro-tumorigenic role of FURIN *in vivo* using a two-stage chemical carcinogenesis model (Fu et al., 2012). Nevertheless, the specific role of FURIN expression in immune cells during tumor development remains unclear.

To investigate whether the expression of FURIN in the cells of the immune system influences the formation of skin tumors, we treated the back skin of LysMcre-fur$^{(fl/fl)}$ (FURIN deleted in myeloid cells) and CD4cre-fur$^{(fl/fl)}$ (FURIN deleted in T cells) mice and their respective wild-type littermates (LysM WT and CD4 WT) with the mutagen DMBA. Next, we applied the tumor-promoting agent TPA for a period of 16 and 21 weeks. The combined treatment of DMBA/TPA induced the formation of papillomas (Perez-Losada & Balmain, 2003).

A histological analysis showed that in normal mouse skin, FURIN was profusely expressed in the epidermis when compared with the dermis. Nevertheless, after a DMBA/TPA treatment the expression of FURIN was higher in the dermal part of the skin ($P<0.001$) when compared to untreated skin (III, supplementary Figure S1).

Surprisingly, the lack of FURIN in T cells correlated with the development of more papillomas ($P<0.0001$, Figure 1A). We detected the first papillomas in both LysMcre-fur$^{(fl/fl)}$and WT controls after 10 – 12 weeks of a DMBA/TPA treatment (III, Figure 1A), whereas CD4cre-fur$^{(fl/fl)}$mice developed the first papillomas after 8 weeks, and after 9 weeks all of the CD4cre-fur$^{(fl/fl)}$mice had developed papillomas on their back skin ($P<0.001$, III Figure 1B).

At the end of the study (17 weeks) the CD4cre-fur$^{(fl/fl)}$mice had developed nearly 20 papillomas per animal in contrast to WT controls, which only developed five

papillomas (III, Figure 1B). In addition, LysMcre-fur$^{(fl/fl)}$, LysM WT and CD4 WT mice developed the same number of tumors at 17 weeks of a DMBA/TPA treatment and after 21 weeks no new tumors were detected (III, Figure 1B).

The incidence of tumor development in CD4cre-fur$^{(fl/fl)}$mice compared to CD4 WT mice was 4.6 fold greater during the experiment. However, despite the significantly higher number of papillomas and the higher rates of papilloma development observed in CD4cre-fur$^{(fl/fl)}$mice compared to the other strains, these papillomas did not continue to grow in size (III, supplementary Figure S2A).

Collectively, these observations demonstrate that the deficiency of FURIN in T cells but not in myeloid cells is associated with carcinogenesis in a multistage chemical carcinogenesis model in mice. These initial results prompted us to deepen our understading of the role of FURIN in T-cells in squamous skin cancer.

5.3.2 Tumor formation in CD4cre-fur$^{(fl/fl)}$mice correlates with enhanced cell proliferation, but not with vascularization

To study the underlying mechanisms associated with the role of T-cell expressed FURIN in the suppression of skin tumors, we applied histological analyses to investigate the thickness of the epidermal and dermal skin layers, cell proliferation (Ki67) and the frequency of apoptosis (TUNEL) in the skin of DMBA/TPA treated and untreated mice. As a result of these analyses, we observed that untreated CD4cre-fur$^{(fl/fl)}$mice displayed an inherent thickening of the epidermis (III, supplementary Figure S3A). Following a DMBA/TPA treatment the epidermal thickness of CD4cre-fur$^{(fl/fl)}$and CD4 WT mice showed a significant increase ($P<0.001$) compared to untreated skin. In addition, the increment of thickness (30%) in the epidermis of CD4cre-fur$^{(fl/fl)}$mice was sustained ($P<0.0001$, III supplementary Figure S3A). In contrast, the dermal thicknesses were similar in both genotypes.

Regarding the level of cell proliferation based on Ki67-positivity, we observed that CD4cre-fur$^{(fl/fl)}$mice exhibited significantly more proliferating cells than CD4 WT mice in the epidermis of the untreated mice skin ($P<0.0001$, III supplementary Figure S3C). After a DMBA/TPA treatment for 17 weeks CD4cre-fur$^{(fl/fl)}$mice displayed more cell proliferation in the epidermal and dermal layers of the skin ($P<0.001$ and $P=0.0056$, III supplementary Figure S3C, D) than did control mice. Nevertheless, we did not detect differences in the cell proliferation rate in the papilloma tissue (III

supplementary Figure S3C, D). Next, a study of apoptotic cells using TUNEL staining revealed less apoptotic cells in the untreated dermis of CD4cre-fur$^{(fl/fl)}$mice ($P<0.05$, III supplementary Figure S4A). At 17 weeks, the number of dying cells in CD4cre-fur$^{(fl/fl)}$ and CD4 WT DMBA/TPA treated mice was significantly reduced (epidermis, $P<0.0001$, dermis $P<0.0001$, III supplementary Figure S4).

Vascular supply is an essential factor for tumor growth, and FURIN has been shown to play an important role in angiogenesis (McColl et al., 2007; Siegfried et al., 2003). In order to address the role of FURIN in the vasculature of the skin we performed an immunohistological analysis of the endothelial cell marker CD31 in the untreated skin of CD4cre-fur$^{(fl/fl)}$ and CD4 WT mice (III, supplementary Figure S5A). The CD31 detection revealed a moderately higher number of blood vessels in CD4cre-fur$^{(fl/fl)}$ compared to CD4 WT mice. In addition, a DMBA/TPA treatment for 17 weeks showed a higher vascular density in the skin (2-fold increase) of CD4 WT mice ($P<0.0001$) compared to CD4cre-fur$^{(fl/fl)}$ mice ($P<0.0001$, III supplementary Figure S5A).

Our results demonstrated that the accelerated tumorigenesis observed in CD4cre-fur$^{(fl/fl)}$ mice was not associated with a dysregulation of angiogenesis.

5.3.3 FURIN deficiency in T cells regulates macrophage extravasation or a differentiation response to a DMBA/TPA treatment

Previously, several reports have demonstrated the induction of acute inflammation as a key factor in the promotion of tumorigenenesis in a multistage chemical carcinogenesis model in mouse skin (May et al., 2015; Swann et al., 2008). In order to investigate whether CD4cre-fur$^{(fl/fl)}$ mice develop an atypical inflammatory response during a DMBA/TPA treatment, we first used immunohistochemistry to assess the number of skin CD3+ T cells, infiltrating F4/80+ macrophages and elastase positive neutrophils. In this study we named F4/80+ cells macrophages, but it is essential to highlight that although F4/80 has been widely used as marker specific for both circulating monocytes and tissue-residing monocytes and macrophages, its expression is highly variable and not limited to these cell populations (Austyn & Gordon, 1981; McGarry & Stewart, 1991). For instance, macrophages, dermal myeloid dendritic cells (DCs) and Langerhans cells

express F4/80 surface markers (Austyn & Gordon, 1981). Thereby, the sole use of F4/80 as a marker does not provide delimitation between monocytes, tissue resident or monocyte-derived macrophages, different macrophages subsets (M1/M2), myeloid dendritic cells, and probably other myeloid-derived cells that could express the marker (Jablonski et al., 2015). This and other limitations have contributed to changing the view of this marker as exclusive for mononuclear phagocytes (Malissen, Tamoutounour, & Henri, 2014). Taking into consideration the discovery that Langerhans cells dowregulate the expression of F4/80 after they uptake the antigen and become migrating DCs (H. H. Lin et al., 2005); myeloid dendritic cells express F4/80 to a lesser extent than macrophages (Merad, Sathe, Helft, Miller, & Mortha, 2013) and since other cells that populate the skin, such as mast cells, NK cells and lymphocytes have not been reported in the literature to express F4/80 we made the assumption (I) that F4/80+ cells were mainly macrophages, although a better designation should be more general (e.g F4/80+ myeloid cells) (H. H. Lin et al., 2005).

Our observations demonstrate the presence of a significantly higher number of CD3+ T cells in CD4cre-fur$^{(fl/fl)}$ mice compared to CD4 WT controls ($P<0.0001$) performed in untreated skin ($P<0.0001$) and at 43 hours post a DMBA/TPA treatment ($P<0.05$). Nevertheless, after 17 weeks of a DMBA/TPA treatment the number of T cell was similar in both genotypes (III, Figure 2A and B).

Interestingly, CD4cre-fur$^{(fl/fl)}$ mice displayed a clear fluctuation in the number of F4/80+ macrophages measured at different stages of chemically induced tumorigenesis. At 43 hours of a DMBA/TPA treatment the number of F4/80+ macrophages was higher in CD4cre-fur$^{(fl/fl)}$mice, whereas after 17 weeks of treatment CD4cre-fur$^{(fl/fl)}$mice displayed a significant reduction in the number of F4/80+ macrophages colonizing the non-tumorous skin **(**$P<0.001$, III Figure 2C, D).

The number F4/80+ macrophages underneath the papillomas was significantly lower in CD4cre-fur$^{(fl/fl)}$ mice compared to CD4 WT mice (III, Figure 2C, D). In contrast, the number of neutrophils was similar in CD4cre-fur$^{(fl/fl)}$ mice and CD4 WT mice at the different stages of tumor formation.

These findings could suggest a specific effector function for FURIN, when expressed in T cells, in the regulation of inflammation at different stages of tumorigenesis.

5.3.4 FURIN-deficient T cells display an activated phenotype at the skin level.

Different T cell populations have been implicated in the promotion or inhibition of skin tumorigenesis. To obtain detailed information concerning T lymphocyte populations in the skin of CD4cre-fur$^{(fl/fl)}$ mice during chemically induced carcinogenesis, we performed a flow cytometric analysis of the skin draining lymph node (dLN) cells of CD4cre-fur$^{(fl/fl)}$ mice and CD4 WT mice. Our results demonstrated non-significant differences in the number of CD4+ T in the skin dLNs of untreated and DMBA/TPA treated (17 weeks) CD4cre-fur$^{(fl/fl)}$mice compared to CD4 WT mice (III, Figure 3). Although untreated CD4cre-fur$^{(fl/fl)}$mice showed a lower number of dLN CD8+ T cells than CD4 WT mice, the difference vanished after 17 weeks of a DMBA/TPA treatment. In contrast, we detected higher percentages of CD4+CD25+Foxp3+ regulatory T (Treg) cells in the skin dLNs of untreated and DMBA/TPA treated (17 weeks) CD4cre-fur$^{(fl/fl)}$ mice compared to CD4 WT mice (III, Figure 3).

Notably, the number of CD4+ and CD8+ T cells expressing the early activation marker CD69 was higher in CD4cre-fur$^{(fl/fl)}$ mice compared to CD4 WT mice (III, Figure 3).

In addition, the quantification of the T cell memory markers CD44 and CD62L revealed a significantly higher percentage of central memory CD62L^{high}CD44^{high} CD8+ T cells in both untreated and 17-week-treated CD4cre-fur$^{(fl/fl)}$ mice (III, Figure 3). These results are in harmony with a recent report that demonstrated how the inhibition of endogenous TGF-β1 signaling in CD8+ T cells promotes their conversion into central memory cells (Takai, Schlom, Tucker, Tsang, & Greiner, 2013). Pro-TGF-β1 is a FURIN target, thus the autocrine TGF-β1 signaling is probably impaired in FURIN-deficient CD8+ T cells favoring the conversion of CD8+ T cells into central memory cells.

Collectively, these data indicate that in the chemical induced skin tumorigenesis model FURIN deficient T cells from the skin dLNs are intrinsically hyperactivated and preferentially differentiate into a Treg phenotype.

5.3.5 CD4cre-fur$^{(fl/fl)}$ mice displayed different types of Th mediated immune-responses at early and late stages of chemical induced skin tumorigenesis

FURIN regulates T-cell activation and the T helper balance in the immune system (A. Oksanen et al., 2014; Pesu et al., 2006). To deepen our understanding of the biology of skin dLN FURIN-deficient T cells in the early phase of tumor development, we investigated the production of cytokines 48 h after a second treatment with TPA using intracellular staining and flow cytometry. We observed that CD4+ and CD8+ effector T cells from CD4cre-fur$^{(fl/fl)}$ mice produced higher levels of pro-inflammatory interferon gamma (IFNγ) (III, Figure 4B, C). In contrast, we did not observe differences in the production of the IL17A and IL-10 cytokines, which are usually described as pro-inflammatory and anti-inflammatory respectively (III, Figure 4B, C).

Next, we investigated the type of immune response displayed by CD4cre-fur $^{(fl/fl)}$ mice and CD4 WT mice during a DMBA/TPA treatment using a multiplex assay, which allowed the analysis of various chemokines in serum samples produced 48 hours after the second application of TPA. The study showed no differences in the levels of CCL2/MCP1, CXCL-1/GROα, CCL11/Eotaxin, CXCL2/MIP-2, CCL7/MCP-3, CCL5/RANTES, CCL3/MIP-1α or CCL4/MIP-1β (data not shown). Interestingly, the level of CXCL10/IP-10 in the sera of CD4cre-fur$^{(fl/fl)}$ mice were two folds higher than in CD4 WT mice sera (p=0.0711, III Figure 4E).

Overall, these findings suggest that in the early stages of skin tumorigenesis the lack of FURIN in T cells promotes a Th1-type-skewed immune response.

In addition, we performed a flow cytometric analysis of the IFNγ, IL-17A and IL-10 cytokines produced by skin dLNs CD4+Foxp3+ Treg cells isolated from the CD4cre-fur$^{(fl/fl)}$ and CD4 WT mice. Intriguingly, Tregs from CD4cre-fur$^{(fl/fl)}$ mice showed a trend towards an increased production of IFN-γ (p=0.0592) accompanied by a significant reduction in the production of IL17A (p=0.0066, III Figure 4D). In contrast, the percentage of skin dLN Treg cells producing IL-10 as well as the mean fluorescence intensity of the CD25 surface marker were similar in both genotypes.

Altogether, the flow cytometry data suggests that the increased susceptibility of CD4cre-fur$^{(fl/f)}$ mice to developing papillomas could be due the tendency of FURIN-deficient T cells to propel the Th1-type immune responses (Xiao et al., 2009).

To study the type of Th immune response developed by CD4cre-fur$^{(fl/fl)}$ mice at the later stages of chemically induced carcinogenesis we determined the levels of

several cytokines and chemokines derived from the skin dLNs cells of CD4cre-fur $^{(fl/fl)}$ mice and CD4 WT mice treated with DMBA/TPA for 17 weeks. In contrast to our observations during the early phase of tumorigenesis, the cells isolated from CD4cre-fur$^{(fl/fl)}$ mice after 17 weeks of treatment did not produce increased levels of IFN-γ. Instead, the production of cytokines switched to the Th2-type cytokine IL-13 and Th17-type cytokine IL-22 (P=0.0219 and P=0.0393, respectively, III, Figure 5). In addition, the production of other Th2/Th17 cytokines such as IL-4, IL-17A, IL-9 or IL-10 showed a tendency to be upregulated in FURIN-deficient T cells (III, Figure 5). The shift towards the Th2 and Th17 type of response in FURIN-deficient T cells in the later stages of carcinogenesis was confirmed at the mRNA level using qRT-PCR in samples from the back skins of CD4cre-fur$^{(fl/fl)}$mice and CD4 WT mice. Our data showed an upregulation of IFN-γ mRNA levels in FURIN-deficient T cells during the early stage of carcinogenesis and high levels of IL-17a mRNA detected during the late stage (III, supplementary Figure S6).

In summary, these results demonstrate that when expressed in Tcells, FURIN regulates the balance between the different types of Th immune responses during the course of carcinogenesis.

6 DISCUSSION

6.1 FURIN regulates the innate immune response (I, II)

Several pathogens escape the host's defense mechanisms by adapting to the host's protein-proteolytic machinery. Thereby, proprotein convertases are critical in the pathogenesis of certain infectious diseases. *Pseudomonas* exotoxin A (Gu, Gordon, Fitzgerald, & Leppla, 1996), *diphtheria* toxin (Tsuneoka et al., 1993), Shiga toxins (Lea, Lord, & Roberts, 1999), anthrax toxin as well as the envelopes of the HIV and Influenza viruses (Thomas, 2002) require a FURIN/PCSK-dependent proteolysis step in order to exercise their pathogenic function (Decroly, Benjannet, Savaria, & Seidah, 1997; Garten et al., 2015; Opal et al., 2005). Consequently, the inhibition of FURIN/PCSKs could protect the host from FURIN/PCSK-dependent viruses and/or bacteria (Shiryaev et al., 2007).

The innate immune system represents the first line of defense against harmful pathogens. Inhibiting FURIN/PCSKs specifically in myeloid cells could thus contribute to constraining infections. The specific role of FURIN in the cells of the innate immune system has remained unclear, hence the present studies (I, II) investigated the effect of FURIN inhibition specifically in myeloid cells during LPS induced endotoxemia (LysMcre-fur$^{(fl/fl)}$ mice model (I)) and a *Mycobacterium marinum* infection in zebrafish (furinA$^{td204e/+}$ mutant (II)). We demonstrated that the absence of FURIN expression in mouse innate immune cells could strengthen host responses (I). Likewise, a *Mycobacterium marinum* infected furinA$^{td204e/+}$ zebrafish mutant displayed an enhancement of the innate immune response characterized by a significant reduction in the bacterial burden in a chronic *M. marinum* infection.

Altogether, these results unveiled an essential/partially non-redundant role for FURIN in the regulation of the innate immune response.

6.1.1 LysMcre-fur$^{(fl/fl)}$ mice display mild splenic abnormalities and elevated levels of IL-1β in their serum

The expression of PCSK1, PCSK2, PCSK5, FURIN and PCSK7 is highly induced in monocytes/macrophages stimulated with LPS (Lansac et al., 2006; Turpeinen et al., 2011). Moreover, previous studies using germ-line deletions have demonstrated that PCSK1 and PCSK7 regulate the production of innate cytokines (Refaie et al., 2012) and regulate the antiviral immune response by processing and activating TLR7 and TLR8 (Hipp et al., 2013). Although these and other findings argue for a role of PCSKs in the regulation of the innate immunity, the specific role of FURIN in the cells of the innate immunity has remained unknown.

In the present study, we used two different *in vivo* models (mice (I) and zebrafish (II)) to determine the role of FURIN in the regulation of the innate immunity. Concretely, we characterized both a novel tissue-specific knockout mouse model (LysMcre-fur$^{(fl/fl)}$), in which FURIN was specifically deleted in myeloid cells, and *Mycobacterium marinum* infected furinA$^{td204e/+}$ zebrafish mutants.

Previously, we have shown that six months old CD4cre-*fur*$^{(fl/fl)}$ mice develop severe inflammatory bowel disease (Pesu et al., 2008). In contrast, LysMcre-*fur*$^{(fl/fl)}$ mice remain healthy as they age. Interestingly, recent findings have implicated a role for the splenic PCSK enzymes in the response to LPS (Lansac et al., 2006). FURIN is expressed in the splenic red pulp, a zone enriched with macrophages, which regulate important functions, such as the extramedullary myelopoiesis, the clearance of senescent red cells, the antigen presentation as well as tolerance to self-antigens (Dutta et al., 2015; Martinez-Pomares & Gordon, 2012; McGaha, Chen, Ravishankar, van Rooijen, & Karlsson, 2011). In addition, PCSK1 knockout mice display splenic abnormalities, for example disorganization of the marginal zone and red pulp (Refaie et al., 2012). We found that the spleens of LysMcre-*fur*$^{(fl/fl)}$ mice develop a normal size and weight. However, we detected significantly reduced numbers of splenocytes compared to controls. This finding could indicate a role for FURIN expression in myeloid cells in hematopoiesis, or in cellular homeostasis in the spleen. Although the mechanism(s) involved in the reduction of splenocyte numbers in LysMcre-*fur*$^{(fl/fl)}$ mice need further clarification, the fact that cells in the bone marrow of LysMcre-*fur*$^{(fl/fl)}$ expressed 60% less *furin* mRNA than cells from control mice, suggest that the delicate microenvironment is dirupted probably due to a reduction of the proteolytic maturation of hematopoietic growth factors or

extracellular matrix receptors, which would thereby affect the extramedullary hematopoiesis (Krebsbach, Kuznetsov, Bianco, & Robey, 1999). Further, a flow cytometric analysis performed in the spleen reveiled normal proportions of macrophages (F4/80+CD11b+), neutrophils (Gr1+CD11b+), CD3+ T and B220+ B cells, but a significant reduction in the numbers of CD3+CD4+CD8- T helper cells and an elevated proportion of CD3+CD4-CD8+ cytotoxic T cells in LysMcre-*fur*$^{(fl/fl)}$ mice. We argue that a FURIN deficiency in myeloid cells might be implicated in the regulation of the homing, proliferation and/or apoptosis of splenic lymphocytes hence affecting the CD4+/CD8+ T cell ratio in the spleen (which has been shown to correlate with inflammation) (Ohsugi & Kumasaka, 2011). A flow cytometric analysis performed in the peritoneal cavity and bone marrow corroborated that the lack of FURIN did not significantly alter the gross numbers of macrophages, neutrophils, T cells and B cells. Interestingly, the altered proportions of CD3+CD4+CD8- T helper and CD3+CD4-CD8+ cytotoxic T cells in the spleen was not detected in the peritoneal cavity or bone marrow. Altogether, these findings reveal that FURIN expression in myeloid cells is not essential for the development of neutrophils or macrophages, but appears to be implicated in cellular development and the CD4+/CD8+ T cell balance in the spleen. *In vitro* and *in vivo* studies in macrophages and lymphocytes have uncovered a role for FURIN/PCSK in the production of important mediators of the immune response (Duhamel et al., 2015; Pesu et al., 2008; Refaie et al., 2012). Notably, one in vitro study demonstrated that the lack of PCSK1 in resting peritoneal macrophages promotes the production of IL-1β (Duhamel et al., 2015). Likewise, the analysis of LysMcre-*fur*$^{(fl/fl)}$ mouse serum under steady state conditions showed elevated levels of IL-1β, which is indicative of an inherent inflammatory phenotype and strongly suggests a role for FURIN/PCSKs expressed in myeloid cells in the regulation of IL-1β production.

6.1.2 FURIN deficient peritoneal macrophages inherently upregulate the expression of pro-inflammatory genes

Macrophages are essential components of the innate immune response and perform a critical role in the maintenance of tissue homeostasis. Importantly, macrophages are involved in a wide spectrum of pathologies (Buchmann, 2015; A. E. Ward & Rosenthal, 2014). During an infection these cells phagocytose pathogens and secrete several molecular mediators such as cytokines and chemokines, which

amplify the immune response through the activation of other immune cells. Commonly, macrophages are classified as classically activated (M1-pro-inflammatory) or alternatively activated (M2-anti-inflammatory) according to the type of cytokines they produce upon different stimuli. PCSKs have been shown to be expressed in resting and LPS-stimulated macrophages and their deletion generates aberrant gene expression signatures (Ortutay et al., 2015; Pesu et al., 2008; Refaie et al., 2012; Turpeinen et al., 2013). In the present study we demonstrated, for the first time, that when expressed in peritoneal macrophages, FURIN has an intrinsic suppressive effect on the expression of genes related to the activation of pro-inflammatory M1 type macrophages (Murray et al., 2014). Futher studies in other LysM-positive cells will contribute to deepen the understandng of the physiological role of Furin.

Our findings show that, similarly to human CD14+ monocytes (Turpeinen et al., 2011), the expression of FURIN is efficiently upregulated in the peritoneal macrophages of mice upon activation with LPS+/- IFN-γ. Moreover, deleting FURIN in macrophages does not affect the expression of other PCSK enzymes. Consequently, the compensatory expression of other biochemically redundant PCSKs caused by the lack of FURIN (Roebroek et al., 2004) does not interfere with the analysis of FURIN's biological function in macrophages.

In addition, the analysis of the global gene expression patterns in resting FURIN-deficient peritoneal macrophages isolated from LysMcre-fur$^{(fl/fl)}$ mice clearly shows the reproducible upregulation of several genes typically expressed in macrophages that have been activated such as *Serpinb1a, Serpinb2, Hcar2 , Egr1, Il6, Il1β, Ptgs2, Ccl2, Ccl7 and C5ar1* (Feingold et al., 2014; Gong et al., 2011; Murray et al., 2014; Piao et al., 2015; Udofa et al., 2013). We also detected the downregulation of the *Atf7* gene, whose deletion promotes the constitutive activation of resident peritoneal macrophages and increases susceptibility to LPS-induced septic shock in mice, suggesting that ATF7 might be a negative modulator of LPS-induced gene activation (K. Yoshida et al., 2015). Interestingly, resting FURIN-deficient macrophages upregulate the expression of genes such as *Dusp6* and *Fcgr1*, which are poorly expressed in alternatively activated macrophages (Gundra et al., 2014), indicating that the lack of FURIN in macrophages promotes a pro-inflammatory phenotype.

Due to their plasticity macrophages display key roles in both immunoprotection and tissue homeostasis. However, the powerful capacity of macrophages to redesign their functions can lead to the development of several pathologies (Biswas,

Chittezhath, Shalova, & Lim, 2012; Liu, Zou, Chai, & Yao, 2014; Mantovani & Allavena, 2015; Porta et al., 2011). Several factors regulate macrophage heterogeneity, such as intricate networks of cell signaling cascades, metabolic pathways, transcription factors, and epigenetic modifications. Hence, the study of the genetic and molecular factors involved in macrophage biology, specifically in the regulation of the balance between the different phenotypes, will greatly contribute to the development of effective strategies in the treatment of several human diseases (Mantovani & Allavena, 2015; Murray & Wynn, 2011; Wynn et al., 2013). Here we showed, for the first time, that resting FURIN-deficient macrophages display a specific transcriptional signature characterized by the upregulation of genes preferentially expressed during the activation of pro-inflammatory M1 type macrophages (Murray & Wynn, 2011). Since tissue-resident macrophages display different transcriptional signatures (Gautier et al., 2012a; Gosselin et al., 2014; Lavin et al., 2014), it is important to highlight that our study introduces FURIN as a regulator of gene expression in macrophages isolated from the peritoneal cavity (I).

6.1.3 FURIN expression in myeloid cells attenuates inflammation.

The classification of macrophages into the M1 and M2 type of phenotypes is based on *in vitro* experiments, and the relevance of these phenotypes *in vivo* is unclear. Moreover, pure M1 and M2 macrophages probably do not occur *in vivo.* To induce the M1 phenotype *in vitro,* macrophages are generally incubated with interferon-γ (IFN-γ) and the TLR4 ligand lipopolysaccharide, which is a component of the cell wall of Gram-negative bacteria. Resting macrophages stimulated with this combination of molecules produce high levels of pro-inflammatory cytokines, including interleukin-1β (IL-1β), IL-6, IL-12, and tumor necrosis factor-α (TNF-α), chemokines such as MCP-1 or CCL2 as well as reactive oxygen species and inducible nitric oxide synthase (NOS2), which produces high amounts of nitric oxide, contributing to the clearance of pathogens. Hence, this type of macrophage activation markedly promotes inflammation and the elimination of pathogens.

Among our initial findings, the high IL-1β levels in the serum of LysMcre-*fur*$^{(fl/fl)}$ mice and the upregulation of pro-inflammatory genes in FURIN-deficient peritoneal macrophages suggested a marked pro-inflammatory phenotype in LysMcre-*fur*$^{(fl/fl)}$ mice. Hence, to test whether the lack of FURIN in myeloid cells also restricts inflammation *in vivo* we performed an *in vivo* model of LPS-induced endotoxemia, which is considered a systemic inflammation induced by a Toll-like receptor 4

agonist. This model has been widely used to study the ability of distinct animal models to respond to an inflammatory stimulus, and it is characterized by a dysregulation of the secretion of pro-inflammatory cytokines, which generates several symptoms including systemic fever and inducible nitric-oxide synthase-dependent vasodilatation (MacMicking et al., 1995).

As expected, LysMcre-*fur*$^{(fl/fl)}$ mice exhibited a higher rate of mortality 24 hours after the induction of endotoxemia (62% mortality in KO vs. 38% mortality in WT). The aforementioned results were further explained by the significantly enhanced levels of the pro-inflammatory cytokines TNF-α and IL-6, the reduced production of anti-inflammatory IL-10 and the significant augmentation in the proportion of F4/80+CD11b+Ly6C+Ly6G- "inflammatory" monocytes in the spleen after an LPS challenge in LysMcre-*fur*$^{(fl/fl)}$ mice. During acute inflammation F4/80+CD11b+Ly6C+Ly6G- cells constitute one of the dominant population of recruited cells (Serbina et al., 2008; Zigmond et al., 2012) and typically enhance inflammatory effector functions through the elevated production of the pro-inflammatory molecules IL-6, IL-1β, TNF-α, NOS2 and CCL2 (Grainger et al., 2013; Swirski et al., 2009). This publication stressed FURIN's role in the generation/recruitment of splenic F4/80+CD11b+Ly6C+Ly6G- "inflammatory" monocytes during LPS-induced endotoxemia.

Some studies have previously shown an association between a M1 macrophage phenotype and susceptibility to septic shock (López-Bojórquez et al., 2004; Mehta et al., 2004), as was observed in LPS-challenged LysMcre-*fur*$^{(fl/fl)}$ mice. Moreover, PCSK1 KO mice display a dysregulation in their response to endotoxemia. This is partially explained by the presence of macrophages with a pro-inflammatory phenotype (Refaie et al., 2012)**.** Nevertheless, the aforementioned study was performed in mice lacking overall expression of the *Pcsk1* gene, which interferes with the assessment of the individual contribution of cells lacking PCSK1 to the LPS-induced endotoxemia response. Here, we overcome this particular limitation using mice, in which FURIN is deleted only in myeloid cells. Consequently, the increased sensitivity to a pro-inflammatory stimulus observed in LysMcre-*fur*$^{(fl/fl)}$ mice can be primarily attributed to the type of activation of FURIN-deficient myeloid cells. Altogether, our results reveal that, when expressed in macrophages, FURIN takes part in modulating the response to LPS-induced endotoxemia.

During endotoxemia, the recognition of LPS by cells of the innate immune system (*i.e.* macrophages) via TLR4 initiates an overactive immune response typically characterized by the uncontrolled production of pro-inflammatory cytokines (Rossol

et al., 2011; Yamamoto & Akira, 2009). Hence, the regulation of TLR4 expression has an essential role in the activation of systemic inflammation during endotoxemia. Recently, PCSK1 was shown to play a role in the regulation of TLR4 signaling and cytokine secretion (Refaie et al., 2012). In addition, PCSKs regulate the level of expression and activation of human TLR7 by direct proteolysis (Hipp et al., 2013).

Notably, our findings demonstrate that the expression levels of LPS/TLR4-dependent genes are not affected by the lack of FURIN in peritoneal macrophages. In addition, the dynamics in response to LPS were similar in FURIN deficient macrophages compared to controls. Likewise, the analysis of genes of the TLR pathway using microarray data revealed only minor differences between the dynamic expression patterns of WT and FURIN KO peritoneal macrophages. In contrast, the dynamic expression of several other pro-inflammatory genes, including *Trem1, Nos2, Il15, Il33, and Il12rb1,* was upregulated in FURIN-deficient peritoneal macrophages, whereas genes typically induced in alternatively activated macrophages, such as *Ch25h, Olr1 and Arg1,* were suppressed. In addition, the stimulation of FURIN-deficient macrophages with various TLR ligands and IFN-γ indicated that the anti-inflammatory function of FURIN is not associated with a specific stimulus, but can be detected in TLR2, TLR4 and TLR7/8 activated macrophages. Collectively, these data suggest that FURIN is nonessential for the immediate TLR4 responses, but highlight the relevance of FURIN as regulator of the anti-inflammatory response in both resting and activated macrophages.

6.1.4 The lack of FURIN in macrophages modulates the production of the bioactive TGFβ-1 cytokine, TNF-α Converting Enzyme (TACE) and Caspase-1 p20:

Cytokines are essential for macrophage biology and are mediators in the transition from the innate to the adaptive immunity. In concert with other molecules, cytokines determine the fate of macrophages into the different activation phenotypes. The dysregulation of cytokine production in macrophages is a typical factor in several pathologies ranging from chronic inflammation to allergy (Pollard, 2009).

The maturation of several cytokines produced by macrophage, including TNF-α, IL-1β and TGF-β1, require proteolytic processing in the cell (Biswas & Mantovani,

2010) . For instance, TGF-β1 is one of FURIN's substrates (Dubois et al., 2001; Pesu et al., 2008) and TGF-β1 signaling has been associated with an imbalance between the macrophage phenotypes (M1/M2) that causes a sustained inflammatory response and delayed wound healing (Al-Mulla, Leibovich, Francis, & Bitar, 2011). Likewise, TNF-α Converting Enzyme (TACE), a critical enzyme for the endotoxin-triggered release of TNF-α from myeloid cells *in vivo* in mice (Horiuchi et al., 2007)**,** is proteolytically activated by a FURIN-like proprotein convertase (Endres et al., 2003). The deletion of TACE results in the development of an anti-inflammatory phenotype in macrophages (Driscoll, Vaisar, Tang, Wilson, & Raines, 2013).

Another essential enzyme in macrophage biology is Caspase-1, which processes and releases the pro-inflammatory IL-1β in a highly regulated process involving a multiprotein complex called the capase-1 inflammasome, which has been linked to the development of autoinflammatory and autoimmune diseases (e.g multiple sclerosis, Alzheimer's disease and Parkinson's disease) as well as metabolic disorders such as atherosclerosis and type 2 diabetes and obesity (Martinon et al., 2006; Strowig, Henao-Mejia, Elinav, & Flavell, 2012). Inflammasomes assemble in the cytosol after detecting PAMPs or DAMPs and act as a scaffold to recruit pro-caspase-1 (Lamkanfi & Dixit, 2014; Martinon, Burns, & Tschopp, 2002)**.** Numerous families of Patterns Recognition Receptors (PRRs) are important structures in the inflammasome complex, including NLRs and ALRs in both mice and humans (Takeuchi & Akira, 2010). The Capase-1 inflammasome regulates the activation of Caspase-1 and promotes the inflammatory response triggered by pathogens and molecules derived from host proteins. The most studied structure of the inflammasome is NLRP3, which is induced by stimuli such as ATP, pore-forming toxins, crystalline substances, nucleic acids, hyaluronan, and fungal, bacterial or viral pathogens (Guo, Callaway, & Ting, 2015). Caspase-1 is constitutively expressed in both M1 and M2 macrophages and it is activated in M1 macrophages through self-cleavage in the inflammasome complex.

The modulation of the Caspase-1 cascade plays a role in switching a macrophage from the pro-inflammatory towards its anti-inflammatory phenotype (Pelegrin & Surprenant, 2009). Furthermore, previous data also suggest a functional link between PCSK activity and IL-1β. For instance, the proteolytic cleavage of the anthrax lethal toxin by FURIN activates Caspase-1 in macrophages (Bugge & Leppla, 2006) and the inhibition of FURIN in an experimental model of arthritis in mice is associated with the production of high levels of IL-1β (H. Lin et al., 2012).

In this study, we addressed the role of FURIN in the proteolytic maturation of the anti-inflammatory cytokine TGF-β1 as well as the enzymes TACE and Caspase-1 which are essential for the secretion of the pro-inflammatory cytokines TNF-α and IL-1β in macrophages. Our findings indicated that FURIN KO macrophages secreted significantly lower levels of bioactive TGF-β1 than cells from wild-type controls (without affecting the mRNA levels of *Tgfb1*). This could explain at least in part the pro-inflammatory phenotype displayed by resting and LPS-stimulated FURIN-deficient peritoneal macrophages. In marked contrast, the lack of FURIN increased the relative levels of the mature TACE protein in LPS-stimulated peritoneal macrophages, which correlated with the high levels of TNF-α production in LPS-challenged LysMCre-fur$^{(fl/fl)}$ mice *in vivo*. These data do not support the idea that FURIN is the bona-fide PCSK that activates TACE. Instead, they suggest that other macrophage-expressed and LPS-induced PCSKs, such as PCSK6 or PCSK7, could be more important in the maturation of TACE *in vivo*. Similar to TACE, the analysis of Caspase-1 processing demonstrated a higher production of Caspase-1p20 in FURIN-deficient macrophages, which is consistent with the elevated levels of serum IL-1β**.** Remarkably, this study reveals that FURIN is a novel proteolytic regulator of the key pro-inflammatory cytokine IL-1β.

Overall, a FURIN deficiency in myeloid cells reduces the production of the anti-inflammatory TGF-β1, but upregulates the activation of the TACE and Caspase-1 enzymes, which activate the key pro-inflammatory cytokines TNF-α and IL-1β, respectively. These findings along with the intrinsic upregulation of pro-inflammatory mRNAs contribute to the pro-inflammatory phenotype of the LysMcre-fur$^{(fl/fl)}$ mice.

Although a deeper understanding of the mechanisms involved in the aberrant cytokine expression in FURIN deficient macrophages requires further investigation, our observations suggest that the maturation of TGF-β1 and the altered production of TACE and Caspase 1 are likely contributing events**.** Interestingly, an intriguing possibility that could explain the upregulation of Caspase-1 in FURIN deficient macrophages is the negative regulation of the NLRP3 inflammasome.

We conclude that the inhibition of FURIN specifically in innate immune cells might strengthen host responses during infections. Accordingly, this effect, together with the plausible reduction in the infectivity of PCSK-dependent pathogens, could potentially be applied to the treatment and prevention of PCSK-dependent infections.

6.1.5 FURIN-deficient zebrafish infected with *Mycobacterium marinum* exhibited a pro-inflammatory phenotype (II).

Mycobacterium marinum is a fish and amphibian pathogen, which is a relative of *Mycobacterium tuberculosis* and causes a mycobacterial disease with striking pathological and histological similarities to human tuberculosis (Parikka et al., 2012). Accordingly, an *M. marinum* infection in zebrafish is a valuable, cost-effective and ethical model for studying the human mycobacterial disease.

In publication I, we demonstrated a role for FURIN expressed in myeloid cells in the modulation of the response to LPS-induced endotoxemia, whereas in study II, the Furin-deficient zebrafish allowed us to address the role of FURIN in the regulation of immune responses in the context of an infection.

Our findings demonstrated the upregulation of *furinA* during a mycobacterial infection in adult zebrafish. In addition, similar to our observations in peritoneal and bone marrow macrophages in LysMcre-fur$^{(fl/fl)}$ mice, the infected *furinA*$^{td204e/+}$ mutants exhibited an enhancement of the early innate immune response characterized by elevated expression levels of pro-inflammatory cytokine genes (*tumor necrosis factor a* (*tnfa*), *lymphotoxin alpha* (*lta*) and *interleukin 17a/f3* (*il17a/f3*). In addition, the deficient expression of *furinA* in adult zebrafish correlated with a reduction in the number of copies of *M. marinum* (low-dose infection model), suggesting that FurinA weakened the protective host responses against mycobacteria.

This study confirmed the critical role for FURIN in the regulation of the innate immune response in the context of a mycobacterial infection. Further studies of a *mycobacterium tuberculosis* infection in Furin deficient cells in mice models could definitely contribute to deepening our understanding of the immune regulatory role of this enzyme in the context of a TB infection.

6.2 T-cell-expressed proprotein convertase FURIN inhibits the development of DMBA/TPA-induced skin cancer (III).

FURIN and other PCSKs have been implicated in the pathogenesis of tumorigenesis and metastasis due to their role in the maturation/activation of important substrates associated with carcinogenesis, such as growth factors, receptors, matrix metalloproteinases, and adhesion molecules (Bassi et al., 2005). The high expression of PCSKs in several human cancers and cancer cell lines has been positively correlated with the proliferation and invasiveness of the tumor in experimental settings (Bassi et al., 2001; Bassi et al., 2005). Consequently, the inhibition of PCSK as a cancer treatment has raised considerable interest, since the suppressed activity of PCSKs imply limited production of important molecules associated with tumorigenesis and metastasis, such as matrix metalloproteinases and growth factors. FURIN inhibitors that silence FURIN mRNA systemically, for example bi-shRNAi(furin)/GMCSF DNA/autologous tumor cell (FANG) (Senzer et al., 2012), have been successfully applied to patients with Ewing's Sarcoma (Ghisoli et al., 2016), an advanced stage of ovarian cancer and colorectal cancer with liver metastasis (Nemunaitis et al., 2014; Oh et al., 2016). In contrast, some studies in liver cancers associated FURIN overexpression with the supression of tumor growth and a better postoperative survival (Declercq et al., 2015; Huang et al., 2012), indicating that the systemic inhibition of FURIN could have a detrimental effect on the treatment of certain types of cancers.

Despite the eagerness of the scientific community to apply FURIN inhibitors to the treatment of cancer, contradictory results have increasingly demonstrated the importance to delve into the role of FURIN at the molecular and cellular level during carcinogenesis. Here (III), we demonstrated that when expressed in T cells, FURIN plays and essential role in the T-helper response balance during tumor development in a multistage chemical skin carcinogenesis model.

6.2.1 CD4cre-fur$^{(fl/fl)}$ mice display a distinctive papilloma pathogenesis

Previously we have shown that the lack of FURIN in T cells and macrophages (I, II) generates an inherent activated phenotype in both cell types (Pesu et al., 2008). These observations could imply that FURIN deficient immune cells are more efficient in promoting the antitumoral response. Unexpectedly, the opposite was confirmed after CD4cre-fur$^{(fl/fl)}$ mice exposed to DMBA/TPA treatment were shown to display higher numbers of papillomas that arose earlier when compared to CD4 WT control mice. In contrast to CD4 WT mice, CD4cre-fur$^{(fl/fl)}$ mice showed an inherently thicker epidermis characterized by higher epidermal cell proliferation that was sustained after 17 weeks of a DMBA/TPA treatment.

Our findings revealed that CD4cre-fur$^{(fl/fl)}$ mice, but not LysMcre-fur$^{(fl/fl)}$ mice, developed more papillomas in accordance to several studies that have demonstrated the essential role of αβ T cells in promoting tumor development (Girardi et al., 2003; Yusuf et al., 2008). Our investigation at the cellular level in the skin draining lymph nodes of CD4cre-fur$^{(fl/fl)}$ mice unveiled no major effects of the DMBA/TPA treatment concerning the numbers of CD4+ or CD8+ T cell populations. Nevertheless, the skin dLNs of CD4cre-fur$^{(fl/fl)}$ mice exhibited an inherently higher number CD4+CD25+Foxp3+ T regulatory cells and elevated numbers of activated CD4+CD69+ and CD8+CD69+ T cells that persisted after treatment. In accordance with the described role of IFN-γ in tumor promotion at the early stages of papilloma development (Xiao et al., 2009), we found that during the early phase of tumor development CD4cre-fur$^{(fl/fl)}$ mice harbored higher numbers of pro-inflammatory IFN-γ producing T cells than did wt mice. Furthermore, our findings that FURIN deficient T cells produced more Th2/Th17 type cytokines after 17 weeks of a DMBA/TPA treatment support the observation that IL-17A plays an essential role in tumor promotion in both human non-melanoma skin cancer and in mouse models of skin cancer (Nardinocchi et al., 2015; L. Wang, Yi, Zhang, Pardoll, & Yu, 2010). Consequently, the Th2/Th17 type cytokine profile exhibited by FURIN-deficient T cells could promote carcinogenesis at later stages.

LysMcre-fur$^{(fl/fl)}$ mice and the inactivating mutation in the *furin*A gene in zebrafish infected with *Mycobacterium marinum* have demonstrated a role for FURIN in the regulation of the pro-inflammatory response displayed by the components of the innate immunity. Nevertheless, the pro-inflammatory phenotype observed in FURIN-deficient myeloid cells (I) did not show to have major effects on papilloma

development in the multistage chemical carcinogenesis skin cancer model. It is important to highlight that we have not characterized the skin macrophages in LysMcre-fur$^{(fl/fl)}$ mice. Consequently, we have not throughly assessed the real impact of Furin-expressed macrophages in the context of skin carcinogenesis.

6.2.2 FURIN has a multifaceted role in T-cell-dependent immunity

FURIN has a versatile effect on T-cell-dependent immunity due to its role in the maturation of numerous substrates, which display different roles in T cell biology, such as TGF-β1, TACE, NOTCH1, etc. (Ortutay et al., 2015). For example, FURIN has been shown to regulate the peripheral tolerance through the control of the bioavailability of TGF-β1 in Tregs (Pesu et al., 2008). TGF-β1 is a pleiotropic growth factor with a dual role in immunosurveillance as demonstrated by its function as a promoter or suppressor of tumorigenesis (M. L. Chen et al., 2005). Notably, a TGF-β1 deletion in T cells has been shown to reduce the invasiveness of a B16-OVA tumor, suggesting that TGF-β1 derived from activated CD4+ T cells plays a role in immunosurveillance (Donkor, Sarkar, & Li, 2012).

Furthermore, we have demonstrated that FURIN can regulate the balance of a T helper type response in CD4cre-fur$^{(fl/fl)}$ mice infected with the intracellular *Toxoplasma gondii* parasite. We observed that CD4cre-fur$^{(fl/fl)}$ chronically infected with the *Toxoplasma gondii* parasite displayed a diminished Th1 type response, which indicates that FURIN-deficient T cells are unable to generate an appropriate protective immune response. In addition, OVA-specific FURIN KO CD4+ T cells favored the polarization toward a Th2 type response (A. Oksanen et al., 2014). These results are in accordance with the shift from the Th1 to the Th2/Th17 type immune response that occurred in CD4cre-fur$^{(fl/fl)}$ mice after 17-weeks of a DMBA/TPA treatment. Consequently, the inhibition of FURIN correlates with the promotion and modulation of immune responses mediated by T-helper cells in the context of tumor development.

In addition, although the biological role of FURIN in CD8+ cytotoxic lymphocytes have been incompletely addressed, our results showed that FURIN-deficient CD8+ T cells upregulate the activation marker CD69 and produce high levels of IFN-γ (III).

Collectively, we demonstrated that FURIN expression in T cells has a role in the immune modulation of skin carcinogenesis in a mouse model of multistage chemical carcinogenesis. Thus the inhibition of FURIN at a systemic level or specifically in T

cells may boost the development of certain cancer types that are mainly caused by chronic insults to the immune system. These results highlight the importance of careful consideration when using FURIN inhibitors in the treatment of human cancers.

In studies I and III we use conditional knockout animals based on the Cre-*loxP* system. It is important to address some of the limitations of this system that could impact the results of the investigation. For instance, the efficiency of the Cre-*loxP* system depends on several factors, such as the activity of Cre, the nature of floxed genes, and the role of the floxed genes in cell proliferation and/or survival. In addition, the expression levels of a tissue-specific Cre are determined by the activity regulatory factors that control an endogenous lineage-specific gene. The efficiency of a gene deletion is also determined by the floxed gene to be deleted. The efficiency of the deletion may thereby vary from gene to gene (S. Sharma & Zhu, 2014). The CD4-cre and LysM-cre lines used in publications I and III display a high deletion efficiency in the cell populations that were studied and the efficiency of the deletion was monitored by *in vitro experiments*.

Another limitations that has been reported for the Cre-*loxP* system is the unexpected deletion of genes due to ectopic Cre expression or the loss of enhancers or repressors that regulate the promoter activity and Cre-mediated DNA damage (Schmidt-Supprian & Rajewsky, 2007). Maintaining the copy number of Cre when designing breeding strategies in CD4-cre and LysM-cre lines reduces this effect (Vannella et al., 2014).

Recently, one study using LysM-cre lines demonstrated that during inflammation and in a autoimmune disease setting some specific populations of macrophages modify the Cre-mediated deletion efficiency and specificity compared to naïve conditions (Vannella et al., 2014).

Future analyses using technologies such as CRISPR/CAS will likely help to overcome some of the limitations we face using the Cre-*loxP* systems.

7 CONCLUSIONS

FURIN has been shown to display functional versatility in the cells of the immune system. First, our functional analysis of FURIN-deficient T cells showthat FURIN acts as a key regulator of T cell biology *in vivo* by maintaining peripheral tolerance (Pesu et al., 2008). In addition, the role of FURIN in the regulation of the polarization T helper cells demonstrate this enzyme as an important factor in the orchestration of the immune response (A. Oksanen et al., 2014). Next, the investigation (I, II) of the role of FURIN in the innate immunity, specifically in resting and LPS-challenged LysMcre-fur$^{(fl/fl)}$ mice as well as in *M. marinum* infected zebrafish *furinA* mutants convincingly confirms a critical role for FURIN also in the myeloid-cell dependent regulation of immunity and consequently in the adequate orchestration of the response against infections.

Finally, our recent study on the role of FURIN expressed in T and myeloid cells in skin cancer (III) demonstrates that the effect of FURIN inhibition is cell-type and context dependent. Consequently, the systemic inhibition of FURIN must be addressed with extreme caution when it is considered for the treatment of human malignancies.

To deepen our understanding of the mechanisms involved in the immune regulatory function of FURIN, further studies need to be performed. Certainly, the application of novel genome editing technologies such as CRISPR-Cas9 will provide better insights concerning the regulation of FURIN/PCSKs, their redundancy *in vivo* and their specific molecular and cellular roles in health and disease.

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10 ORIGINAL COMMUNICATIONS

Research Paper: Immunology

Myeloid cell expressed proprotein convertase FURIN attenuates inflammation

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ABSTRACT

The proprotein convertase enzyme FURIN processes immature pro-proteins into functional end- products. FURIN is upregulated in activated immune cells and it regulates T-cell dependent peripheral tolerance and the Th1/Th2 balance. FURIN also promotes the infectivity of pathogens by activating bacterial toxins and by processing viral proteins. Here, we evaluated the role of FURIN in LysM+ myeloid cells *in vivo*. Mice with a conditional deletion of FURIN in their myeloid cells (LysMCre-*fur*$^{(fl/fl)}$) were healthy and showed unchanged proportions of neutrophils and macrophages. Instead, LysMCre-*fur*$^{(fl/fl)}$ mice had elevated serum IL-1β levels and reduced numbers of splenocytes. An LPS injection resulted in accelerated mortality, elevated serum pro-inflammatory cytokines and upregulated numbers of pro-inflammatory macrophages. A genome-wide gene expression analysis revealed the overexpression of several pro-inflammatory genes in resting FURIN-deficient macrophages. Moreover, FURIN inhibited *Nos2* and promoted the expression of *Arg1*, which implies that FURIN regulates the M1/M2-type macrophage balance. FURIN was required for the normal production of the bioactive TGF-β1 cytokine, but it inhibited the maturation of the inflammation-provoking TACE and Caspase-1 enzymes. In conclusion, FURIN has an anti-inflammatory function in LysM+ myeloid cells *in vivo*.

INTRODUCTION

The components of the innate immunity implement the recognition and elimination of microbes through a complex machinery involving factors that require a post-translational proteolytic activation step to perform their effector functions. The functional maturation of dormant pro-proteins is catalyzed by the proprotein convertase subtilisin/kexin (PCSK) enzymes (PCSK1-2, FURIN, PCSK5-7, membrane-bound transcription factor site 1, PCSK9) [1]. The first seven PCSKs operate at a similar target site consisting of basic amino acids [K/R)-(X) n-(K/R)↓, with *n* being 0, 2, 4 or 6 and X any amino acid], which results in a significant degree of functional redundancy in target recognition and processing *in vitro*. However, humans bearing mutations in the PCSK genes as well as PCSK-deficient experimental animal models have gene-specific phenotypes, which argues for substrate specificity *in vivo* [2].

FURIN was the first discovered and is thus the most studied conventional PCSK enzyme [3]. FURIN is ubiquitously expressed, and in cells it catalyzes the maturation of its targets in the secretory pathway, endosomes and on the cell surface. Due to its widespread expression, FURIN has a plethora of reported targets including cytokines, chemokines and growth factors as

well as other proteases like matrix metalloproteinases. In mice, the expression of *Furin* is essential for embryonic development, which has imposed limitations to our understanding of its cell-type specific function *in vivo* [3, 4]. However, the phenotypes of tissue-specific *Furin* deficient mice have demonstrated that FURIN cannot be compensated for by other PCSK enzymes in endothelial cells (Tie2Cre) or in T lymphocytes (CD4Cre) [5, 6].

FURIN's regulatory role is also implicated in multiple human pathologies. For example, FURIN processes the beta-secretase enzyme in Alzheimer's disease, SNPs in the *Furin* gene are associated with blood pressure levels, and elevated FURIN expression promotes metastatic activity in various cancer types, and the protein is found in advanced atherosclerotic plaques [7-10]. FURIN is an important modulator of the T-cell-dependent adaptive immunity; it becomes upregulated by T-cell-receptor-mediated signaling and through the IL-12/STAT4 pathway in T helper type 1 cells [11, 12]. A conditional deletion of FURIN in T cells results in the aberrant polarization of T helper cells, a lack of a protective cell-mediated host-defense as well as the spontaneous development of autoimmunity in aging animals due to a breakage in peripheral CD4+Foxp3+ T-regulatory-cell-dependent immune tolerance [6, 13]. Consequently, targeting the activity of FURIN/PCSK has been reported to be beneficial for the experimental treatment of, for example, malignancies and rheumatoid arthritis [9, 14-16].

Previous reports have also implicated a role for PCSK enzymes in spleen [17]. FURIN is expressed in splenic red pulp, a zone enriched with macrophages, which regulate extramedullary myelopoiesis, the removal of senescent red cells, the cross-presentation of antigens as well as tolerance to self-antigens [18-20]. In addition, PCSK1 deficient mice show a marked disorganization of the marginal zone and red pulp [21]. In addition to the cellular pro-proteins also the components of several infectious agents, including the envelopes of the HI and Influenza viruses as well as the toxins of *Bacillus antracii* and *Pseudomonas aeruginosa* require a PCSK-dependent proteolysis step to exert their pathogenic function [22-24]. Therefore, inhibitors can protect the host from invading PCSK-dependent pathogens and serve as adjuvants to antibiotics [25]. As the innate immune system forms the first line of defense, targeting PCSKs specifically in myeloid cells could be a potent and well-tolerated strategy to block infections. However, the consequences of PCSK inhibition specifically in the cells of the innate immune system *in vivo* have remained ambiguous. To address this conundrum we have here characterized a novel tissue-specific knock-out mouse model, in which FURIN is deleted in Lysozyme M positive cells, i.e. chiefly in activated macrophages and granulocytes (LysMCre-*fur*$^{(fl/fl)}$) [26].

RESULTS AND DISCUSSION

LysMCre-*fur*$^{(fl/fl)}$ mice have a reduced number of splenocytes and elevated levels of the pro-inflammatory IL-1β cytokine in their serum

Previous studies using germ-line deletions and siRNA have demonstrated that PCSK1 and PCSK7 modulate the secretion process of innate cytokines [21] and rescue unstable MHCI molecules on dendritic cells, respectively [27]. Others and we have further shown that FURIN is upregulated in the LPS activated CD14+ cells [10], and in the plasma of chronic typhoid carriers [28]. To directly address whether FURIN expression in myeloid cells regulates immunity *in vivo*, we generated a mouse model with a conditional deletion of FURIN in LysM+ cells. LysMCre-*fur*$^{(fl/fl)}$ mice were born under normal Mendelian ratios, and unlike T-cell specific CD4Cre-*fur*$^{(fl/fl)}$ mice [6], they did not show age-related health problems such as inflammatory bowel disease (supplementary Figure S1A, B). Notably, in contrast to the reportedly large spleens of PCSK1 KO mice [21] LysMCre-*fur*$^{(fl/fl)}$ animals had significantly reduced numbers of splenocytes compared to controls, which could indicate that FURIN regulates hematopoiesis in LysM+ cells, or cellular homeostasis in the spleen (Figure 1A, supplementary Figure S1C, D). The mechanism(s) leading to the reduction in splenocyte numbers in LysMCre-*fur*$^{(fl/fl)}$ animals remains unclear, but the lowered FURIN levels in the bone marrow (ca. 60% less mRNA expression, supplementary Figure S2A) could disrupt the delicate microenvironment by reducing the proteolytic maturation of hematopoietic growth factors or extracellular matrix receptors [29].

As expected, FURIN was almost completely (>95% less mRNA) absent from both the neutrophils and macrophages of LysMCre-*fur*$^{(fl/fl)}$ mice (supplementary Figure S2B, C). A flow cytometric analysis demonstrated that the lack of FURIN did not significantly alter the proportions of macrophages ($F4/80^+CD11b^+$) and neutrophils ($Gr1^+CD11b^+$) in the peritoneal cavity (Figure 1B), spleen (supplementary Figure S3A) or bone marrow (supplementary Figure S4A). We next analyzed whether deleting FURIN in myeloid cells imposed secondary effects on lymphoid cells, and found that the gross numbers of splenic $CD3^+$ T and $B220^+$ B cells were unaffected (supplementary Figure S3B). Instead, there was a small, but significant reduction in the numbers of $CD3^+CD4^+CD8^-$ T helper cells in the spleen while the proportion of $CD3^+CD4^-CD8^+$ cytotoxic T cells was elevated (supplementary Figure S3C). The lower ratio of splenic $CD4^+/CD8^+$ T cells has previously been associated with inflammation [30], but the quantification of $CD62L^+$ T cells did not reveal marked differences in the numbers

of memory T cells between LysMCre-*fur*$^{(fl/fl)}$ and littermate control animals (supplementary Figure S3C). Collectively, the analyses of different cell compartments indicate that FURIN expression in LysM+ cells is not essential for the development of neutrophils or macrophages, but it promotes the development of splenocytes and may also indirectly affect the CD4$^+$/CD8$^+$ T cell ratio in the spleen.

We have previously demonstrated that deleting FURIN in T cells results in the upregulation of both the Th1 and Th2 signature cytokines in serum [6]. To address how FURIN expression in LysM+ cells controls the production of innate cytokines in steady state we measured the levels of pro-inflammatory IL-1β, TNF-α, IL-6 and MCP-1 cytokines as well as the anti-inflammatory IL-10 in serum (Figure 1C). These data demonstrated that FURIN expression in myeloid cells attenuates the production of pro-inflammatory IL-1β, which is indicative of an auto-inflammatory phenotype [31, 32].

Genome-wide RNA microarray analysis reveals the upregulation of several pro-inflammatory genes in FURIN deficient macrophages

Although proprotein convertases process their target molecules post-translationally, deleting PCSKs also results in aberrant gene expression signatures, which gives clues about the mechanism of the actions of PCSKs in a given cell type [6, 12, 33]. To address how FURIN regulates the genetic signature in macrophages we profiled their gene expression using qRT-PCR and microarray analyses. Similarly to human CD14+ monocytes [10] FURIN was efficiently upregulated in mouse peritoneal macrophages that were activated with LPS+/- IFNγ *in vitro*, (supplementary Figure S2B). The lack of FURIN in endothelial cells (Tie2Cre) causes the compensatory expression of other biochemically redundant PCSKs [5], which interferes with the interpretation of FURIN's biological significance. However, we observed that deleting FURIN in macrophages did not affect the expression of other PCSK enzymes (supplementary Figure S2D). These data indicate that the LysMCre-mediated deletion can be reliably used to assess the specific role of FURIN in LysM+ cells.

In order to characterize the FURIN-dependent global gene expression patterns we performed a genome-wide microarray analysis using resting peritoneal macrophages from LysMCre-fur$^{(fl/fl)}$ and littermate WT animals (Figure 2A, 2B). FURIN deficient peritoneal macrophages displayed a reproducible upregulation of many genes expressed in activated macrophages, such

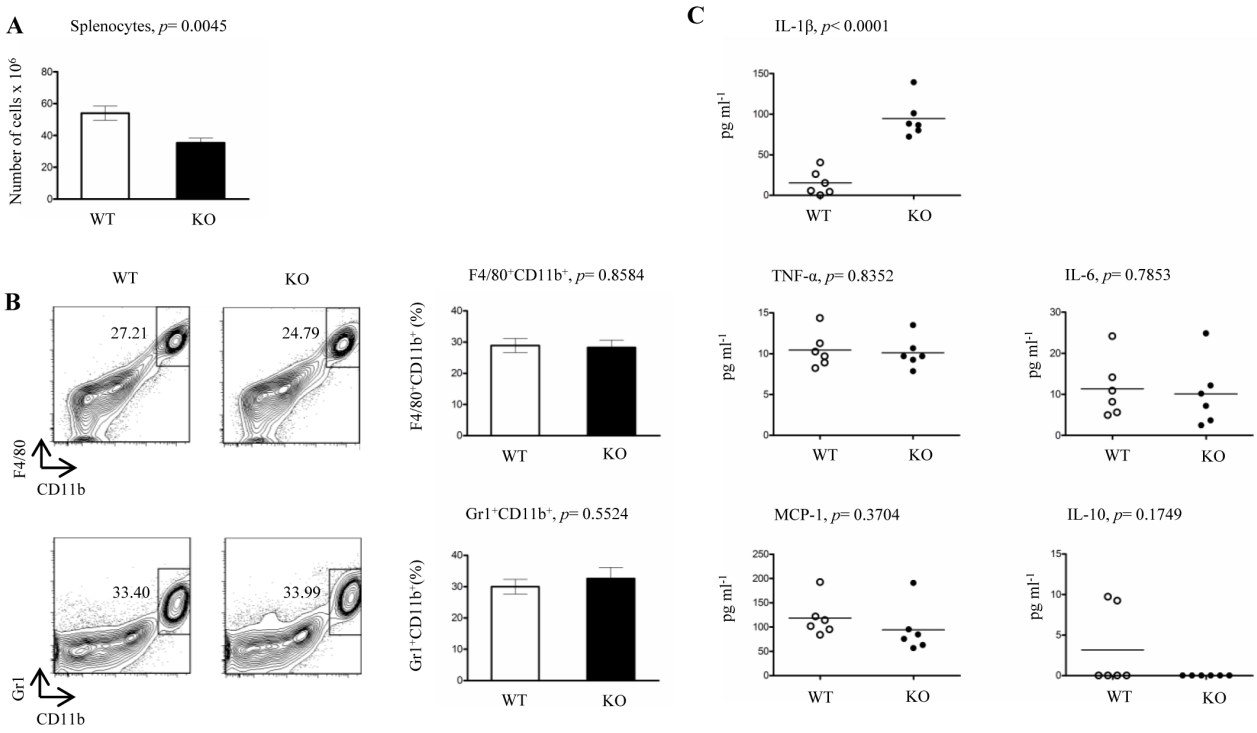

Figure 1: Proportions of splenocytes, peritoneal macrophages, neutrophils and serum cytokines in steady state LysMCre-*fur*$^{(fl/fl)}$ mice. A. Total numbers of live splenocytes from LysMCre-*fur*$^{(fl/fl)}$ (KO) and WT littermate control mice were calculated using trypan blue staining (n = 7/genotype, 6-8 weeks of age). **B.** Flow cytometric analyses of F4/80$^+$CD11b$^+$ (macrophages) and Gr1$^+$CD11b$^+$ (neutrophils) peritoneal cells were performed from LysMCre-*fur*$^{(fl/fl)}$ and WT littermate control mice (n = 4/genotype, 6-8 weeks of age). **C.** Levels of serum cytokines from steady state LysMCre-*fur*$^{(fl/fl)}$ and WT littermate control mice (n = 6/genotype, 6-8 weeks old mice). Plots represent average ± SEM. Statistics were calculated with the two-tailed unpaired Student's t-test.

as *Serpinb1a, Serpinb2, Hcar2 , Egr1, Il6, Il1β, Ptgs2, Ccl2, Ccl7 and C5ar1* [34-38]. In addition, we observed an enhanced expression of *Dusp6* and *Fcgr1,* which are downregulated in alternatively activated macrophages [39]. In contrast, among the downregulated genes of FURIN deficient macrophages, we detected for example *Atf7* whose deletion is associated with the constitutive activation of macrophages [40]. FURIN is induced by its substrate TGF-β1 [41]. Accordingly, the expression of *Ccnd1* was enhanced in the absence of FURIN, similarly to what was seen in TGF-β1 null cells [42]. These data suggest that FURIN has an intrinsic inhibitory function on the expression of genes that associate with the activation of pro-inflammatory M1 type macrophages [43].

LysMCre-*fur*$^{(fl/fl)}$ mice show increased lethality, upregulated pro-inflammatory cytokines and elevated numbers of Ly6-C+ macrophages after an LPS injection

Both the high IL-1β levels in serum and the results from the microarray analysis pointed to a pro-inflammatory phenotype in LysMCre-*fur*$^{(fl/fl)}$ mice. To test if FURIN in LysM+ cells also restrains inflammatory responses *in vivo* we subjected mice to lipopolysaccharide (LPS) triggered inflammation. LysMCre-*fur*$^{(fl/fl)}$ and littermate WT mice were first challenged with a single i.p. injection of 25 mg/kg (LPS) and monitored for 72 hours (Figure 3A). We observed a significantly higher mortality in LysMCre-*fur*$^{(fl/fl)}$ mice 24 hours post-injection (62% mortality in KO *vs*. 38% mortality in WT) (p =

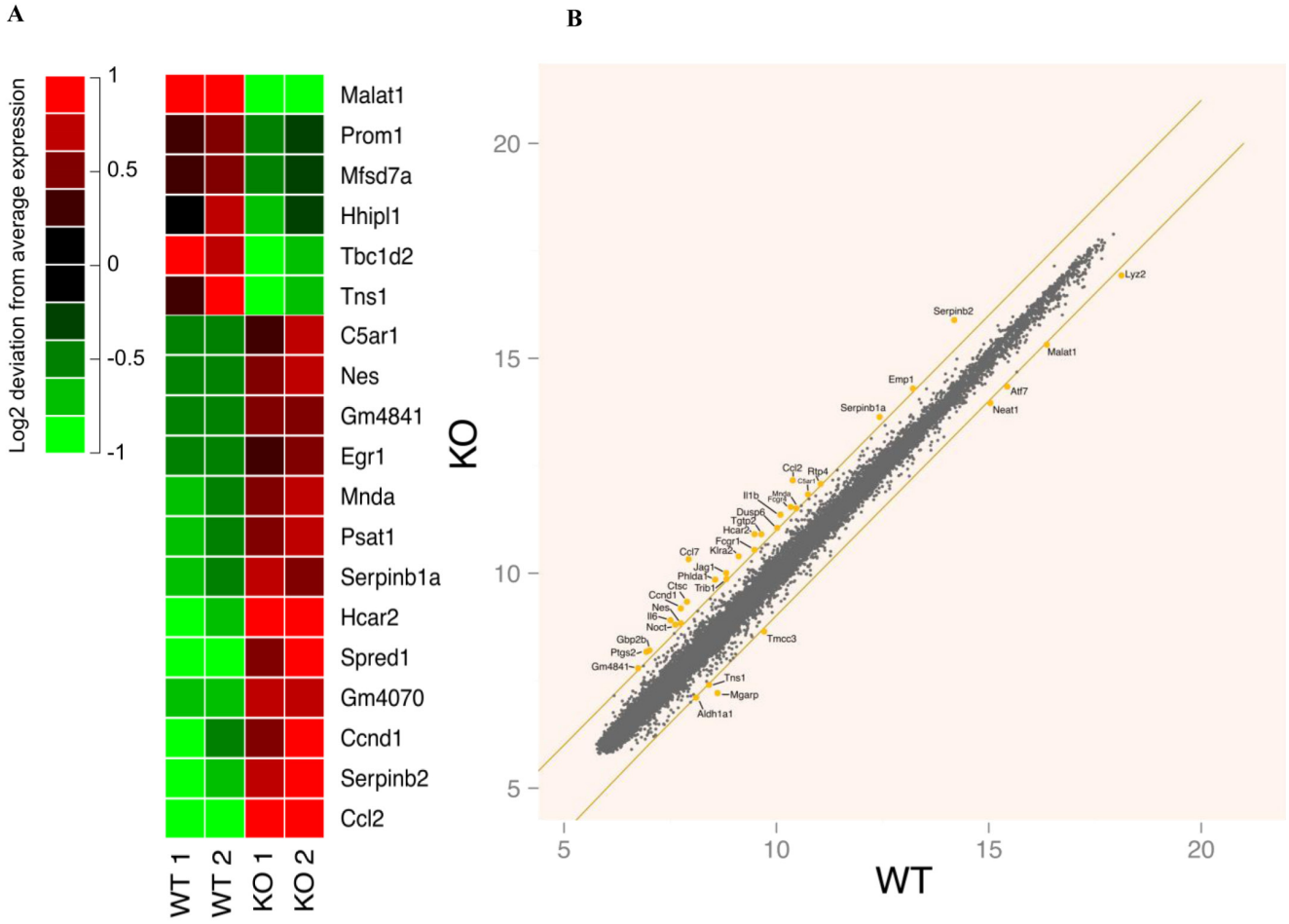

Figure 2: Genome-wide gene expression analyses in resting wild type and FURIN deficient peritoneal macrophages. **A.** The 19 most differentially (log fold change > |1| and multiple testing corrected p-value < 0.05) expressed genes between wild type and FURIN deficient unstimulated peritoneal macrophages. For visualization purposes, the mean of each gene's expression has been subtracted from individual expression values to highlight the differences between the samples. The red and green colors indicate an induction or suppression of gene expression, respectively, relative to the mean across samples. The figure shows two biological replicates for both genotypes. **B.** A scatter plot of the mean gene expression across replicates of wild type and FURIN deficient unstimulated peritoneal macrophages. Selected genes with a log fold change > |1| have been highlighted. The orange lines show the limit where the log fold change equals 1 or -1.

0.0109), which indicates an increased sensitivity to a pro-inflammatory stimulus.

A lower dose of LPS (100 µg/kg) was then used to evaluate how the myeloid-cell-expressed FURIN regulates cytokine levels in the serum and the generation/migration of pro-inflammatory F4/80^{+}CD11b^{+}Ly6C^{+}Ly6G^{-} monocytes/macrophages, which are characterized by accelerated inflammatory effector functions including the high production of IL-6, IL-1β, TNF-α, NOS2 and CCL2 [44, 45]. LPS-injected LysMCre-*fur*$^{(fl/fl)}$ mice had significantly enhanced levels of the pro-inflammatory cytokines TNF-α and IL-6 1 and 3 hours post-infection, respectively, whereas the production of anti-inflammatory IL-10 was reduced at the 3-hour time-point (Figure 3B-3D).

In addition, at 3 hours post-injection there was a significant augmentation in the proportion of F4/80^{+}CD11b^{+}Ly6C^{+}Ly6G^{-} M1 type macrophages in the spleen (Figure 3E). A qRT-PCR analysis on *ex vivo* activated FURIN KO macrophages further showed an upregulation in the expression of *Nos2* (M1 marker gene) upon an LPS/IFN-γ stimulus, whereas the IL-4 induced expression of *Arg1* (M2 marker gene) was clearly reduced [43] (Figure 3F). Previously, the prominence of the M1 over the M2 macrophage phenotype has been associated with an increased susceptibility to a septic shock [46, 47], as was observed in LPS-challenged LysMCre-*fur*$^{(fl/fl)}$ mice.

PCSKs control the quantity and activation of the human Toll-like receptor 7 (TLR7) host responses by direct proteolysis [48]. We next tested if the expression levels of LPS/TLR4-dependent genes are affected by the FURIN deficiency in peritoneal macrophages. First, the time course changes in the mRNA expression of the *Il1b, Tnfa*, *Il6* and *Il10* genes indicated an upregulated base line expression but roughly similar dynamics in response to LPS in FURIN deficient macrophages compared to

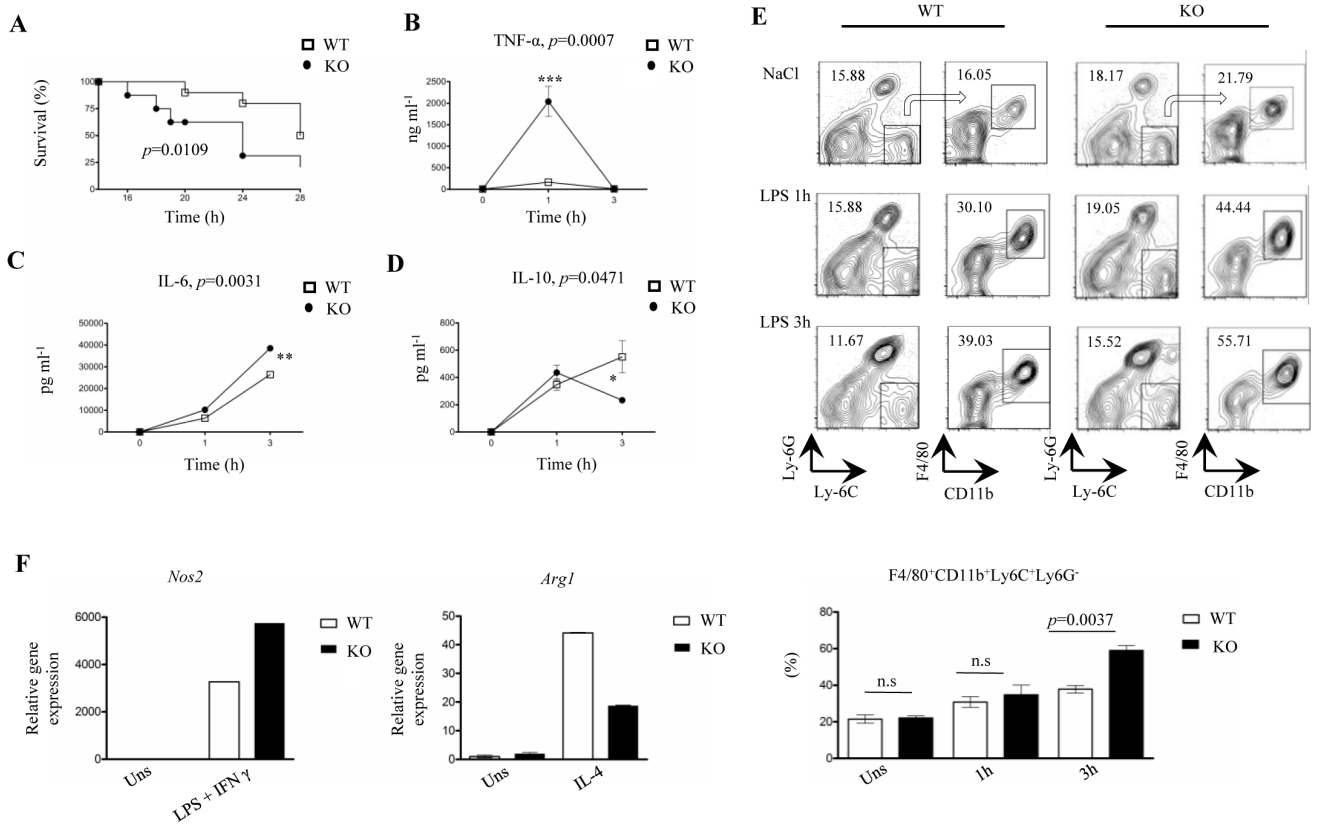

Figure 3: LPS-challenged LysMCre-*fur*$^{(fl/fl)}$ mice exhibit higher mortality, accelerated inflammation and an upregulated pro-inflammatory macrophage response. A. Kaplan Meier survival curve for LysMCre-*fur*$^{(fl/fl)}$ and WT littermate control mice (n = 8/genotype, 6-8 weeks of age, all male) after the intraperitoneal injection of a single lethal dose of LPS (25 mg/kg). LysMCre-*fur*$^{(fl/fl)}$ mice exhibited a significantly higher mortality than WT littermate controls during the first 24 h. **B.-D.** Level of serum cytokines TNF-α, IL-6, IL-10 in LysMCre-*fur*$^{(fl/fl)}$ and WT littermate controls mice (n = 4/genotype, 6-8 weeks of age) after an intraperitoneal injection with NaCl (0.9%) or 100 µg/kg of LPS (plots represent average ± SEM). Statistics were calculated with the two-tailed unpaired Student's t-test. **E.** Flow cytometric analysis of a splenic F4/80^{+}CD11b^{+}Ly6C^{+}Ly6G^{-} cell population in LysMCre-*fur*$^{(fl/fl)}$ mice and WT littermate control mice (n = 3/genotype, 6-8 weeks of age) injected with NaCl (0.9%) or LPS (100 µg/kg) at 0, 1h and 3h. The arrows indicate the gating strategy. (Plots represent average ± SEM). Statistics were calculated using the two-tailed unpaired Student's t-test. **F.** *Nos2* and *Arg1* mRNA expression was assessed by quantitative RT-PCR in wild type and FURIN deficient peritoneal macrophages treated with LPS+IFN-γ (1 µg/ml+20 ng/ml) or IL-4 (50 ng/ml). One representative experiment out of three independent experiments is shown.

controls (Figure 4A). Likewise, a focused analysis of TLR-associated mRNAs using microarray data showed only minimal differences between the dynamic expression patterns of WT and FURIN KO macrophages (Figure 4B). However, the dynamic expression of several other pro-inflammatory genes, including *Trem1, Nos2, Il15, Il33, and Il12rb1*, was inhibited by FURIN in LPS activated macrophages (Figure 4C) [49-52]. These findings were accompanied by the repression of the genes *Ch25h, Olr1 and Arg1*, which are typically induced in alternatively activated macrophages [38]. Finally, we evaluated the expression of pro- and anti-inflammatory cytokines in peritoneal macrophages that were stimulated with various TLR ligands and IFN-γ (supplementary Figure S5). These results show that the anti-inflammatory function of FURIN cannot be attributed to a specific stimulus, but can be seen in TLR2, TLR4 and TLR7/8 activated macrophages. Collectively, these data suggest that FURIN is dispensable for the immediate TLR4 responses, but underscore the importance of FURIN as an anti-inflammatory factor in both resting and activated macrophages.

FURIN deficient macrophages secrete less bioactive TGFβ-1 cytokine but show elevated expression levels of the activated TNF-α Converting Enzyme (TACE) and Caspase-1 p20

The production of several cytokines secreted by macrophages, including TNF-α, IL-1β and TGF-β1, is dependent on proteolytic processing in the cell [53]. Specifically, TGF-β1 is initially produced as an inactive

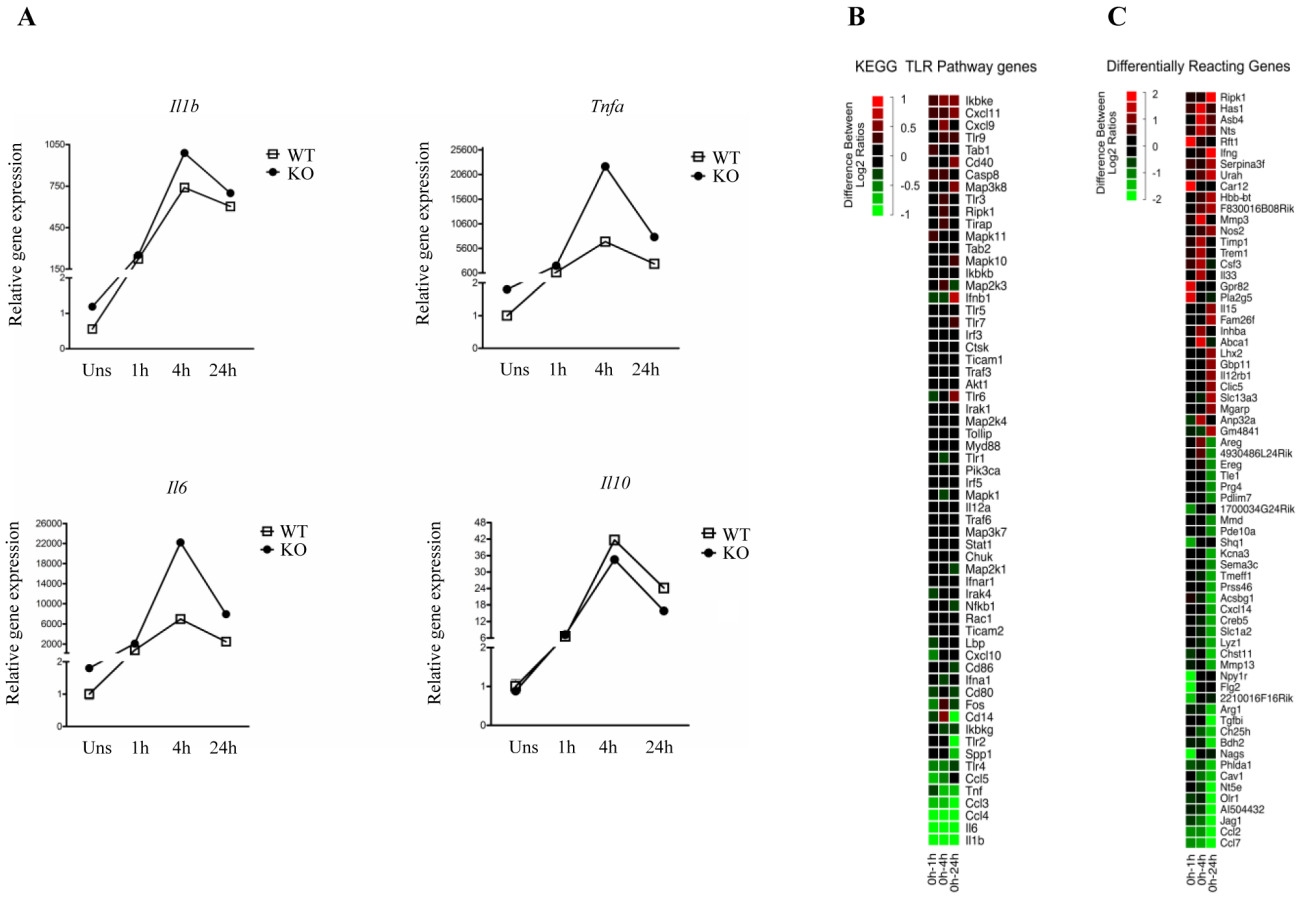

Figure 4: LPS-induced gene expression dynamics in wild type and FURIN deficient peritoneal macrophages. A. mRNA expression levels were determined using quantitative RT-PCR, and the normalized expression in the unstimulated WT sample was arbitrarily set to 1. Curves show changes in the expression of *Il1b*, *Tnfa*, *Il6* and *Il10* mRNAs in WT and FURIN KO peritoneal macrophages that were left unstimulated or were stimulated with LPS (1μg/ml) for 1-24h as indicated. The housekeeping gene 18S was used to normalize the gene expression. (The figure shows one representative experiment of two independent experiments ± SEM). **B.** Microarray analysis of the TLR signaling pathway genes. Log2 fold changes for all genes in both WT and FURIN KO peritoneal macrophages were computed for each time point with an LPS (1μg/ml) stimulation, respective to the unstimulated condition. At each time point, the ratio of the fold changes illustrates the degree of difference in the LPS response over time. The figure shows the average of two independent experiments. The red color indicates an increase and the green a decrease in the log2 fold change difference. **C.** Genome wide analysis of the genes with the strongest differential response to a LPS stimulation between WT and FURIN KO peritoneal macrophages. A selection of differentially responding genes showing greatest differences between ratios at one or several time points are presented. Data were processed as in B.

pro-cytokine, which is converted into an active factor via a complex post-translational cascade involving a cleavage step catalyzed by FURIN [6, 54]. Further, undermined TGF-β1 signaling has been associated with an impaired transit between the macrophage (M1/M2) phenotypes, sustained inflammation and delayed wound healing [55]. Also, TNF-α Converting Enzyme (TACE), which releases soluble TNF-α from its membrane-bound precursor, is proteolytically activated by a FURIN-like proprotein convertase [56] whose deletion results in the development of an anti-inflammatory phenotype in macrophages [57]. In addition, the activation/deactivation of the Caspase-1 cascade, which directly processes IL-1β, plays an important role in the dynamics of macrophage polarization [58]. Previous data also imply a functional connection between PCSK activity and IL-1β; the proteolytic cleavage of the anthrax toxin by FURIN activates Caspase-1 in macrophages [59] and high levels of IL-1β have been observed in an experimental model of arthritis in mice that were treated with a FURIN inhibitor [60].

In order to find out if FURIN regulates the proteolytic maturation of the aforementioned inflammatory cytokines in macrophages we analyzed the secretion of bioactive TGF-β1 as well as the proteolytic activation of TACE and Caspase-1. First, FURIN KO macrophages were found to secrete significantly lower levels of bioactive TGF-β1 in ELISA analyses than cells from wild-type controls, whereas the *Tgfb1* mRNA levels were not affected (Figure 5A, supplementary Figure S6). In contrast, we found that the lack of FURIN upregulated the mature TACE protein in LPS-stimulated peritoneal macrophages (Figure 5B), which is in line with the elevated TNF-α production in LPS-challenged LysMCre-*fur*$^{(fl/fl)}$ mice *in vivo* (Figure 3B). These data also indicate that FURIN is not the bona-fide PCSK that activates TACE, and suggest that another macrophage-expressed and LPS-induced PCSK enzyme, such as PCSK6 or PCSK7 (supplementary Figure S2D), could be more important for the maturation of TACE *in vivo* [61]. Finally, an analysis of Caspase-1 processing also showed a higher production of Caspase-1p20 in FURIN deficient macrophages (Figure 5C). These data are consistent with the observed higher levels of serum IL-1β and reveal a novel proteolytic regulator (FURIN) for this key pro-inflammatory cytokine.

The analyses of proteolytically activated macrophage cytokines thus showed that a FURIN deficiency reduces the production of anti-inflammatory TGF-β1 but upregulates the activation of the TACE

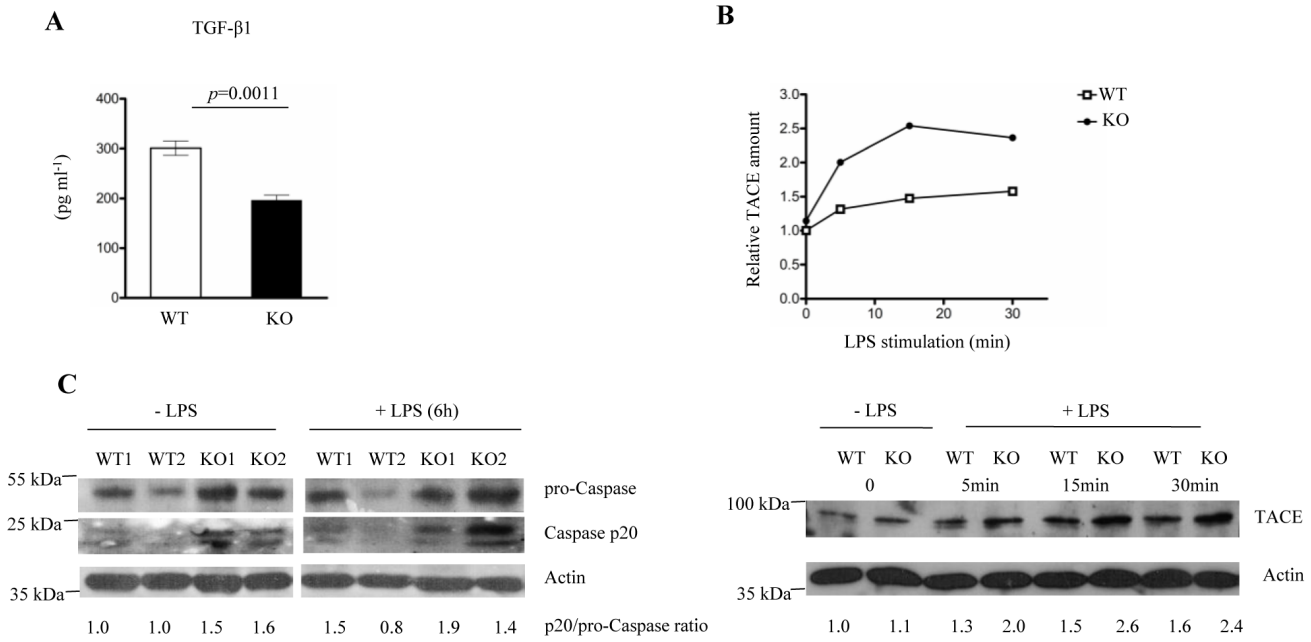

Figure 5: FURIN deficient macrophages produce less bioactive TGF-β1 and show hyperactivation of TACE and Caspase-1 p20. A. The production of bioactive TGF-β1 was measured in the supernatants of unstimulated wild type and FURIN deficient peritoneal macrophages using an enzyme linked immunosorbent assay (ELISA) (n = 3/genotype). Plots represent average ± SEM. Statistics were calculated with the two-tailed unpaired Student's t-test. **B.** WT and FURIN deficient peritoneal macrophages were left unstimulated or were stimulated with LPS (1 μg/ml) for 5-30 minutes as indicated. Mature Tumor Necrosis Factor-α-Converting Enzyme (TACE) (93 kDa) and β-Actin (43 kDa) were detected by western blotting. Normalized (TACE/Actin) levels are presented in the upper panel. Shown is one representative experiment out of three replicates with similar results. **C.** FURIN deficient or littermate wild type bone marrow macrophages (n = 2/genotype) were left unstimulated or were stimulated with ultrapure LPS (500 ng/ml) for 2 hours, then ATP (1mM) was added to the cells for 15 min. Pro-Caspase (55 kDa), Caspase-1 p20 (20 kDa) and actin (43 kDa) were detected with western blotting.

and Caspase-1 enzymes. These findings together with the inherent upregulation of pro-inflammatory mRNAs collectively contribute to the pro-inflammatory phenotype of LysMCre-*fur*$^{(fl/fl)}$ mice. Understanding the underlying mechanism(s) of aberrant cytokine expressions by FURIN deficient macrophages clearly requires further studies, but at least reduced TGFβ-1 maturation is likely to play a role [62]. In conclusion, our data indicate that inhibiting FURIN specifically in innate immune cells could strengthen host responses. This, together with the plausible reduction in the activation of pathogens, could be beneficial for treating and preventing PCSK-dependent infections.

MATERIALS AND METHODS

Experimental animals

Mice bearing floxed *fur* alleles [4] were backcrossed six times with C57BL/6 mice. LysMCre mice with the C57BL/6 background were purchased from Taconic. LysMCre mice were bred with *fur*$^{(fl/fl)}$ animals to generate myeloid-specific FURIN knockout mice LysMCre-*fur*$^{(fl/fl)}$. Mice were housed under pathogen-free standard conditions. All mouse experiments were performed in accordance with the National Animal Experiment Board, Finland, (Permit# ESAVI/2837/04.10.07/2015).

Isolation, culture and *ex vivo* activation of macrophages from the mouse peritoneal cavity and of neutrophils from the bone marrow

Peritoneal cells were extracted from the peritoneal cavity and cultured following a previously published protocol [63] After 1 h incubation WT and FURIN deficient peritoneal macrophages were stimulated with LPS (1 μg/ml, *E. coli* 0127:B8 serotype; Sigma-Aldrich, St Louis, MO, USA), TLR ligands (Zymosan: 10 μg/ml, R848: 1 μg/ml; both from InvivoGen, San Diego, CA, USA) and/or cytokines (IFN-γ: 20 ng/ml, IL-4: 50 ng/ml; PeproTech, Rocky Hill, NJ, USA). After stimulation, the cells were scraped to dislodge them and collected for RNA extraction (RNAeasy, Qiagen, Düsseldorf, Germany). Neutrophils were isolated from the bone marrow of LysMCre-*fur*$^{(fl/fl)}$ and wild-type mice with the Anti-Ly-6G MicroBead Kit (Miltenyi Biotec Norden AB, Lund, SE) and were left unstimulated or were stimulated with 100 μM of fMLP for 1 hour at 37 °C.

Flow cytometric analyses

Peritoneal, splenic and bone marrow cells were analyzed using flow cytometry (BD FACSCanto™ II) and the FlowJo software (Treestar Inc, Ashland, OR, USA). Anti-CD16/CD32 (Clone: 93) (eBioscience, San Diego, CA, USA) was used to block Fc receptors and cell populations were surface stained following the eBioscience FACS protocol with PerCP-Cy5.5- labeled anti- F4/80 (BM8), PE-labeled anti-Gr-1 (RB6-8C5), PE-Cy7-labeled anti-CD11b (M1/70), FITC-labeled anti-B220 (RA3-6B2), APC-labeled anti-CD3, PE-Cy7 labeled anti-CD4 (RM4-5), APC-H7 labeled anti-CD8 (53-6.7), PE-labeled anti-CD62L (MEL-14), PE-labeled anti-Ly-6G (RB6-8C5), APC labeled anti-Ly-6C (HK1.4), PE-Cy7-labeled anti-CD11c (N418), FITC-labeled anti-CD11b (M1/70) (eBioscience, San Diego, CA, USA).

Cytokine measurements

Serum cytokines were measured in both steady state and LPS injected mice. Briefly, LysMCre-*fur*$^{(fl/fl)}$ and WT littermate mice (6-8 weeks of age) were injected intraperitoneally with NaCl (0.9%) or LPS (100 μg/kg, *E. coli* 111:B4 serotype; Sigma-Aldrich) and serum was collected at the 0, 1, 3 h time points. The serum cytokines TNF-α, IL-6, MCP-1 and IL-10 were quantified using the BD™ Cytometry Bead Array Mouse Inflammation Kit (Catalog # 552364) according to the manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ, USA). IL-1β levels were measured with mouse IL-1 beta ELISA Ready-SET-Go!® (eBioscience, San Diego, CA, USA) (Catalog # 88-7013-22). Bioactive TGF-β1 was measured in the supernatants of cultured peritoneal macrophages isolated from LysMCre-*fur*$^{(fl/fl)}$ and WT littermate mice using Human/Mouse TGF beta 1 ELISA Ready-SET-Go! (2nd Generation) (Catalog # 88-8350-76) (eBioscience, San Diego, CA, USA).

LPS induced endotoxemia

LysMCre-*fur*$^{(fl/fl)}$ and WT littermate mice (6-8 weeks of age) were injected intraperitoneally with a lethal dose of LPS (25 mg/kg, *E. coli* 0111:B4 serotype; Sigma Aldrich, St Louis, MO, USA) or with NaCl (0.9%) as a control. Survival of the mice was monitored for 72 h at 3-hour intervals.

Quantitative real-time polymerase chain analyses

The mRNA expression in stimulated and non-stimulated peritoneal macrophages was assessed using qRT-PCR. Briefly, total RNA was extracted using the RNeasy kit (Qiagen, Düsseldorf, Germany), quantified using a NanoDrop ND-2000 (Thermo Fisher Scientific, Massachusetts, USA) and transcribed into cDNA with the iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Gene expression levels were examined using

the Bio-Rad CFX96 Real-Time System and the Sso Fast Eva Green Supermix (Bio-Rad, Hercules, CA, USA). The primers used for quantitative real-time PCR are shown in the supplementary Table S1. Gene expression was normalized to 18s levels; the normalized threshold cycle (Ct) values were subtracted from the target Ct values of each sample (deltaCt). The relative levels of the target mRNA were calculated as 2-deltadeltaCt.

Microarray data analysis

Two biological replicates of FURIN KO and WT peritoneal macrophages were left unstimulated or were stimulated for 1, 4 and 24 hours with LPS (1 μg/ml)(*E. coli* 0127:B8 serotype; Sigma Aldrich, St Louis, MO, USA). The samples were collected and the RNA was isolated using Qiagen RNeasy on-column DNase (Qiagen, Düsseldorf, Germany). 50 ng of purified RNA was amplified and dye labeled using Agilent's Low Input Quick Amp Labeling kit and RNA Spike In kit (Agilent Technologies, California, USA). Initial and purified RNA contents along with dyed and amplified cRNA contents were measured with a NanoDrop ND-2000 (Thermo Fisher Scientific, Massachusetts, USA). RNA and cRNA quality was inspected using the Agilent 2100 Bioanalyzer RNA 6000 Nano kit (Agilent Technologies, California, USA). 300ng of Cy-3 and 300ng of Cy-5 labeled sample were hybridized together on an Agilent Mouse Chip 8x60K (Design ID 028005) (Agilent Technologies, California, USA) overnight at 65°C using the Gene Expression Hybridization kit. The chips were washed with the Gene Expression Wash Pack according to the instructions. The chips were scanned using an Agilent Technologies Scanner model G2565CA using the scan profile AgilentG3_GX_2Color. Scan results were converted into numerical data by the Agilent Feature Extraction software version 10.7.3. The data was analyzed using the R software [64]. Raw probe level intensity values were normalized with loess regression and quantile normalization using R package "limma" to robustly generate sample-wise comparability [65].

Differential gene expression was evaluated using the log2-transformed fold change difference and statistical testing using the two-sample Student's *t*-test. Genes showing an average expression lower than the log2 transformed intensity value 6 were filtered out as non-informative. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database was used to obtain a gene set involved in Toll-like receptor signaling. [66]. The raw microarray data are available at Gene Expression Omnibus (GEO): http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ililkmqqvvqlryn&acc=GSE84117.

Western blot analyses

For the Caspase-1 experiment bone marrow macrophages were cultured using a standard protocol [63] and were left unstimulated or were stimulated for 6 hours with ultrapure LPS (*E. coli* 0111:B4, 500 ng/ml; InvivoGen, San Diego, CA, USA). Cells were washed twice with PBS and incubated with ATP (1mM, Sigma Aldrich, St Louis, MO, USA). Next, the cells were collected and lysed in a buffer specific for Caspase-1 (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM Ethylenediaminetetraacetic acid (EDTA) pH: 8.2 mM Ethyleneglycoltetraacetic acid (EGTA) pH: 7.5, 10% Glycerol, 1% Triton X-100, 50 mM Sodium fluoride, 200 μM Sodium vanadate) [67]. For the TACE-1 experiment, peritoneal macrophages were left unstimulated or were stimulated with LPS for 5-30 minutes (1 μg/ml, *E. coli* 0127:B8 serotype; Sigma Aldrich, St Louis, MO, USA). Next, the cells were collected and lysed in a buffer specific for TACE (1% Triton X-100, 15 mM NaCl, 50 mM Tris HCL pH 7.4, protease inhibitors cocktail (Roche), 10mM 1,10-phenanthroline) [68]. Equal amounts of proteins were separated on 12% polyacrylamide gels and transferred onto nitrocellulose membranes (Whatman, GE Healthcare, Pollards Wood, UK). The proteins were probed with a primary antibody, either anti-ADAM17-cytoplasmic domain (ab39162; abcam) or anti-caspase-1-t/ICE (AHZ0082; Invitrogen), followed by a HRP-conjugated secondary antibody (RnD systems, Minneapolis, MN, USA). Anti-actin (MAB1501R; Merck Millipore, Billerica, MA, USA) was used as a loading control. Immunoblots were visualized with an ECL detection system (GE Healthcare, Pollards Woods, UK). Signal intensities were analyzed using the NIH ImageJ software.

Statistical analysis

Statistical analyses were performed using the two-tailed unpaired Student's *t*-test. $P < 0.05$ indicates statistical significance and the variability is depicted using the standard error of the mean (SEM). The Kaplan Meier test was applied to estimate survival in the LPS induced endotoxemia experiment.

Conventions and Abbreviations

PCSK: Proprotein Convertase Subtilisin/Kexin type; FBS: Fetal Bovine Serum; ELISA: Enzyme-Linked Immunosorbent Assay; CBA: Cytometric Bead Array; qRT-PCR: quantitative Reverse Transcription Polymerase Chain Reaction; IL: Interleukin; TLR: Toll Like Receptor; LPS: Lipopolysaccharide; R848: Resiquimod; IFN-γ: Interferon gamma; TNF-α: Tumor Necrosis Factor alpha; MCP-1: Monocyte Chemotactic Protein 1; TACE:

Tumor Necrosis Factor Alpha Converting Enzyme; ADAM17: ADAM Metallopeptidase Domain 17; *Ptgs2*: Cyclooxygenase-2; CXCL: C-X-C motif Chemokine; CCL: Chemokine (C-C motif) Ligand; *Serpinb1a*: Serine (or cysteine) peptidase inhibitor, clade B, member 1a; *Serpinb2*: Serpin Peptidase Inhibitor, Clade B (Ovalbumin), Member 2 ; *Hcar2*: Niacin Receptor 1 ; *Egr1*: Early Growth Response 1; *C5ar*: Complement Component 5a Receptor 1; *Dusp6*: Dual specificity phosphatase 6; *Fcgr1*: Fc Fragment of IgG, High Affinity Ia, Receptor; *Atf7*: Activating Transcription Factor 7; *Ccnd1*: Cyclophilin C; CD11b: Cluster of Differentiation molecule 11b, Integrin alpha M; Ly6C: Lymphocyte antigen 6C; Ly6G: Lymphocyte antigen 6G; *Trem1*: Triggering receptor expressed on myeloid cells 1; *Il12rb1*: Interleukin 12 Receptor, Beta 1; *Ch25h*: Cholesterol 25-Hydroxylase; *Olr1*: Oxidized Low Density Lipoprotein (Lectin-Like) Receptor 1 ; *Nos2*: inducible Nitric Oxide Synthase 2; *Arg*1: Arginase 1.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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Author Contributions

Z.M., conducted and designed experiments, analyzed the data and wrote the manuscript and A.G., V.T., W.N. S.A. and S.H. assisted with the experiments. I.J. contributed to the design of the flow cytometry and cell culture experiments. V.K. and A.Y. designed and performed the microarray analyses. M.N. designed, organized and supervised the microarray analysis. M.P. conceived and supervised the project, designed experiments and edited the manuscript. All authors have reviewed the manuscript.

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Myeloid cell expressed proprotein convertase FURIN attenuates inflammation

Supplementary Material

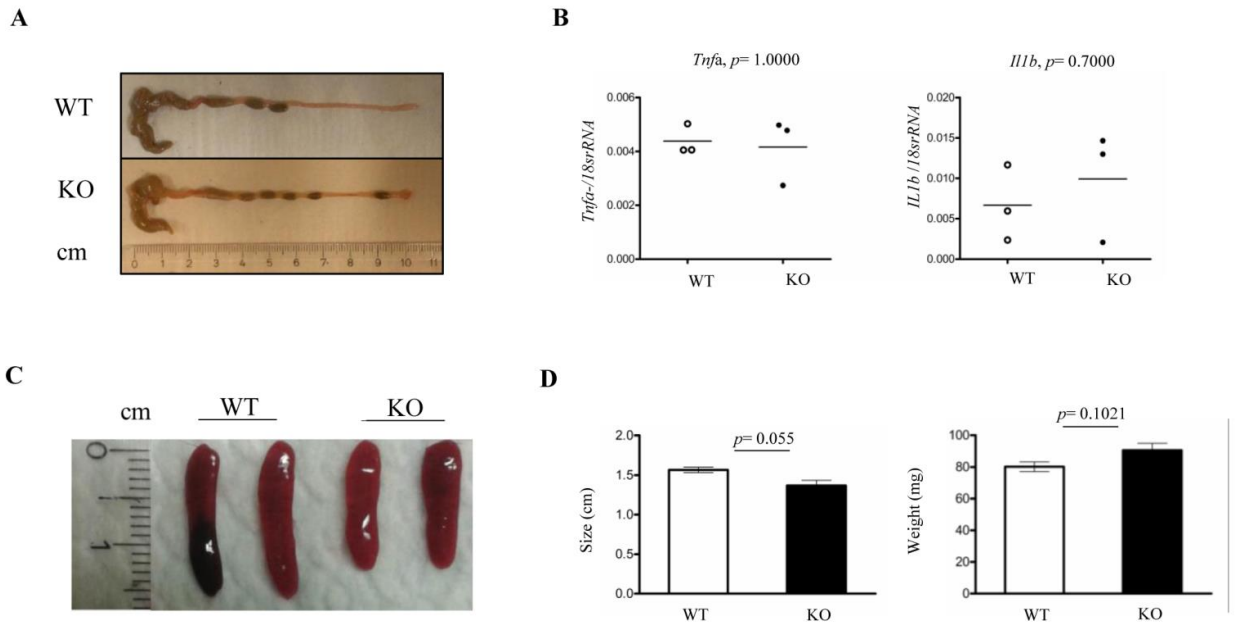

Figure S1 Gut and spleen morphology and cytokine gene expression in the large intestine. **A**. A representative picture of the gut gross morphology in LysMCre-*fur*$^{(fl/fl)}$ and WT littermate controls mice at 16-19 weeks of age. **B.** mRNA expression was assessed by quantitative RT-PCR to determine the expression of the *Tnfa* and *Il1b* genes in the large intestines of LysMCre-*fur*$^{(fl/fl)}$ and WT littermate control mice, n=3/genotype, 16-19 weeks of age. **C.** A representative picture of the spleen gross morphology in LysMCre-*fur*$^{(fl/fl)}$ and WT littermate controls (n=2+2). **D.** Bar graphs represent size and weight averages ± SEM, n=3/genotype, 6-8 weeks of age. Statistics were calculated using the two-tailed unpaired Student's t-test.

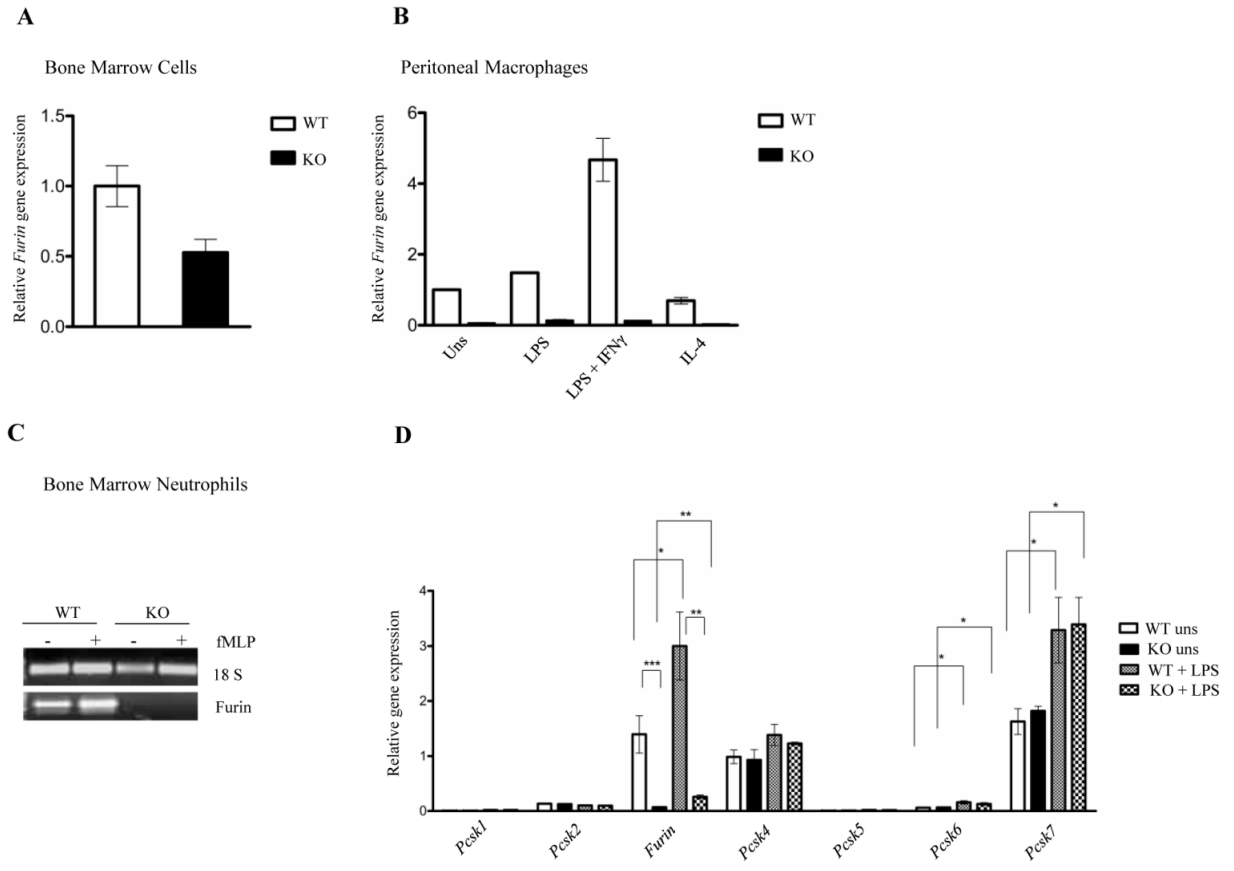

Figure S2 The *Pcsk* gene expressions in LysMCre-*fur*$^{(fl/fl)}$ mice. A. Bone marrow cells were isolated from the femurs and tibias of LysMCre-*fur*$^{(fl/fl)}$ and WT littermate control mice (n=2/genotype, 6-8 weeks of age). *Furin* mRNA expression was assessed by quantitative RT-PCR. Plots represent average ± SEM. **B.** WT and FURIN KO peritoneal macrophages were left unstimulated or were stimulated *in vitro* with LPS (1 µg/ml) and/or cytokines: IFN-γ (20 ng/ml); IL4 (50 ng/ml). *Furin* mRNA expression was assessed by quantitative RT-PCR. The figure shows one representative experiment out of three performed. **C.** Neutrophils were isolated from the bone marrow of LysMCre-*fur*$^{(fl/fl)}$ and WT littermate control mice and were left unstimulated or stimulated with 100 µM of fMLP. *Furin* mRNA expression was assessed by quantitative RT-PCR. The figure shows the gel electrophoresis of *Furin* amplification products. The housekeeping gene 18S was used to normalize the gene expression. **D.** mRNA expressions of the

Pcsk genes were assessed by quantitative RT-PCR in unstimulated and LPS stimulated (1 µg/ml for 4 hours) WT and FURIN KO peritoneal macrophages. The normalized expression of *Furin* in WT samples was set to 1. The housekeeping gene 18S was used to normalize the gene expressions. Statistics were calculated using the two-tailed unpaired Student's t-test. Error bars=SEM (n=4/genotype, *p<0.05, **p<0.01***, p<0.001).

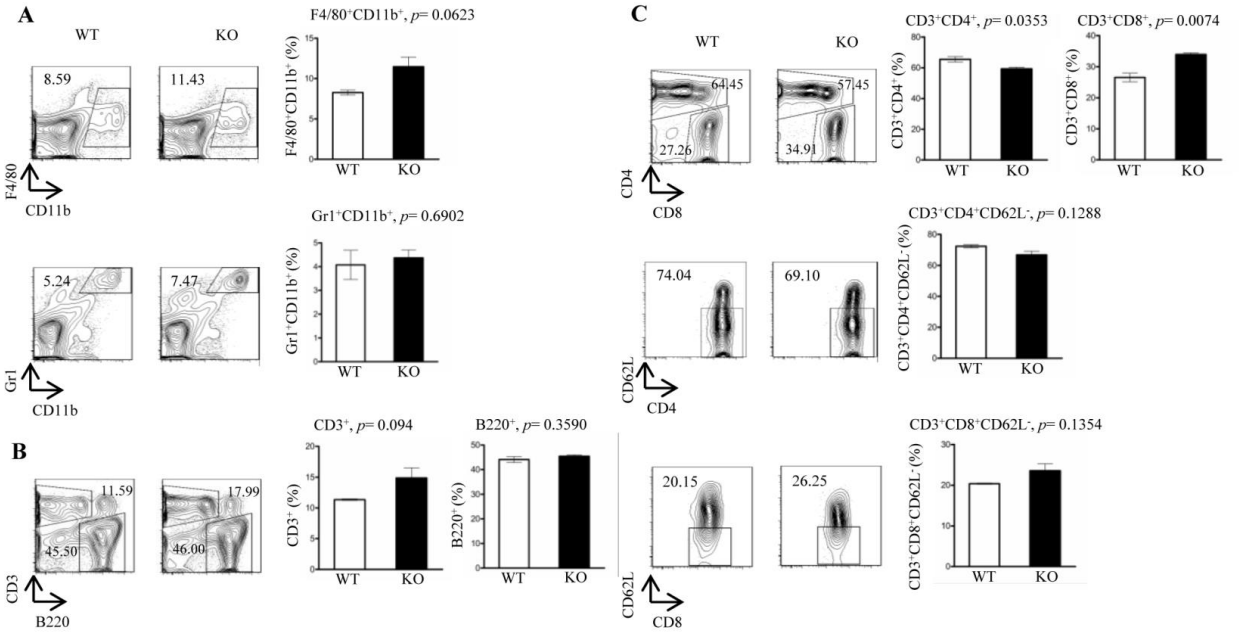

Figure S3. Proportions of immune cell populations in the spleens of LysMCre-*fur*$^{(fl/fl)}$ and littermate wild type mice. A–C. Representative flow cytometry contour plots and plotted mean values of splenic F4/80$^+$CD11b$^+$, Gr1$^+$CD11b$^+$, CD3$^+$, B220$^+$, CD3$^+$CD4$^+$, CD3$^+$CD8$^+$, CD3$^+$CD4$^+$CD62L$^-$ and CD3$^+$CD8$^+$CD62L$^-$ cell populations are shown. (n=4/genotype, 6-8 weeks of age) (Plots represent average ± SEM). Statistics were calculated using the two-tailed unpaired Student's t-test.

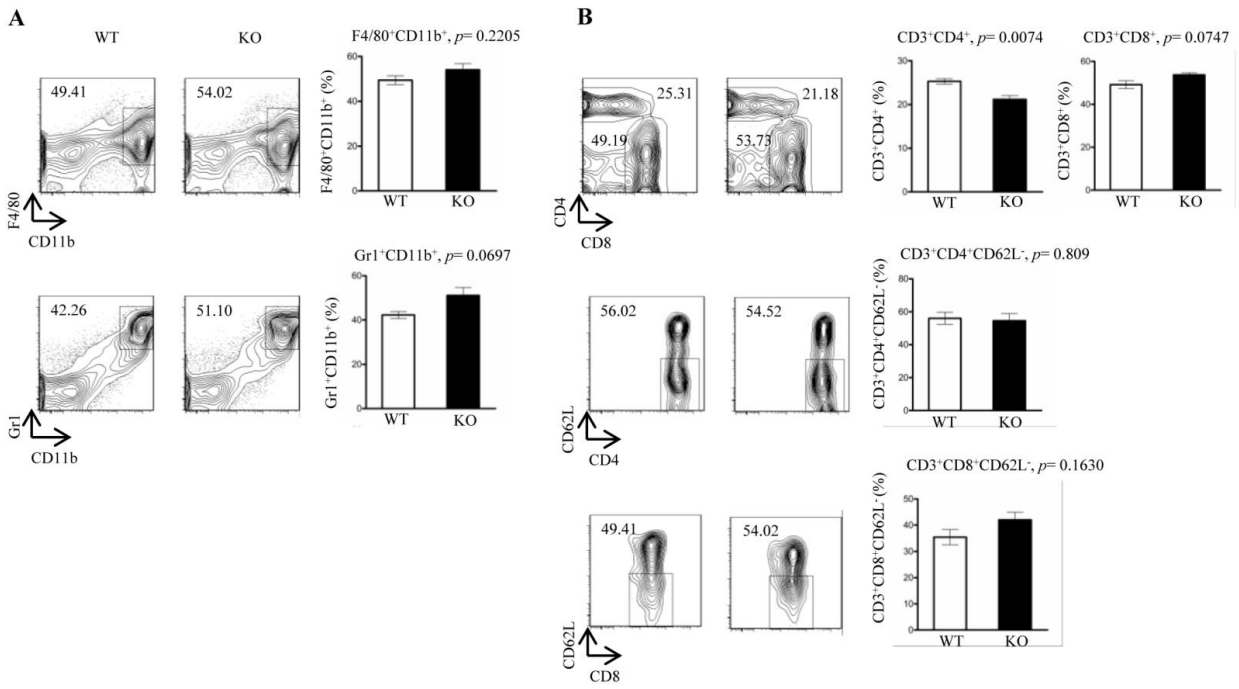

Figure S4. Proportions of immune cell populations in the bone marrow of LysMCre-*fur*$^{(fl/fl)}$ and littermate wild type mice. A-B. Representative flow cytometry contour plots and plotted mean values of bone marrow $F4/80^+CD11b^+$, $Gr1^+CD11b^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+CD62L^-$ and $CD3^+CD8^+CD62L^-$ cell populations are shown. (n=4/genotype, 6-8 weeks of age) (Plots represent average ± SEM). Statistics were calculated using the two-tailed unpaired Student's t-test.

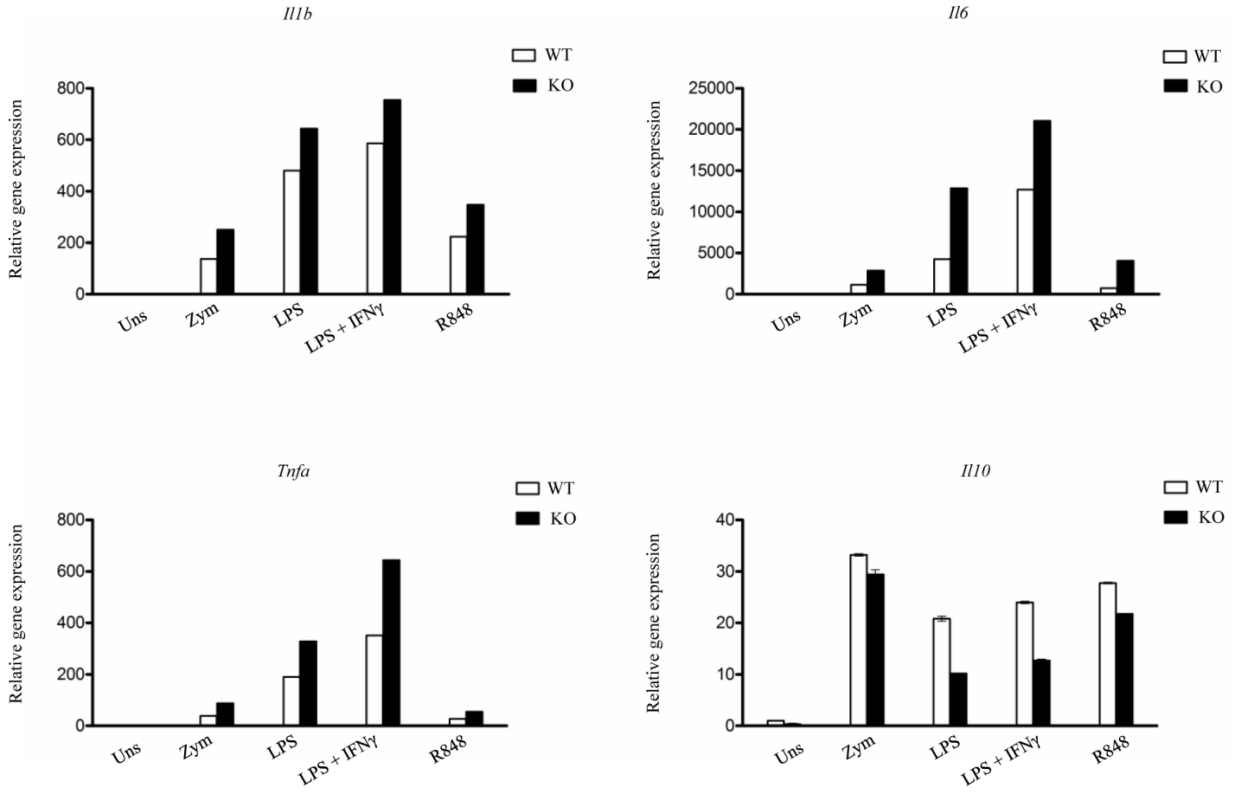

Figure S5. Cytokine mRNA expression in FURIN deficient and wild type macrophages activated with different TLR ligands. WT and FURIN KO peritoneal macrophages were left unstimulated or were stimulated for 4h with TLR ligands (Zymosan: 10 μg/ml, LPS: 1 μg/ml, R848: 1 μg/ml) and/or cytokines (IFN-γ: 20 ng/ml). Gene expression levels were determined using quantitative RT-PCR, and the relative expression in the untreated WT sample was arbitrarily set to 1. The housekeeping gene 18S was used to normalize the gene expression. One representative experiment out of three identical experiments is shown. Error bars=SEM.

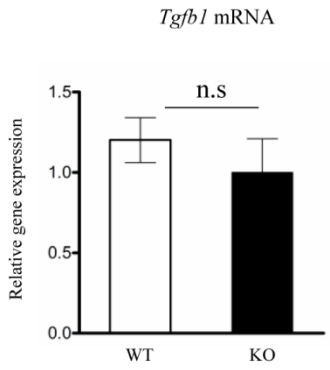

Figure S6. *Tgfb1* mRNA expression in resting wild type and FURIN deficient peritoneal macrophages. *Tgfb1* mRNA levels were determined in unstimulated WT and FURIN KO peritoneal macrophages using quantitative RT-PCR. The relative expression in the WT sample was arbitrarily set to 1. The housekeeping gene 18S was used to normalize the gene expression. (n=4/genotype) (Plots represent average ± SEM). Statistics were calculated using the two-tailed unpaired Student's t-test.

Table S1. Sequences of the primers used for the RT-PCR.

Primers for the RT-PCR analyses were designed using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and the Ensembl database (http://www.ensembl.org).

Gene	Forward	Reverse
Il1b	5'-CGT GGA CCT TCC AGG ATG AG-3'	5'-CAT CTC GGA GCC TGT AGT GC-3'
Tnfa	5'-CTT CTG TCT ACT GAA CTT CGG G-3'	5'-CAG GCT TGT CAC TCG AAT TTT G-3'
Il6	5'- TGT GCA ATG GCA ATT CTG AT- 3'	5'- CTC TGA AGG ACT CTG GCT TTG- 3'
Nos2	5'-GGG CAG TGG AGA GAT TTT GC-3'	5'-CCA GAG GGG TAG GCT TGT CT-3'
Il10	5'-GCC CAG AAA TCA AGG AGC AT-3'	5'-TGT AGA CAC CTT GGT CTT GGA G-3'
Arg1	5'-AAG AAT GGA AGA GTC AGT GTG G-3'	5'-GGG AGT GTT GAT GTC AGT GTG-3'
Pcsk1	5'-GTA CAC ATC CTA CAA TAC AGT CCA G-3'	5'-TCC CTT CTA CCC TCC ACA TT-3'
Pcsk2	5'-AGG TGT GCA GGA GAA GTT TC-3'	5'-GTC TGT CAT AAA GGG CTG GTC-3'
Furin	5'-CAG AAG CAT GGC TTC CAC AAC-3'	5'-TGT CAC TGC TCT GTG CCA GAA-3'
Pcsk4	5'-TCT TGG ACG ATG GCA TTG AG-3'	5'-TTC CAT GTC GGT TCT CAT CG-3'
Pcsk5	5'-CGC TTT CAA CGC CAA GAT TG-3'	5'-AGT CTT GCC ATC GTC ATC TG-3'
Pcsk6	5'-CTA AAC AAG CTT TCG AGT ATG GC-3'	5'-TGG TGT AGA TGC TGT TGG TG-3'
Pcsk7	5'-TTC TGT GCA GTG GGT GTG-3'	5'-CTG TCA GTA AGT GGT CCA TCC-3'
Tgfb1	5'-CCTGAGTGGCTGTCTTTTGA-3'	5'-CGTGGAGTTTGTTATCTTTGCTG-3'
18s	5'-GTG ATC CCT GAG AAG TTC CAG-3'	5'-TCG ATG TCT GCT TTC CTC AAC-3'

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The Proprotein Convertase Subtilisin/Kexin FurinA Regulates Zebrafish Host Response against *Mycobacterium marinum*

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Tuberculosis is a chronic bacterial disease with a complex pathogenesis. An effective immunity against *Mycobacterium tuberculosis* requires both the innate and adaptive immune responses, including proper T helper (Th) type 1 cell function. FURIN is a proprotein convertase subtilisin/kexin (PCSK) enzyme, which is highly expressed in Th1 type cells. *FURIN* expression in T cells is essential for maintaining peripheral immune tolerance, but its role in the innate immunity and infections has remained elusive. Here, we utilized *Mycobacterium marinum* infection models in zebrafish (*Danio rerio*) to investigate how *furin* regulates host responses against mycobacteria. In steady-state *furinA*$^{td204e/+}$ fish reduced *furinA* mRNA levels associated with low granulocyte counts and elevated Th cell transcription factor expressions. Silencing *furin* genes reduced the survival of *M. marinum*-infected zebrafish embryos. A mycobacterial infection upregulated *furinA* in adult zebrafish, and infected *furinA*$^{td204e/+}$ mutants exhibited a proinflammatory phenotype characterized by elevated *tumor necrosis factor a* (*tnfa*), *lymphotoxin alpha* (*lta*) and *interleukin 17a/f3* (*il17a/f3*) expression levels. The enhanced innate immune response in the *furinA*$^{td204e/+}$ mutants correlated with a significantly decreased bacterial burden in a chronic *M. marinum* infection model. Our data show that upregulated *furinA* expression can serve as a marker for mycobacterial disease, since it inhibits early host responses and consequently promotes bacterial growth in a chronic infection.

Tuberculosis (TB) is an epidemic infectious disease caused by the mycobacterial species *Mycobacterium tuberculosis* (1, 2). Circa 13% of the individuals with active TB were simultaneous carriers of the human immunodeficiency virus (HIV), and almost one-third of TB-associated deaths occurred among HIV$^+$ patients, demonstrating the critical role of cluster of differentiation 4 (CD4$^+$) T lymphocyte-mediated immunity in the control of *M. tuberculosis* infection (3, 4). More specifically, the adaptive immunity against TB is primarily mediated by T helper (Th) type 1 cells, as is suggested by the gene expression profile upon infection (5), as well as the infection-induced mortality of gamma interferon-deficient (6, 7) and interleukin-12 (IL-12)-deficient (8) mice.

The proprotein convertase subtilisin/kexin (PCSK) enzymes are a family of serine endoproteases with nine members in humans: PCSK1 and -2, FURIN, PCSK4 to -7, membrane-bound transcription factor peptidase site 1 (MBTPS1), and PCSK9 (9). Typically, PCSKs convert precursor proteins (proproteins) into their biologically active forms by cleaving them at specific target motifs made up of the basic amino acids lysine and arginine (9, 10). FURIN was the first identified mammalian PCSK and is present in vertebrates and many invertebrates (11, 12). A series of *in vitro* experiments have suggested a central role for FURIN in host defense because it proteolytically activates several immunoregulatory proproteins, such as membrane-inserted matrix metallopeptidase 14 (13) and integrins (9), as well as tumor necrosis factor (TNF) and transforming growth factor beta (TGFB) family cytokines (e.g., the TNF superfamily, member 13b, and TGFB1) (12). In addition, infectious agents, including bacterial toxins (anthrax) and viral proteins (HIV gp160), are processed by FURIN (12).

Previously, we and others have shown that *FURIN* is predominantly expressed in Th1 cells and that *FURIN* expression is induced in activated CD4$^+$ T lymphocytes and myeloid cells (14–17). Our functional analyses using mice with a tissue-specific deletion of *Furin* in T cells (CD4cre-fur$^{fl/fl}$) further demonstrated that FURIN is essential for the adequate maturation of pro-TGFB1 and for T regulatory (Treg) cell-mediated immune suppression *in vivo* (18). The breakage of peripheral immune tolerance in CD4cre-fur$^{fl/fl}$ mice resulted in an age-related progression of a systemic autoimmune disease characterized by excessive numbers of overtly activated CD4$^+$ and CD8$^+$ T cells and an increase in proinflammatory cytokine production. In line with the critical role of FURIN in immune suppression, the administration of exogenous recombinant FURIN can alleviate autoimmunity in an experimental arthritis model (19). Notably, as a germ line *Furin* gene knockout (KO) in mice is lethal during embryonic development (20), the systemic role of FURIN in immune regulation and infections is still poorly understood.

Zebrafish (*Danio rerio*) is a small nonmammalian vertebrate model organism, with humoral and cellular components of the

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innate and adaptive immune systems similar to those of humans (21–24). *Mycobacterium marinum*, a close relative of *M. tuberculosis*, is a natural zebrafish pathogen and causes a mycobacterial disease, which shares the main pathological and histological features of human TB (25, 26). Consequently, an *M. marinum* infection in fish is considered a relevant, cost-effective and ethical tool for studying the human mycobacterial disease. Both embryo and adult zebrafish infection models are now well established; while embryos can be used to specifically investigate innate immune responses (27, 28), the adult model enables the study of a chronic progressive mycobacterial infection, as well as spontaneous latency (25, 29).

Genetic variation affects TB susceptibility in humans. To study mutant phenotypes of selected host genes, a large collection of mutant zebrafish strains is available (Zebrafish Mutation Project, Wellcome Trust Sanger Institute, Cambridge, United Kingdom). Zebrafish has two *FURIN* orthologs: *furinA* and *furinB*. *furinA*, like the mammalian *FURIN* gene, has a critical, nonredundant role in organism development (30). Here, we have silenced the expression of *furin* genes in developing fish and used a *furinA*$^{td204e/+}$ mutant zebrafish strain to study how FurinA regulates the development of adult zebrafish immune cells and the host response against mycobacteria.

MATERIALS AND METHODS

Zebrafish lines and maintenance. Nine- to 16-month-old zebrafish were used in the adult experiments. *furinA*^{td204e} mutation-bearing zebrafish in an AB genetic background (Zebrafish Information Network [ZFIN] ID: ZDB-GENO-080606-310) were purchased from the Zebrafish International Resource Center (Oregon). The genotypes of the *furinA*$^{td204e/+}$ mutant zebrafish and their wild-type (WT) siblings were confirmed by sequencing (30). The fish were kept in a standardized flowthrough system (Aquatic Habitats, Florida, USA) with a light/dark cycle of 14 h and 10 h and fed with SDS 400 food twice a day. Until 7 days postfertilization (dpf), embryos were grown according to standard protocols in embryo medium (E3) at 28.5°C. The zebrafish housing, care, and all experiments have been approved by the National Animal Experiment Board of Finland (permits LSLH-2007-7254/Ym-23, ESAVI/6407/04.10.03/2012, ESAVI/733/04.10.07/2013, ESAVI/2267/04.10.03/2012, and ESAVI/8125/04.10.07/2013).

Flow cytometry. Zebrafish were euthanized in a 0.04% 3-aminobenzoic acid ethyl ester anesthetic (pH 7.0; Sigma-Aldrich, Missouri, USA), and kidneys were isolated and homogenized into a single-cell suspension of phosphate-buffered saline with 0.5% fetal bovine serum (Gibco/Invitrogen, California, USA). Relative amounts of blood cell precursors, erythrocytes, granulocytes, and lymphocytes were determined by flow cytometry in steady-state (uninfected) *furinA*$^{td204e/+}$ mutants and WT controls by using a FACSCanto II (Becton Dickinson, New Jersey, USA). The data were analyzed with the FlowJo program (v7.5; Tree Star, Inc., Oregon, USA). Hematopoietic cell types were identified based on granularity (side scatter [SSC]) and particle size (forward scatter [FSC]) (31). Granulocytes and lymphocytes for *furinA* expression analyses were purified from WT AB zebrafish kidneys by using flow cytometric sorting with a FACSAria I apparatus (Becton Dickinson).

Experimental infections in adult zebrafish. *M. marinum* (ATCC 927 strain) was cultured and the inoculation performed as described previously (25). In brief, the zebrafish were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester and various amounts of *M. marinum*, together with 0.3 mg/ml phenol red (Sigma-Aldrich), were injected intraperitoneally (i.p.) using an Omnican 100 (30-gauge) insulin needle (Braun, Melsungen, Germany). The *M. marinum* CFU used in the infections were verified by plating serial dilutions on 7H10 agar plates. Infected fish were tracked daily, and humane endpoint criteria of the national ethical board were monitored.

MO and *M. marinum* coinjections. Oligonucleotide sequences for *furinA* and *furinB* gene silencing morpholinos (MOs) and the injection protocol have been previously described (32). The injection volume was set to 2 nl, and 0.25 pmol of both *furinA* and *furinB* MOs or 0.5 pmol of RC MO was used. *M. marinum* was simultaneously coinjected into the yolk sac, and 2% polyvinylpyrrolidone was used as a carrier solution in the suspension (27, 33). Survival was analyzed daily with a visual inspection with an Olympus IX71 microscope.

Histology. The presence of *M. marinum* in infected adult zebrafish was verified with a histological analysis and Ziehl-Neelsen staining (25, 34). Uninfected controls were included to exclude background mycobacterial infection. Sections were visualized with an Olympus BX51 microscope and Olympus ColorView IIIu camera using a ×100 magnification or with a fully automated Objective Imaging Surveyor virtual slide scanner (Objective Imaging, Cambridge, United Kingdom). Digitization of scanned sample sections was done at a resolution of 0.4 μm per pixel using a 20× Plan Apochromatic microscope objective, and image data were converted to JPEG2000 format as described previously (35).

qRT-PCR. RNA and/or DNA was isolated from kidneys, lymphocytes, granulocytes, and the tissue homogenates of organs in the abdominal cavity using an RNeasy RNA purification kit (Qiagen, Hilden, Germany) or with an RNA-DNA coextraction method for TRIreagent (Molecular Research Center, Ohio, USA). The relative mRNA levels of target genes were quantified from cDNA with quantitative real-time PCR (qRT-PCR). The reverse transcription was done with an iScript Select cDNA synthesis kit (Bio-Rad, California, USA). Maxima SYBR green qPCR master mix (Fermentas, Burlington, Canada) and a CFX96 qPCR machine (Bio-Rad) were used. Primer sequences and ZFIN identification codes for the qRT-PCR-analyzed genes are listed in Table S1 in the supplemental material. The expression of target genes was normalized to the expression of *eukaryotic translation elongation factor 1 alpha 1, like 1* (*eef1a1l1* or *ef1a*) (36). Whenever the RNA-DNA coextraction method was used, the total DNA was isolated simultaneously with the RNA to quantify the *M. marinum* load in the fish with qRT-PCR (25). The results were analyzed with the Bio-Rad CFX Manager software v1.6 (Bio-Rad). No template control samples (H_2O) were included in all experiments to monitor contamination. Melting curve analyses, followed by 1.5% agarose (Bioline, London, United Kingdom) gel electrophoresis, were done to validate the qRT-PCR products of the target genes.

Statistical analysis. Statistical analyses were performed with the Prism v5.02 program (GraphPad Software, Inc., California, USA). A log-rank (Mantel-Cox) test was used in the survival experiments and a nonparametric Mann-Whitney analysis in the flow cytometry and qRT-PCR experiments. *P* values of <0.05 were considered significant.

RESULTS

***furinA* is upregulated in a mycobacterial infection and it controls granulopoiesis and Th cell transcription factor expression.** In the *furinA*^{td204e} mutant fish, a specific thymidine (T)-to-adenosine (A) splice site mutation results in a skipped exon 9 during the transcription of the *furinA* gene (see Fig. S1 in the supplemental material) (30). This leads to a loss-of-function FurinA mutant protein and enables the design of qRT-PCR primers, which can be used to specifically quantify native *furinA* mRNA molecules. In accordance with the developmental lethality of other homozygous *furinA* zebrafish mutants (>98% lethality of *furinA*$^{tg419/tg419}$ mutant) (30), no homozygous *furinA*$^{td204e/td204e}$ mutant fish could be obtained in our fish crosses (up to ~450 genotyped fish), suggesting that in homozygous form this allele is also lethal. In contrast, the heterozygous *furinA*$^{td204e/+}$ mutants were born in normal Mendelian ratios and did not show signs of developmental defects or spontaneous autoimmunity. First, to determine the effect of a heterozygous *furinA*^{td204e} mutation on mRNA levels, *furinA* expression in uninfected and *M. marinum*-infected adult fish (4 and

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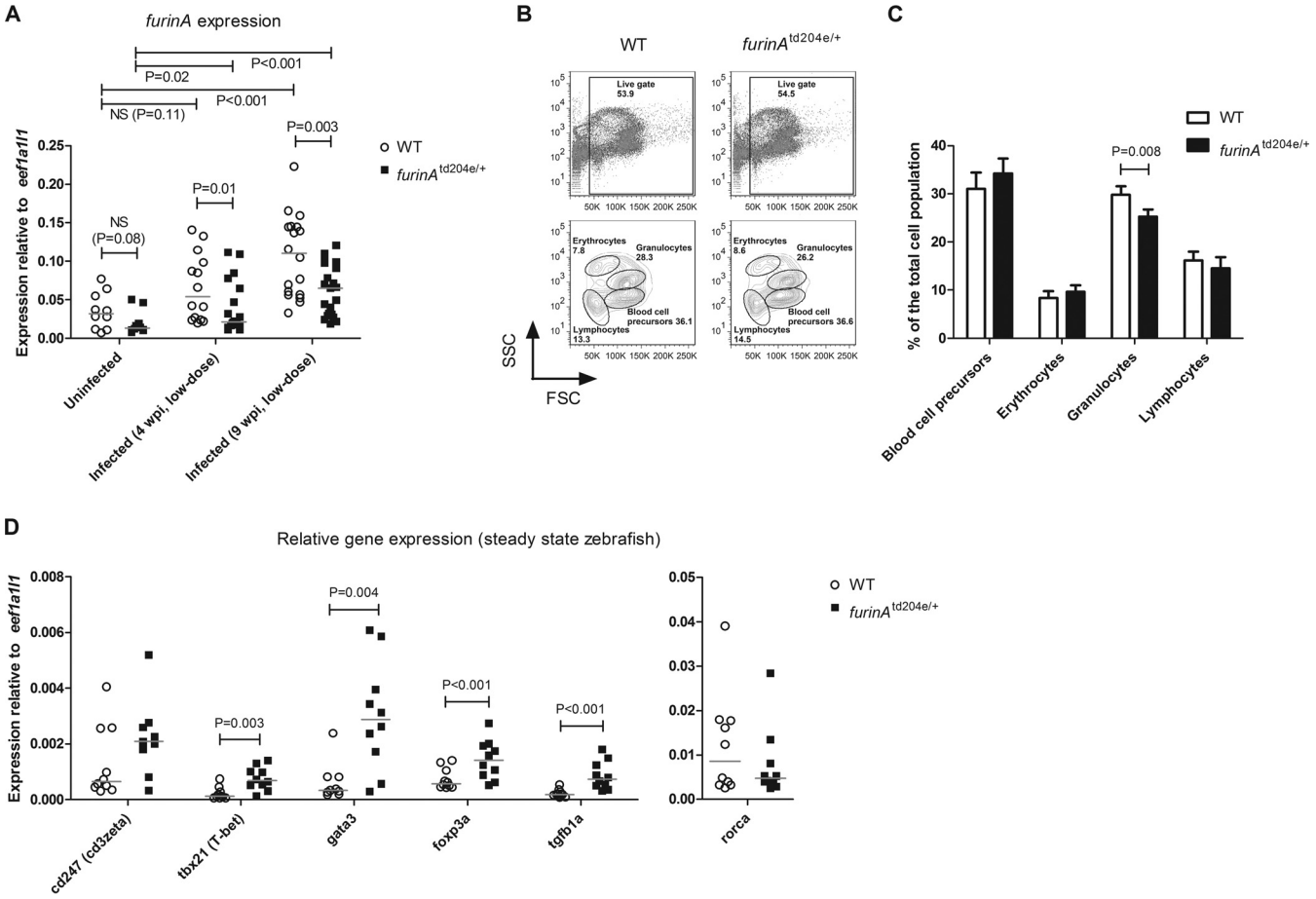

FIG 1 *furinA* expression is reduced in *furinA*$^{td204e/+}$ zebrafish and associates with decreased granulocyte counts, as well as altered T helper cell subtype transcription factor expression. (A) Relative *furinA* expression was measured in uninfected ($n = 10$) and *M. marinum*-infected (at 4 and 9 wpi, low-dose, $n = 13$ to 21) *furinA*$^{td204e/+}$ mutant adult zebrafish and WT controls with qRT-PCR. Samples were run as technical duplicates. (B and C) The relative percentages of blood cell precursors, erythrocytes, granulocytes, and lymphocytes were determined in the kidneys of steady-state (uninfected) *furinA*$^{td204e/+}$ mutants and WT zebrafish ($n = 5$ in both groups) with flow cytometry, based on granularity (SSC) and cell size (FSC). Representative flow cytometry plots are shown in panel B. Gated populations are outlined, and the cell counts inside the gates are given as the percentages of the total viable cell population. The average relative percentages of different hematopoietic cell populations in mutants and controls are plotted in panel C (error bars indicate the standard deviations). (D) Relative expressions of different Th cell-associated genes (*cd247*, *tbx21*, *gata3*, *foxp3a*, and *rorca*), as well as *tgfb1a*, were quantified in *furinA*$^{td204e/+}$ mutants and WT controls ($n = 10$ in both groups) with qRT-PCR. Gene expressions in panels A and D were normalized to *eef1a1l1* expression and represented as a scatter dot plot and median. In panel A, a one-tailed Mann-Whitney test was used in the statistical comparison of differences between *furinA*$^{td204e/+}$ zebrafish and WT controls, and a two-tailed Mann-Whitney test was used in panels C and D, as well as in the comparisons between uninfected and infected experimental groups in panel A.

9 weeks postinfection [wpi], low dose; 34 ± 10 CFU) was quantified with qRT-PCR (Fig. 1A). Previously, *in vitro* analyses have shown that *FURIN* expression is upregulated as a result of CD4$^+$ T cell activation and in lipopolysaccharide (LPS)-stimulated CD14$^+$ myeloid cells (14, 15). In accordance with this, the *M. marinum* infection caused an induction in *furinA* mRNA expression in both *furinA*$^{td204e/+}$ (1.6-fold at 4 wpi, $P = 0.02$, and 4.9-fold at 9 wpi, $P < 0.001$) and WT zebrafish (1.7-fold at 4 wpi, not significant [NS], $P = 0.11$; 3.4-fold at 9 wpi, $P < 0.001$) demonstrating that immune activation *in vivo* upregulates this convertase. Furthermore, in the infected groups, *furinA*$^{td204e/+}$ fish had on average 39% ($P = 0.01$) and 43% less ($P = 0.003$) *furinA* mRNA compared to WT controls at 4 and 9 wpi, respectively. A similar trend was also observed in uninfected zebrafish with a 44% decrease in *furinA* expression (NS, $P = 0.08$). Put together, the data indicate that *furinA* is upregulated in response to a mycobacterial infection, and that the *furinA*$^{td204e/+}$ zebrafish can be used to explore the functional role of this PCSK in a mycobacterial infection *in vivo*.

The development of hematopoietic cells in zebrafish is highly similar to that in humans (31, 37). To assess the effect of the reduced *furinA* expression on hematopoiesis in the *furinA*$^{td204e/+}$ fish, we studied their blood cell composition with flow cytometry (Fig. 1B and C) (31). The flow cytometric analysis revealed no marked differences in blood cell precursor, erythrocyte or lymphocyte populations in *furinA*$^{td204e/+}$ zebrafish compared to WT controls. However, the amount of granulocytes in *furinA*$^{td204e/+}$ fish was significantly decreased, by an average of 15.4% ($P = 0.008$), compared to controls, indicating a role for FurinA in granulopoiesis.

Previously, we showed that FURIN is critical for normal mammalian Th polarization and CD4$^+$ Treg cell function; CD4cre-fur$^{fl/fl}$ mice have abnormally large effector CD4$^+$ and Treg cell populations accompanied with an excessive production of Th1 and Th2 cytokines (15, 18). To address whether FurinA regulates the generation of Th subsets in zebrafish, we assessed the expression of different T cell markers (*CD247 antigen*; *cd247*, *T-box 21*;

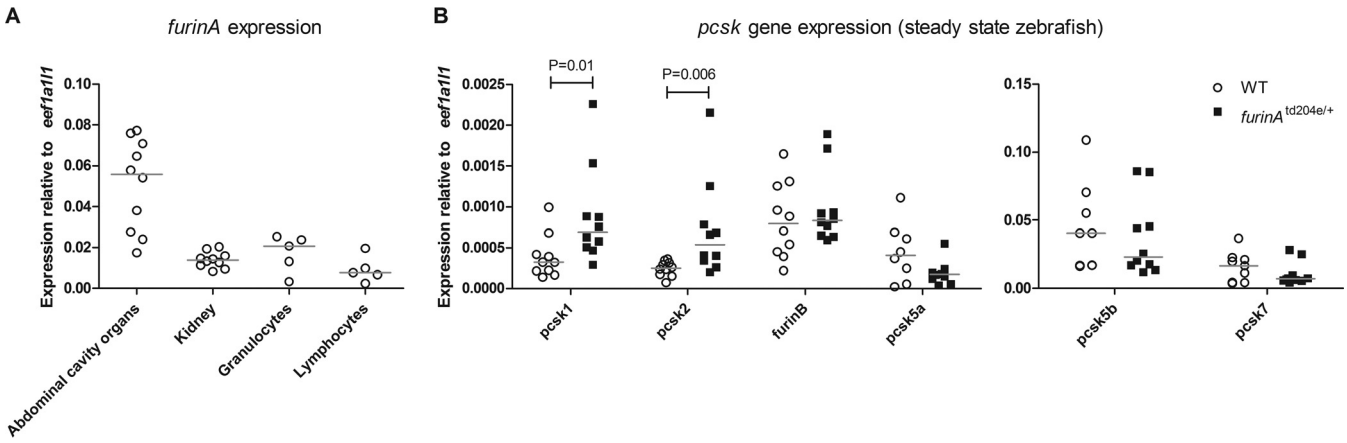

FIG 2 Expression of zebrafish *pcsk* genes in *furinA*$^{td204e/+}$ mutants and WT controls. (A) Relative *furinA* expression was measured with qRT-PCR in the tissue homogenates of organs in the abdominal cavity ($n = 10$) and kidney ($n = 10$) as well as in purified granulocytes ($n = 5$) and lymphocytes ($n = 5$) isolated from steady-state WT AB zebrafish. Samples were run as technical duplicates. (B) The relative expressions of zebrafish *pcsk* genes (*pcsk1*, *pcsk2*, *furinB*, *pcsk5a*, *pcsk5b*, and *pcsk7*) were quantified in the tissue homogenates of organs in the abdominal cavities of steady-state adult *furinA*$^{td204e/+}$ mutant ($n = 10$) and WT ($n = 8$ to 10) zebrafish by using qRT-PCR. Gene expressions were normalized to *eef1a1l1* expression and are represented as a scatter dot plot and median. A two-tailed Mann-Whitney test was used in the statistical comparison of differences.

tbx21, *gata3*, *forkhead box P3a*; *foxp3a*, *retinoic acid receptor-related orphan receptor C a*; *rorca*) in *furinA*$^{td204e/+}$ mutants and WT controls (Fig. 1D). As in T cell-specific FURIN conditional KO (cKO) mice, the mRNA levels of Th1, Th2, and the Treg cell markers *tbx21* (*T-bet*, $P = 0.003$), *gata3* ($P = 0.004$), and *foxp3a* ($P < 0.001$) were elevated in *furinA*$^{td204e/+}$ zebrafish. In contrast, there was no significant difference in the expression of the Th17 cell marker *rorca* between *furinA*$^{td204e/+}$ and WT zebrafish, which is in line with the normal IL-17 production previously observed in CD4cre-fur$^{fl/fl}$ mice (18).

TGFB1 directly induces *Furin* expression in rodents, which is a prerequisite for its functional maturation and anti-inflammatory function (18, 38). Consequently, the autoimmune phenotype of CD4cre-fur$^{fl/fl}$ mice can be chiefly attributed to a lack of bioavailable, T cell-produced TGFB1. In our present study, zebrafish FurinA was found to regulate *tgfb1a* expression *in vivo* (Fig. 1D), which could result from an attempt to compensate for the defective maturation of the Tgfb1a cytokine by increasing the efficiency of *tgfb1a* transcription.

The quantification of the *furinA* mRNA expression in WT zebrafish demonstrated that it is expressed in both innate and adaptive immune cells (Fig. 2A), which is in line with the previously reported ubiquitous expression pattern of *FURIN* orthologues in vertebrates (9, 32). In mammals, the first seven PCSK enzymes have been demonstrated to exhibit a significant functional redundancy and shared substrate molecules, which interferes with the interpretation of a PCSK specific phenotype (39). Therefore, we next addressed the expression of the zebrafish *pcsk* genes (*pcsk1*, *pcsk2*, *furinB*, *pcsk5a*, *pcsk5b*, and *pcsk7*) in *furinA*$^{td204e/+}$ mutants and WT controls (Fig. 2B). The *pcsk* genes *furinB*, *pcsk5a*, *pcsk5b*, and *pcsk7* showed comparable expression levels between *furinA*$^{td204e/+}$ and WT zebrafish, whereas *pcsk1* and *pcsk2* were significantly upregulated in the *furinA*$^{td204e/+}$ fish ($P = 0.01$ and $P = 0.006$, respectively), which theoretically could partially compensate for the effect of reduced *furinA* expression.

Furin regulates the survival of *M. marinum*-infected zebrafish embryos. Whereas upregulated T cell gene expression in *furinA*$^{td204e/+}$ zebrafish indicates enhanced immune responses, granulopenia can result in immunodeficiency. To study the net effect of FurinA on mycobacterial host defense in adult zebrafish, we infected *furinA*$^{td204e/+}$ and WT zebrafish with a high-dose of *M. marinum* (8,300 ± 1,800 CFU) and followed their survival for 11 weeks (Fig. 3A). WT fish exhibited ca. 60% mortality during the first 5 weeks and about one-third of them were alive at the study endpoint (Fig. 3A). *furinA*$^{td204e/+}$ mutants showed similar lethality, and no statistical difference in gross survival between mutant and WT fish could be detected. In addition, a histopathological examination revealed that the two fish groups had similarly organized granulomas at both 3 and 11 wpi, and there were no obvious differences in the numbers of granulomas (Fig. 3B). Uninfected WT and *furinA*$^{td204e/+}$ zebrafish controls did not show background mycobacteriosis in a Ziehl-Neelsen staining (data not shown).

Morpholino (MO)-based expression silencing in developing zebrafish embryos can be used to study a gene's function specifically in innate immune responses (22, 40). Since *furinA* regulated the granulopoiesis, we addressed its role in innate immunity by inhibiting the expressions of *furinA* and *furinB* in the embryonic *M. marinum* infection model (27, 32, 33). Infecting either control (random control MO injected [RC]) or the double *furin* gene knockdown embryos with *M. marinum* (131 ± 125 CFU) resulted in substantial lethality by 7 days postinfection (dpi; 93 and 100%, respectively, Fig. 3C). The survival of infected *furinA*+*B* morphants was, however, significantly reduced compared to controls (*furinA*+*B* versus RC, $P < 0.01$). Notably, as FurinA is essential for zebrafish development the increased lethality of *M. marinum*-infected *furin* morphant embryos could result from general developmental defects.

FurinA inhibits the early expression of proinflammatory cytokine genes in a mycobacterial infection. The containment of a mycobacterial disease is critically dependent on adaptive Th1 type responses but also on adequate innate immune responses. The significance of the innate immunity is perhaps best illustrated by an increased susceptibility to TB in patients receiving anti-TNF

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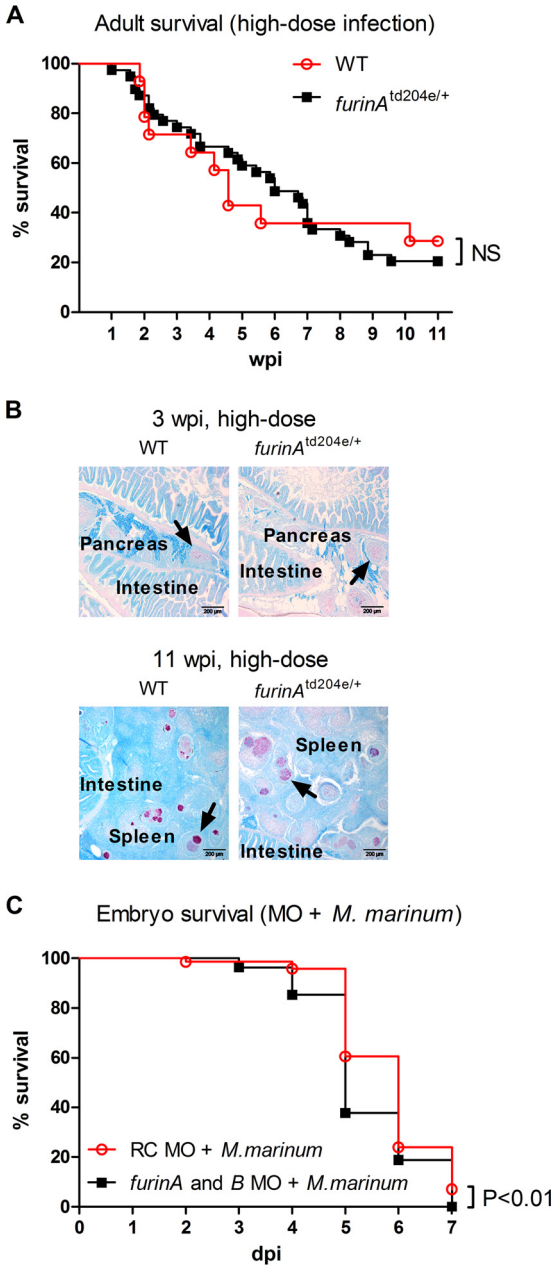

FIG 3 Role of *furin* in zebrafish survival during *M. marinum* infection. (A) The survival of adult *furinA*$^{td204e/+}$ ($n = 39$) and WT ($n = 14$) zebrafish was monitored for 11 weeks after an experimental high-dose *M. marinum* inoculate. The data were collected from a single experiment. (B) *M. marinum* granulomas in adult WT and *furinA*$^{td204e/+}$ zebrafish infected with a high-dose bacterial inoculate were identified with Ziehl-Neelsen staining at 3 wpi (17,300 ± 6,900 CFU) and 11 wpi (8,300 ± 1,800 CFU). Representative images from 4 to 10 individuals per group are shown. Typical granulomas are indicated with arrows. (C) Zebrafish embryos were microinjected before the four-cell stage with RC ($n = 71$) or both *furinA* and *furinB* MOs ($n = 82$) and *M. marinum* (131 ± 125 CFU). At 1 dpf, embryos were screened to identify successfully injected embryos, and survival was monitored up until 7 dpf. Collated data from two separate experiments with 30 and 41 embryos in the RC MO groups and 27 and 55 embryos in the *furinA* and *furinB* MO groups are shown. In panels A and C, a log-rank (Mantel-Cox) test was used for the statistical comparison of differences.

neutralizing antibodies and an association of human Toll-like receptor polymorphisms with an increased disease risk (4, 41, 42). FURIN can process target molecules that are important in innate immunity *in vitro* (e.g., TNF converting enzyme and Toll-like receptor 7) (43, 44), but whether it also regulates innate immune responses in infections *in vivo* has not been addressed. We next analyzed the early immune response against *M. marinum* by measuring the cytokine gene expression in *furinA*$^{td204e/+}$ fish and WT controls. Both *furinA*$^{td204e/+}$ and WT adult zebrafish were infected with a high dose of *M. marinum* (10,300 ± 3,300 CFU) and a qRT-PCR expression analysis of both proinflammatory (*tnfa*, *il1b*, *lta*, *ifng1-1*, *il22*, and *il17a/f3*) and anti-inflammatory (*il10* and *tgfb1a*) cytokine genes was performed at 1, 6, and 12 dpi (Fig. 4).

An analysis of the kinetics of the cytokine gene induction in WT fish (Fig. 4A) demonstrated that the expression levels of *tnfa*, *lta*, and *ifng1-1* were significantly upregulated upon *M. marinum* infection already at 1 dpi (15.1-, 5.5-, and 396.4-fold, respectively), with rising kinetics until 6 dpi (161.4-, 13.1-, and 478.8-fold, respectively). At 12 dpi, the induction of *tnfa* had declined to 110.3-fold, whereas *lta* and *ifng1-1* expressions had returned to their baseline levels. *il17a/f3* was also significantly induced at 1 dpi (18.0-fold), but its expression decreased during the following days (6 dpi, 7.7-fold; 12 dpi, baseline expression). In contrast, both *il1b* and *il22* showed a delayed expression pattern by peaking at 12 dpi (*il1b*, 2.3-fold; *il22*, 10.8-fold). The induction of the anti-inflammatory cytokine genes *il10* and *tgfb1a* was evident already by day 6 postinfection (*il10*, 2.4-fold; *tgfb1a*, 10.7-fold), and the expression of both genes was even more pronounced at 12 dpi (4.4- and 19.0-fold, respectively). In conclusion, an *M. marinum* infection in zebrafish results in an enhancement in the levels of various macrophage, natural killer cell, γδ T cell, and lymphoid tissue inducer cell-associated cytokines already during the first 12 days after infection, indicating an efficient activation of pro- and anti-inflammatory processes.

To determine how FurinA contributes to the early cytokine levels induced by *M. marinum*, we compared the expression of the aforementioned cytokine genes in infected *furinA*$^{td204e/+}$ and WT zebrafish (Fig. 4B to I). *furinA*$^{td204e/+}$ mutants showed a significantly higher relative expression of the proinflammatory cytokine genes *tnfa* ($P = 0.007$), *lta* ($P = 0.03$), and *il17a/f3* ($P = 0.002$) at 1 dpi compared to WT fish. Interestingly, the inherent relative upregulation of *tgfb1a* in *furinA*$^{td204e/+}$ mutants was completely abolished by the 12th postinfective day, and this was accompanied by a relative reduction in *il10* gene expression. The low *furinA* expression also associated with a sustained upregulation of the *il17a/f3* cytokine gene. Collectively, these results could indicate that inflammation-accelerating innate cytokine responses dominate in *M. marinum*-infected *furinA*$^{td204e/+}$ mutant fish. To demonstrate that FURIN attenuates proinflammatory responses specifically in innate immune cells, we used cultured macrophages from WT and LysMcre-fur$^{fl/fl}$ mice (Fig. 5) (45, 46). In these experiments we saw that in activated macrophages reduced *Furin* mRNA levels (77% decrease, $P = 0.004$) are associated with significantly upregulated transcription of the proinflammatory cytokine gene *Tnf* ($P = 0.03$).

***furinA*$^{td204e/+}$ mutants have decreased bacterial burden and *cd247* expression in a chronic *M. marinum* infection model.** We have recently established a model for studying a latent mycobacterial infection in adult zebrafish (25). A low-dose i.p. *M. mari-*

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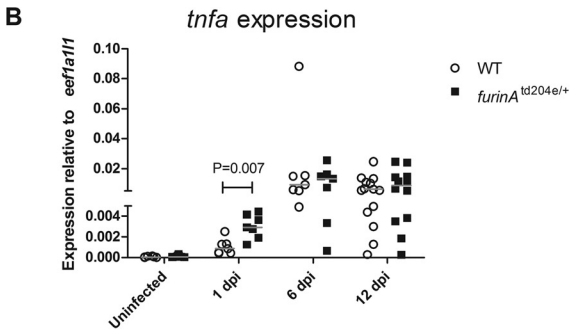

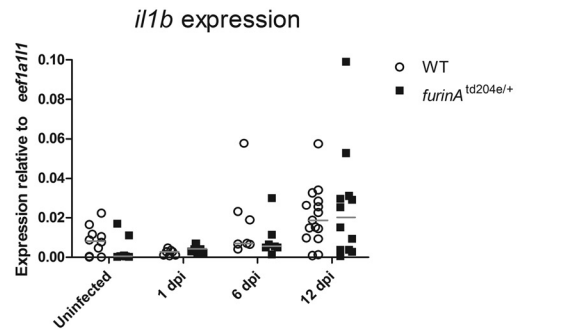

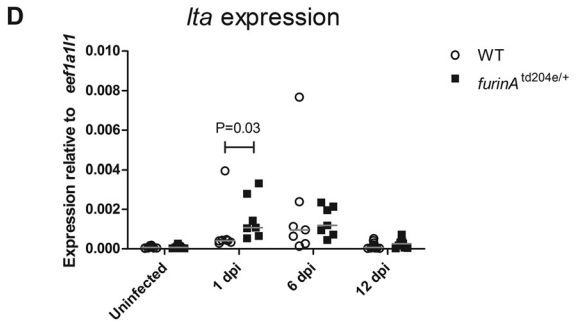

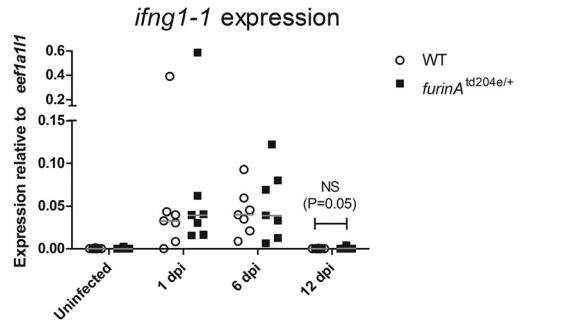

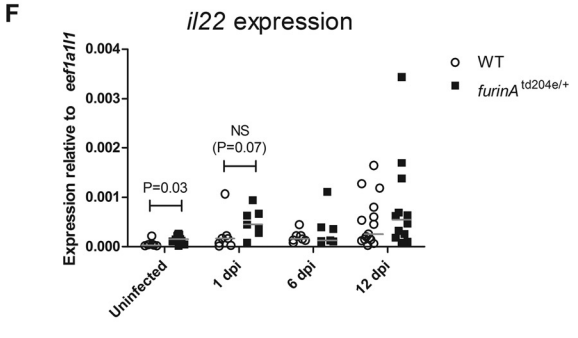

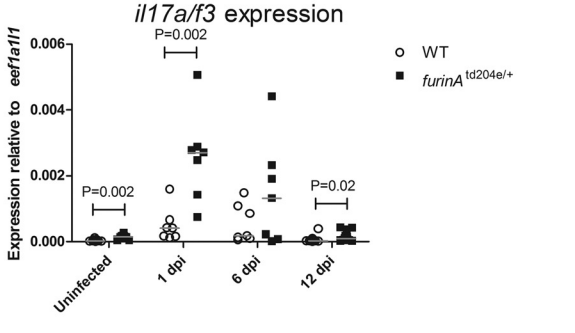

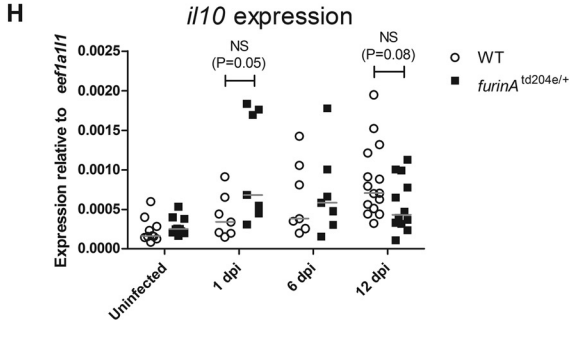

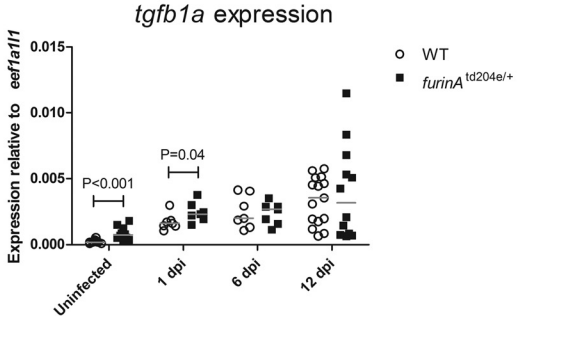

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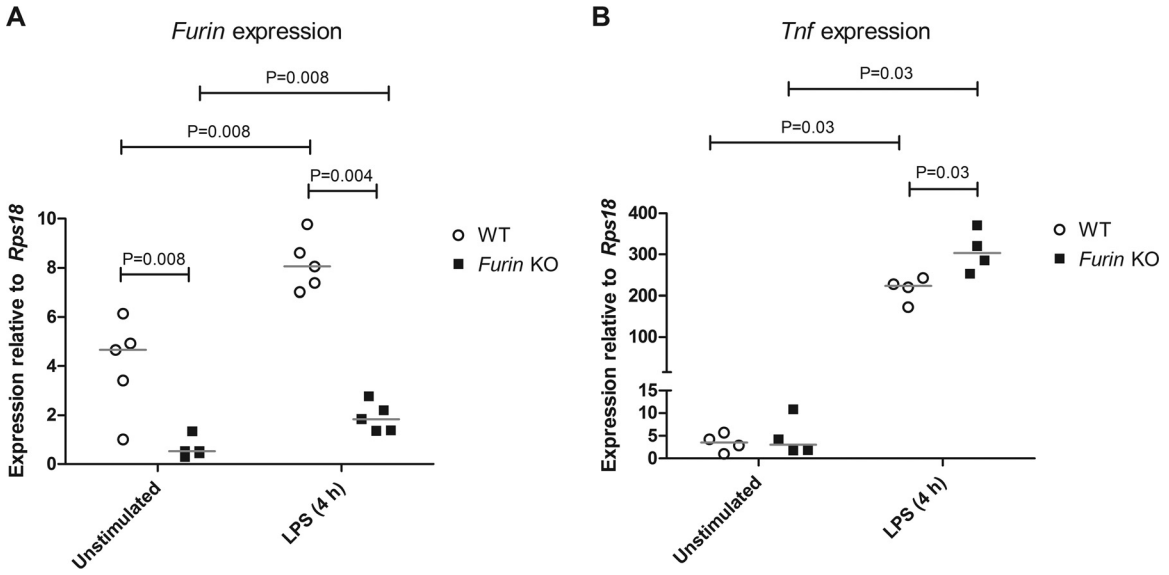

FIG 5 Reduced *Furin* expression is associated with an upregulated expression of *Tnf* in activated mouse macrophages. Bone marrow-derived macrophages were cultured from *Furin* KO (LysMcre-fur$^{fl/fl}$) and WT littermate mice (n = 4 to 5) as described previously (46). The relative expressions of *Furin* (Ensembl ID ENSMUSG00000030530) (A) and *Tnf* (Ensembl ID ENSMUSG00000024401) (B) were determined in unstimulated and LPS-stimulated (4 h) samples with qRT-PCR. Gene expressions were normalized to *ribosomal protein S18* (*Rps18*, Ensembl ID ENSMUSG00000008668) expression and are represented as a scatter dot plot and median. A two-tailed Mann-Whitney test was used in the statistical comparison of differences. The qRT-PCR primers used for the mouse genes were as follows: *Furin*, 5′-CAGAAGCATGGCTTCCACAAC-3′ and 5′-TGTCACTGCTCTGTGCCAGAA-3′; *Tnf*, 5′-CTTCTGTCTACTGAACTTCGGG-3′ and 5′-CAGGCTTGTCACTCGAATTTTG-3′; and *Rps18*, 5′-GTGATCCCTGAGAAGTTCCAG-3′ and 5′-TCGATGTCTGCTTTCCTCAAC-3′.

num inoculate (~35 CFU) results in static bacterial burdens, a constant number of granulomas, and low mortality. We thus utilized this model to investigate how FurinA contributes to the adaptive mycobacterial immunity and the development of mycobacterial latency. When *furinA*$^{td204e/+}$ mutant and WT control fish were infected with small amounts of *M. marinum* (46 ± 8 CFU) and survival was monitored for 9 weeks, statistically significant difference between the groups could not be observed (Fig. 6A). However, mycobacterial quantification revealed a trend of smaller bacterial amount in the *furinA*$^{td204e/+}$ mutants at 4 wpi (34 ± 10 CFU, NS) (Fig. 6B), but significantly reduced *M. marinum* copy numbers from the internal organ isolates of infected *furinA*$^{td204e/+}$ zebrafish compared to WT fish at 9 wpi (1.9-fold reduction, $P = 0.04$) (Fig. 6B). On average, bacterial copy number medians at 9 wpi were 11,000 (13 copies in 100 ng of zebrafish DNA) in *furinA*$^{td204e/+}$ mutants and 21,000 (50 copies in 100 ng of zebrafish DNA) in WT zebrafish, which suggests that *furinA* inhibits host responses in chronic mycobacterial infection.

The reduced mycobacterial load in latency could be a result of the upregulation of proinflammatory cytokines upon the *M. marinum* infection in *furinA*$^{td204e/+}$ mutant fish (Fig. 4B to G) but also a consequence of inherently accelerated T cell responses (Fig. 1D). To evaluate the T cell responses in latency, we quantified the relative expression of a general T cell marker *cd247* (*cd3zeta*) and Th cell subtype-associated transcription factors (*tbx21*, *gata3*, *foxp3a*, and *rorca*) in *furinA*$^{td204e/+}$ mutant and control fish at both 4 and 9 wpi (see Fig. S2 in the supplemental material and Fig. 6C). As expected, an infection-induced upregulation of these genes was seen in both WT and mutant zebrafish (at 4 wpi, 2.9- to 23.9-fold and 1.5- to 8.1-fold, respectively, and at 9 wpi, 4.2- to 19.0-fold and 2.5- to 8-fold, respectively), suggesting T cell activation. Interestingly, at 9 wpi, *cd247* expression was significantly lower in infected *furinA*$^{td204e/+}$ mutants compared to WT controls ($P = 0.03$), whereas the expression of the Th subset-associated transcription factors *tbx21*, *gata3*, *foxp3a*, and *rorca* did not differ between infected *furinA*$^{td204e/+}$ fish and controls. In addition, the expression levels of innate immunity cytokine genes (*tnfa*, *il1b*, *il10*, *tgfb1a*, *lta*, *ifng1-1*, and *il17a/f3*) were found to be similar between *furinA*$^{td204e/+}$ and WT fish (see Fig. S2 and S3 in the supplemental material), indicating that innate immune cell activity during a chronic *M. marinum* infection is FurinA independent.

Together, the reduced relative expression of *cd247* at 9 wpi and loss of upregulation of *tbx21*, *gata3*, and *foxp3a* in *furinA*$^{td204e/+}$ zebrafish compared to WT controls indicate that FurinA enhances T cell responses in a mycobacterial infection. However, *furinA*$^{td204e/+}$ mutants had lower *M. marinum* copy numbers,

FIG 4 FurinA attenuates the early expression of proinflammatory cytokine genes in an experimental high-dose mycobacterial infection. The relative expression of proinflammatory cytokine genes (*tnfa*, *il1b*, *lta*, *ifng1-1*, *il22*, and *il17a/f3*) and anti-inflammatory cytokine genes (*il10* and *tgfb1a*) was determined in adult *furinA*$^{td204e/+}$ (n = 7 to 12) and WT (n = 7 to 15) zebrafish with qRT-PCR after a high dose of an *M. marinum* inoculate at 1, 6, and 12 dpi. (A) Fold gene expression induction median shown for all of the aforementioned genes in infected WT zebrafish. The fold induction was normalized to the gene expression median in uninfected zebrafish. *, $P < 0.05$; **, $P < 0.01$. (B to I) Relative gene expression in *furinA*$^{td204e/+}$ and WT zebrafish represented as a scatter dot plot and median. Note the different scales of the y axes and the divided y axis in panels B and E. Gene expressions were normalized to *eef1a1l1* expression. At 1 and 6 dpi, samples were run as technical duplicates and uninfected, as well as 12-dpi, samples once. A two-tailed Mann-Whitney test was used in the statistical comparison of differences.

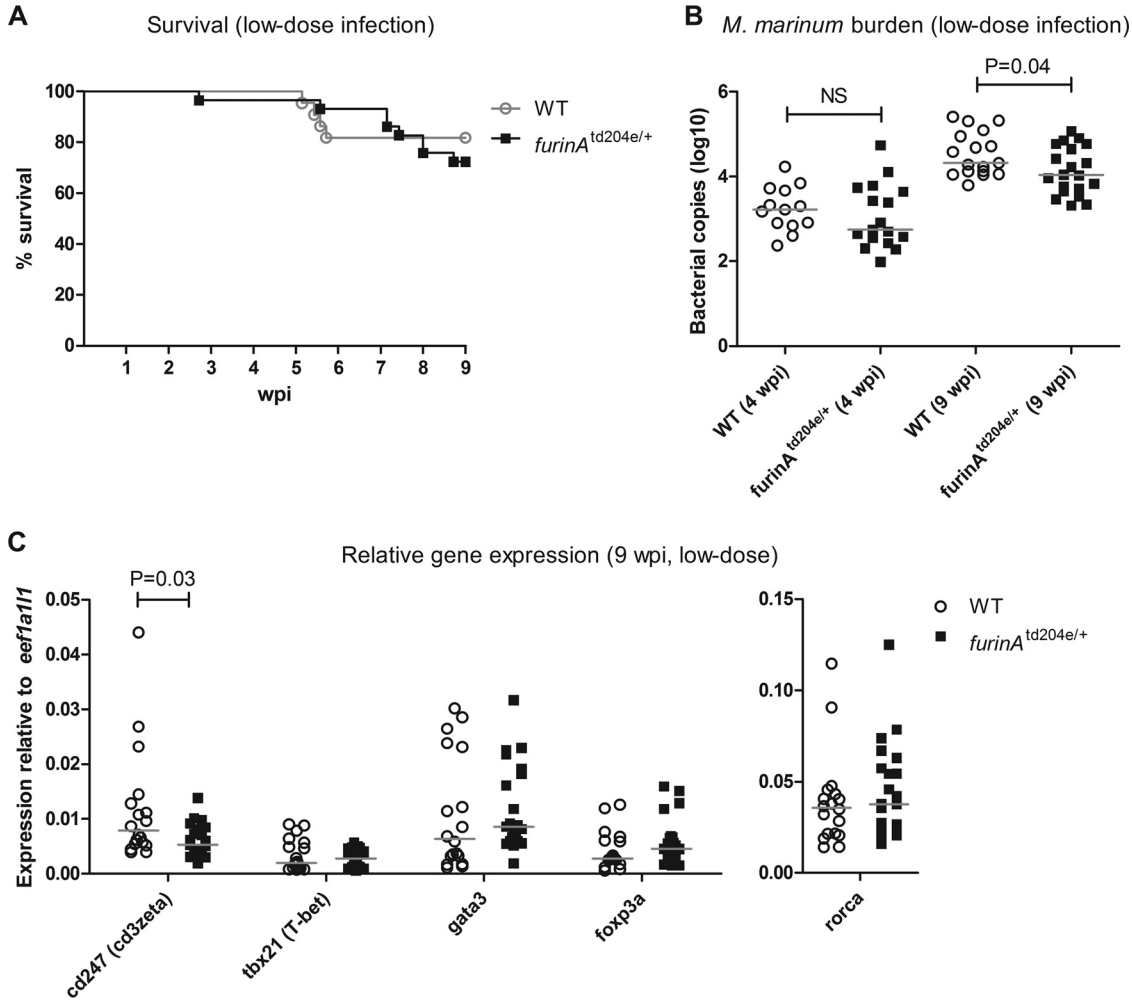

FIG 6 Downregulation of *furinA* expression decreases the *M. marinum* burden and the T cell marker *cd247* mRNA level in an experimental low-dose mycobacterial infection. A latent mycobacterial infection was induced with a low-dose *M. marinum* inoculate. (A) Survival of adult *furinA*$^{td204e/+}$ ($n = 29$) and WT ($n = 22$) zebrafish was monitored for 9 weeks. A log-rank (Mantel-Cox) test was used for the statistical comparison of differences. The data were collected from a single experiment. (B) The *M. marinum* burden of *furinA*$^{td204e/+}$ mutants ($n = 17$ to 20) and WT controls ($n = 13$ to 18) was quantified with DNA qRT-PCR at 4 and 9 wpi. Bacterial load is represented as the median of total bacterial copies ($\log_{10}$). *M. marinum* quantifications were run as technical duplicates. (C) The relative expression of Th cell markers (*cd247*, *tbx21*, *gata3*, *foxp3a*, and *rorca*) was quantified with qRT-PCR in *furinA*$^{td204e/+}$ mutants ($n = 18$ to 21) and WT controls ($n = 18$) at 9 wpi. Gene expressions were normalized to *eef1a1l1* expression and represented as a scatter dot plot and median. Expression analyses were run as technical duplicates. In panels B and C, a two-tailed Mann-Whitney test was used in the statistical comparison of differences.

which suggests that FurinA also inhibits antimycobacterial host responses.

DISCUSSION

Despite intensive studies, our understanding of the pathogenesis and host immunity of TB is still incomplete. We found that *furinA* expression is upregulated upon *M. marinum* infection and that inhibiting *furin* genes in developing zebrafish reduces the survival of infected embryos. An analysis of *furinA*$^{td204e/+}$ mutant adult zebrafish demonstrated that FurinA regulates the development of granulocytes and the expression of Th subset-associated genes in steady-state fish. When *furinA*$^{td204e/+}$ mutant fish were infected with a high dose of *M. marinum*, reduced *furinA* mRNA levels were found to correlate with an enhanced expression of the pro-inflammatory cytokine genes *tnfa*, *lta*, and *il17a/f3*. In contrast, experiments using a latent mycobacterial infection model showed that infected *furinA*$^{td204e/+}$ mutants have lowered expression levels of the T cell marker gene *cd247* (*cd3zeta*) compared to controls. The net effect of the reduced *furinA* expression in adult zebrafish was a significant decrease in *M. marinum* copy numbers in a low-dose infection model, suggesting that FurinA attenuates protective host responses against mycobacteria.

Through catalyzing the endoproteolytic cleavage of target molecules, PCSK enzymes regulate the maturation of host defense factors, as well as the activity of invading pathogens (9, 10). *In vitro* analyses have demonstrated that PCSK enzymes have significantly overlapping biochemical functions in substrate processing, and therefore genetic inactivation of PCSKs is instrumental for decoding their specific biological roles (9, 10, 47). We have previously characterized the expression of seven *pcsk* genes in developing embryos and multiple adult zebrafish tissues (32). Two orthologous genes of mammalian *FURIN*, *furinA* and *furinB* (30), were found to be ubiquitously expressed, and biochemical analyses

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showed that FurinA, but not FurinB, is able to proteolytically process pro-Tgfb1a, suggesting that FurinA is the corresponding biological equivalent for human FURIN (32). Germ line *Furin* KO mice die on day 11 of embryogenesis due to severe developmental defects in ventral closure, as well as in heart tube fusion and looping (20). Accordingly, we could not identify any homozygous adult *furinA*$^{td204e/td204e}$ fish, indicating that FurinA has a specific, nonredundant function also during zebrafish development. Importantly, however, in its heterozygous form, the adult *furinA*^{td204e} allele did not interfere with normal development but reduced the levels of *furinA* mRNA. This in turn allowed the use of adult *furinA*$^{td204e/+}$ mutants in the experiments to assess how *furinA* expression regulates host responses. Interestingly, *furinA* downregulation in *furinA*$^{td204e/+}$ mutant zebrafish upregulated *pcsk1* and *pcsk2* expression, which implies an attempt to compensate for the reduced FurinA activity. In mammals, PCSK1 and PCSK2 have restricted gene expression patterns, function chiefly in neuroendocrine tissues, and are not able to compensate for FURIN during development (9, 10). However, the lack of PCSK1 was recently found to associate with a proinflammatory phenotype and increased lethality in LPS-induced septic shock in mice (48). Consequently, the elevated *pcsk1* expression in zebrafish could theoretically also attenuate inflammation in zebrafish and thus partially mask the specific immunoregulatory function of FurinA.

It is well established that protective immunity against TB is mediated by both innate and adaptive immune responses. As in mammals, the cells of the zebrafish immune system include lymphocytes, neutrophils, and macrophages (49), as well as dendritic cells (50), eosinophils (51, 52), human mast cell-like cells (53), and natural killer cells (54). Our flow cytometric analyses of *furinA*$^{td204e/+}$ mutant fish kidneys (the primary site of hematopoiesis in fish) showed normal numbers of lymphocytes, blood cell precursors, and erythrocytes, but low granulocyte counts, indicating that FurinA promotes granulopoiesis. Granulocyte maturation is regulated through a complex network of protein mediators (55), some of which are known substrates for PCSKs (12). For example, granulocyte development is disrupted in mice deficient in integrin alpha 9 (56). Also, functional NOTCH signaling promotes entry into granulopoiesis (57), whereas conditional inactivation of TNF converting enzyme increases granulopoiesis (58). Deciphering the detailed molecular mechanisms by which FurinA regulates granulocyte development, however, would require the spatiotemporal identification of its specific substrates using proteomics analyses, followed by characterizing the function of the substrates in zebrafish.

Although the Th1 type cell immune response is crucial in adaptive immunity against TB (5–8), other Th lymphocyte subsets, including Th2, Th17, and Treg cells, also regulate the magnitude of the host defense and survival (59–62). We have previously shown that FURIN is dispensable for T cell development in mice but that it plays a role in $CD4^+$ T cell activation and polarization (15, 18). When we characterized the expression of Th cell subtype transcription factors in steady-state zebrafish, we found that decreased *furinA* expression associated with the upregulation of *tbx21* (a Th1 cell marker), *gata3* (a Th2 cell marker), and *foxp3a* (a Treg cell marker) expression, suggesting an increase in Th1, Th2, and Treg cell counts in the *furinA*$^{td204e/+}$ mutants. These findings are in line with the previously reported hyperproduction of both Th1 and Th2 hallmark cytokines and increased Treg cell numbers in *FURIN* T cell cKO mice (18) but also indicate that reduced *FURIN* expression (and not only the lack of it) can accelerate Th1 and Th2 responses. In contrast, aging *furinA*$^{td204e/+}$ mutants did not develop overt autoimmunity, which demonstrates that the residual *furinA* expression, accompanied with elevated *tgfb1a* mRNA levels, is sufficient for maintaining adequate peripheral immune tolerance in steady state.

To assess how granulopenia and altered Th subtype gene expressions in *furinA*$^{td204e/+}$ mutants might contribute to the host defense against mycobacteria, adult zebrafish were infected i.p. with *M. marinum* inoculates. *furinA*$^{td204e/+}$ mutants exhibited similar gross survival, and statistically significant differences could not be observed. In contrast, inhibiting *furin* genes during development associated with significantly reduced survival of *M. marinum*-infected embryos. Albeit these findings could be indicative of either immunodeficiency or an unnecessarily strong host response in the lack of Furin, they need to be interpreted cautiously. The expression of *furinA* is critical for zebrafish development, and survival differences in *furinA*+*B* morphant fish could simply result from "failure to thrive." Therefore, we chose to use adult *furinA*$^{td204e/+}$ fish to address how *furinA* regulates the innate immune responses in *M. marinum* infection (30). After a high-dose mycobacterial infection, lower *furinA* mRNA expression levels resulted in a proinflammatory phenotype characterized by enhanced early expression of *tnfa*, *lta*, and *il17a/f3* but declining expression levels of the anti-inflammatory cytokine genes *il10* and *tgfb1a*. Previously, TNF and IL-17 have been linked to a protective, innate immunity against TB (61, 63), and an *LTA* polymorphism has been associated with susceptibility to the disease (64). The role of Tnfa appears, however, complicated; Roca and Ramakrishnan recently showed that either deficient or excess production of this cytokine accelerates TB pathogenesis through reduced microbicidal activity of macrophages or programmed necrosis of macrophages, respectively (65). Since *furinA* downregulation causes a proinflammatory phenotype, FurinA deficiency could be beneficial for protection by increasing the early microbicidal activity of innate cells through upregulated Tnfa levels. In addition, both *furinA*$^{td204e/+}$ mutants and controls showed well-organized granulomas and no free bacteria in Ziehl-Neelsen staining, which suggests relatively normal macrophage function also in controlling the high bacterial loads in the chronic phase.

We have previously shown that infecting zebrafish with a low *M. marinum* dose (~35 CFU) results in a nonprogressive mycobacterial disease that can be reactivated by gamma irradiation (25). In this model, the host survival and the latent state of infection both depend on functional adaptive immune responses and normal lymphocyte numbers. The determination of the mycobacterial burden in latency revealed that reduced *furinA* expression associated with significantly decreased *M. marinum* copy numbers, and this could not be explained by elevated T cell responses. Specifically, we noticed that *furinA*$^{td204e/+}$ mutant fish actually expressed lower levels of the general T cell marker gene *cd247* (*cd3zeta*) and that the overexpression of Th1/2, as well as Treg marker genes in steady-state mutants, was completely abolished in the chronically infected *furinA*$^{td204e/+}$ zebrafish. How *furinA* downregulation affects these responses is not clear but would require a careful kinetic analysis of marker gene expression levels. In summary, we can conclude that a reduction in systemic *furinA* expression associates with enhanced host responses to mycobacteria in zebrafish.

A challenge in TB diagnostics is to specifically identify the activation of latent infection. The present means, such as the tuberculin skin test, the interferon gamma release assay (IGRA), and a chest X-ray, can only reveal the presence of TB-associated memory cells and tissue damage, but there are no markers available for the detection of mycobacterial growth in the host in the clinic. Our data show that *furinA*/*FURIN* expression is upregulated in the host in response to a mycobacterial infection and the Th1 hallmark cytokine IL-12 (15). FURIN is also secreted from macrophages in response to LPS activation (14), and it can be measured from serum (66). Therefore, in the future it will be interesting to assess whether serum FURIN levels can be used as an infection biomarker to mirror mycobacterial growth and the activation of Th1 type immune responses. Furthermore, PCSK inhibitors have relatively recently been suggested as drugs for cancer and infectious diseases (9, 10, 67). Blocking FURIN also associates with accelerated immune responses, as shown by the spontaneous development of autoimmunity in T cell-specific *FURIN* cKO mice and by the prevention of experimental arthritis upon recombinant FURIN administration (18, 19). Our results here demonstrate that diminished *furinA* expression reduces mycobacterial loads in a latent infection model, which suggests that PCSK inhibitors could potentially be used to harness also TB. Adverse effects, such as autoimmunity and developmental defects in stem cells, may pose a significant clinical problem. Investigating the molecular mechanisms by which FURIN regulates mycobacterial immunity further may help us find specific target molecules for future drug development.

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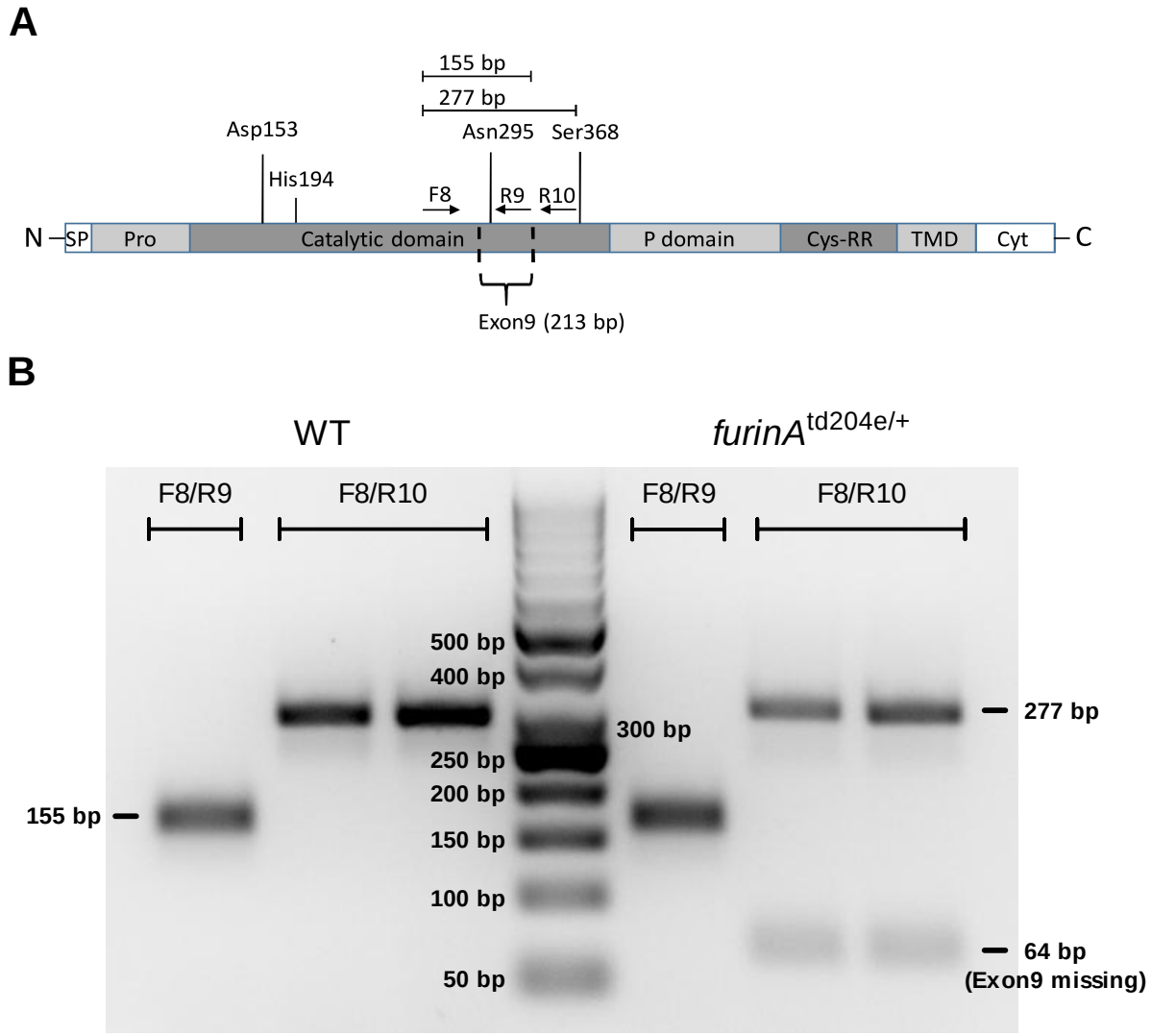
Supplemental Table 1. Primers used in the qRT-PCR analyses.

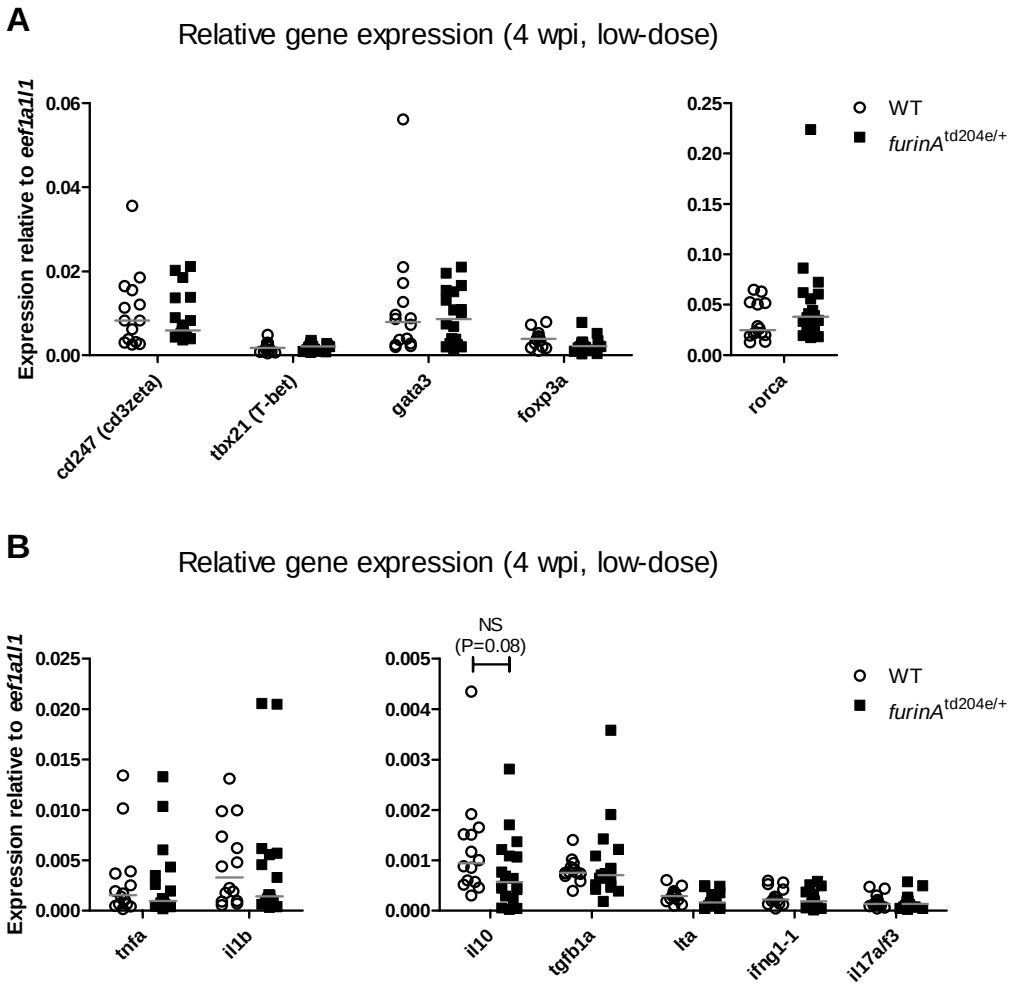
Gene	ZFIN ID	Human gene ortholog (HGNC)	Sequence 5´-3´	Reference
furinA	ZDB-GENE-040901-1	*FURIN*	F CCAAAGAGGCTTTCCAACGC R CGTACTGCTGCTGATGGACAG	-
pcsk1	ZDB-GENE-071009-1	*PCSK1*	F CGGGAAAAGGAGTGGTCAT R GGTGGAGTCGTATCTGGG	Turpeinen et al. 2013
pcsk2	ZDB-GENE-090608-1	*PCSK2*	F CGGATCTGTATGGAAACTGC R GCCGGACTGTATTTTATGAAT	Turpeinen et al. 2013
furinB	ZDB-GENE-040901-2	*FURIN*	F CCAAGGCATCTACATCAACAC R ACACCTCTGTGCTGGAAA	Turpeinen et al. 2013
pcsk5a	ZDB-GENE-060531-130	*PCSK5*	F GGAGTTTCAATGACCCCAA R ACCACAACTCCTTTCCCA	Turpeinen et al. 2013
pcsk5b	ZDB-GENE-070822-7	*PCSK5*	F TGTTCCTCGACCCTTACCAC R ATCTCGCCATGTCAGGAAAG	Turpeinen et al. 2013
pcsk7	ZDB-GENE-030131-7293	*PCSK7*	F AGAGTGTTGGACGGG R TGCCTAATGGATGCGGT	Turpeinen et al. 2013
cd247 (*cd3zeta*)	ZDB-GENE-061130-4	*CD247*	F CATCACCGGCTTCTTTGTGC R CCCCAGTTTATCAATGGCCTGA	Hammarén et al. 2014
tbx21(*T-bet*)	ZDB-GENE-080104-3	*TBX21*	F GGCCTACCAGAATGCAGACA R GGTGCGTACAGCGTGTCATA	Hammarén et al. 2014
gata3	ZDB-GENE-990415-82	*GATA3*	F GGATGGCACCGGTCACTATT R CAGCAGACAGCCTCCGTTT	Hammarén et al. 2014
foxp3a	ZDB-GENE-061116-2	*FOXP3*	F CAAAAGCAGAGTGCCAGTGG R CGCATAAGCACCGATTCTGC	Hammarén et al. 2014
rorca	ZDB-GENE-990415-250	*RORC*	F GAAGGCTGCAAGGGCTTCTT R TGCAGTTCCTCTGCCTTGAG	-
tnfa	ZDB-GENE-050317-1	*TNF*	F GGGCAATCAACAAGATGGAAG R GCAGCTGATGTGCAAAGACAC	Parikka et al. 2012
il1b	ZDB-GENE-040702-2	*IL1B*	F TGGACTTCGCAGCACAAAATG R GTTCACTTCACGCTCTTGGATG	Parikka et al. 2012
lta	ZDB-GENE-050601-3	*LTA*	F CCACAGTTCAGCAGGACCTC R TTTCCTGCGTGCTCTCATGTC	-
ifng1-1	ZDB-GENE-060210-1	*IFNG*	F AAATGGTGCTACTCTGTGGAC R TTCCAACCCAATCCTTTG	Oksanen et al. 2013
il22 (*ifnphi6*)	ZDB-GENE-060209-3	*IL22*	F TCAGACGAGCACACAGATATG R GATGGCTGGAGTAGTCGTG	-
il17a/f3	ZDB-GENE-041001-192	*IL17A* and *IL17F*	F GGCTCTCACGGGTTTTCAG R ACACTTCTTCACACCAGAACATC	-
il10	ZDB-GENE-051111-1	*IL10*	F GCTCTGCTCACGCTTCTTC R TGGTTCCAAGTCATCGTTG	-
tgfb1a	ZDB-GENE-030618-1	*TGFB1*	F TCGTCTTCCAGCAAGCTCAG R TTGGAGACAAAGCGAGTTCC	-
eef1a1l1 (*ef1a*)	ZDB-GENE-990415-52	*EEF1A1*	F CTGGAGGCCAGCTCAAACAT R ATCAAGAAGAGTAGTACCGCTAGCATTAC	Tang et al. 2007
mmits	-	-	F CACCACGAGAAACACTCCAA R ACATCCCGAAACCAACAGAG	Parikka et al. 2012

Zebrafish gene names and qRT-PCR primer sequences are listed accompanied with the ZFIN identification codes and the names of the orthologous genes in humans. HGNC, HUGO Gene Nomenclature Committee.



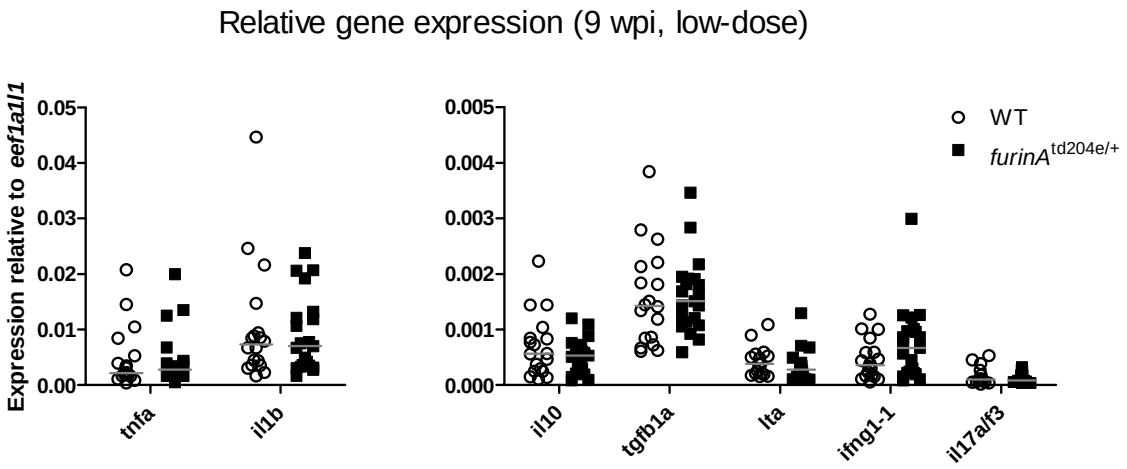
Supplemental Figure 1. *furinA*^{td204e} mutation disrupts the transcription of exon 9.

(A) A schematic presentation of the FURIN/FurinA domain structure and the catalytic amino acid residues (Asp153, His194, Asn295 and Ser 368), as well as the location of exon 9. **(B)** cDNA from both WT and *furinA*$^{td204e/+}$ mutant zebrafish was used as a template in a standard PCR reaction with different primer pairs (F8=Forward primer in exon 8, R9=Reverse primer in exon 9, R10=Reverse primer in exon 10). PCR products were analyzed with agarose gel electrophoresis, which demonstrates the presence of a 64 bp band only in reactions containing cDNA from *furinA*$^{td204e/+}$ mutants. The bands were excised and further verified by DNA sequencing (Data not shown).



Supplemental Figure 2. Comparison of the T cell marker gene expression and the innate cytokine response in *furinA*$^{td204e/+}$ mutants and WT controls in an experimental low-dose mycobacterial infection at 4 wpi.

A latent mycobacterial infection was induced with a low-dose *M. marinum* inoculate. Relative expression of **(A)** Th cell marker genes (*cd247*, *tbx21*, *gata3*, *foxp3a*, *rorca*) as well as **(B)** pro-inflammatory cytokine genes (*tnfa, il1b*, *lta, ifng1-1, il17a/f3*) and anti-inflammatory cytokine genes (*il10*, *tgfb1a*) was determined in *furinA*$^{td204e/+}$ (n=13-14) and WT (n=16-18) zebrafish with qRT-PCR at 4 wpi. Gene expressions were normalized to *eef1a1l1* expression and represented as a scatter dot plot and median. A two-tailed Mann-Whitney was used in the statistical comparison of differences.



Supplemental Figure 3. Comparison of the innate cytokine response in *furinA*$^{td204e/+}$ mutants and WT controls in an experimental low-dose mycobacterial infection at 9 wpi.

A latent mycobacterial infection was induced with a low-dose *M. marinum* inoculate. Relative expression of pro-inflammatory cytokine genes (*tnfa*, *il1b*, *lta*, *ifng1-1*, *il17a/f3*) and anti-inflammatory cytokine genes (*il10*, *tgfb1a*) was determined in *furinA*$^{td204e/+}$ (n=18-21) and WT (n=16-18) zebrafish with qRT-PCR at 9 wpi. Gene expressions were normalized to *eef1a1l1* expression and represented as a scatter dot plot and median. A two-tailed Mann-Whitney was used in the statistical comparison of differences.

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ORIGINAL RESEARCH

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T-cell-expressed proprotein convertase FURIN inhibits DMBA/TPA-induced skin cancer development

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ABSTRACT

Proprotein convertases (PCSK) have a critical role in the body homeostasis as enzymes responsible for processing precursor proteins into their mature forms. FURIN, the first characterized member of the mammalian PCSK family, is overexpressed in multiple malignancies and the inhibition of its activity has been considered potential cancer treatment. FURIN has also an important function in the adaptive immunity, since its deficiency in T cells causes an impaired peripheral immune tolerance and accelerates immune responses. We addressed whether deleting FURIN from the immune cells would strengthen anticancer responses by subjecting mouse strains lacking FURIN from either T cells or macrophages and granulocytes to the DMBA/TPA two-stage skin carcinogenesis protocol. Unexpectedly, deficiency of FURIN in T cells resulted in enhanced and accelerated development of tumors, whereas FURIN deletion in macrophages and granulocytes had no effect. The epidermises of T-cell-specific FURIN deficient mice were significantly thicker with more proliferating Ki67+ cells. In contrast, there were no differences in the numbers of the T cells. The flow cytometric analyses of T-cell populations in skin draining lymph nodes showed that FURIN T-cell KO mice have an inherent upregulation of early activation marker CD69 as well as more $CD4^+CD25^+Foxp3^+$ positive T regulatory cells. In the early phase of tumor promotion, T cells from the T-cell-specific FURIN knockout animals produced more interferon gamma, whereas at later stage the production of Th2- and Th17-type cytokines was more prominent than in wild-type controls. In conclusion, while PCSK inhibitors are promising therapeutics in cancer treatment, our results show that inhibiting FURIN specifically in T cells may promote squamous skin cancer development.

Abbreviations: CTL, cytotoxic T lymphocyte; dLN, draining lymph node; DMBA, 7,12-Dimethylbenz[a]anthracene; IFNγ, interferon gamma; IL, interleukin; KO, knockout; LysM, lysozyme-M; OVA, ovalbumin; PCSK, proprotein convertase; TCR, T-cell receptor; TGF-β1, transforming growth factor-β1; TPA, 12-O-tetradecanoylphorbol-13-acetate; Treg, regulatory T cell; VEGF, vascular endothelial growth factor

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FURIN; proprotein convertase; squamous skin cancer; T cells; transforming growth factor-β1

Introduction

The mammalian proprotein convertase (PCSK) family consists of nine members. The primarily identified seven PCSKs (PCSK1-2, FURIN, PCSK4-7) are closely related and evolutionarily conserved subtilisin/kexin-like serine proteases that convert their immature substrates into functional end-products by cleaving basic amino acid motifs ((K/R)-(X)n-(K/R)↓, where n is 0, 2, 4, or 6 and X is any amino acid).[1] PCSKs operate mainly in the secretory pathway, on the cell surface and in the endosomes. More recently identified and distantly related PCSK family members MBTPS1 and PCSK9 differ from the seven other members in their target sequence specificities. MBTPS1 targets a consensus motif (R/K)-X-(hydrophobic)-X↓ and PCSK9 has only autocatalytic cleavage activity.[2]

PCSK enzymes play an instrumental role in maintaining homeostasis in the body but also in a number of pathological conditions.[2] Various PCSK target proteins are involved in malignant transformation and progression. PCSKs activate for example cell surface-expressed receptors (e.g., integrins), tissue-modifying enzymes like matrix metalloproteinases[3-5] and growth factors needed to support tumor angiogenesis, including vascular endothelial growth factors (VEGF) C and D.[6,7] Many human cancers show high levels of PCSK expression (reviewed in ref.[8]). For example, FURIN expression is elevated in non-small cell lung

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carcinoma as well as in human head and neck squamous cell carcinomas, and the upregulated FURIN activity correlates with accelerated tumor progression.[9-11] Transgenic overexpression of FURIN in mouse epidermal basal layer resulted in increased papilloma and squamous cell carcinoma development and enhanced tumor growth when the mice were subjected to a two-stage chemical carcinogenesis protocol[12]. In line with that data, deleting FURIN from mouse salivary gland cells also delayed PLAG1-induced salivary gland tumorigenesis.[13]

PCSK enzymes are also key regulators of the immune system. By using a conditional, T-cell-specific FURIN deficient mouse (CD4cre-fur$^{flox/flox}$) we have shown that T-cell-expressed FURIN is critical for maintaining peripheral tolerance.[14] T-cell-specific FURIN deletion causes age-related autoimmunity, with expansion and overactivation of effector T cells, excessive production of pro-inflammatory cytokines, and functionally defective regulatory T cells (Treg). The autoimmune phenotype in FURIN T-cell KO mice could be at least partially attributed to reduced levels of bioactive, anti-inflammatory cytokine transforming growth factor-β1 (TGF-β1), which is a known FURIN substrate molecule.[15] Furthermore, we have recently demonstrated that FURIN directly regulates T-cell activation by modifying the TCR-induced transactivation steps.[16]

The implication of PCSKs as regulators of tumor progression and metastasis has provoked an interest to use them as targets of novel anticancer agents. Based on our previous findings, inhibition of FURIN activity in immune cells could be an effective way of boosting antitumor host responses. Thus, we investigated how the immune-cell-expressed FURIN regulates tumorigenesis by using mouse strains with a conditional deletion of FURIN either in T cells or in myeloid cells (CD4cre and LysMcre, respectively) in the two-stage chemical DMBA/TPA-induced skin carcinoma model. Our data demonstrate that FURIN expression in T cells, but not in myeloid cells, constrains the DMBA/TPA-induced development of squamous skin cancer.

Results

T-cell-expressed FURIN inhibits skin tumor induction

Targeting PCSK, and in particular, FURIN activity, has been considered a promising cancer treatment.[17] However, the germ-line deletion of FURIN causes embryonic lethality, which confounds the studies on its *in vivo* functions in cancer research.[18] Therefore, the cell-type-specific function of FURIN in carcinogenesis has remained incompletely understood. To investigate if the immune-cell-expressed FURIN controls skin tumor formation, we treated the back skin of adult mice deficient for FURIN gene expression either in macrophages and granulocytes (designated LysMcre KO[19,40]) or in CD4$^+$ and CD8$^+$ T cells (designated CD4cre KO,[14]) and their respective wild-type littermates (LysM WT and CD4$^+$ WT) once with a local application of the mutagen DMBA, and then with the growth-promoting agent TPA, twice weekly for a period of 16 and 21 weeks. This treatment induces papillomas derived from the interfollicular epidermis.[20]

FURIN protein expression was detected in untreated and DMBA/TPA-treated skin in CD4$^+$ WT mice (Fig. S1). In normal skin, FURIN was expressed abundantly in the epidermis and some resident cells in the dermis were also positive for FURIN expression. DMBA/TPA application induced FURIN mRNA expression and resulted in a strong accumulation of FURIN expressing cells in the dermal part of the skin (Fig. S1).

Unexpectedly, deletion of FURIN specifically from T cells resulted in the development of more papillomas ($p < 0.0001$, Fig. 1A). The first papillomas were observed in the CD4cre KO mice 8 weeks after the beginning of the DMBA/TPA treatment, and after 9 weeks, all of the CD4cre KO mice had developed papillomas on their back skin. The first papillomas were identified in both WT control strains as well as in the LysMcre FURIN KO mice after 10–12 weeks of treatment (Fig. 1A). Furthermore, the CD4cre KO mice also developed significantly more tumors on their back skin than the other strains ($p < 0.001$, Fig. 1B). Prior to euthanization (at 17 weeks due to ethical reasons), the CD4cre KO mice had developed almost 20 papillomas per animal, whereas the WT controls had less than five papillomas on average (Fig. 1B). In addition, both LysMcre KO and LysM WT mice had a similar number of tumors at 17 weeks as CD4$^+$ WT mice. The treatment of LysMcre KO and WT strains was continued for additional 5 weeks, but no differences in tumor formation could be detected (Fig. 1B). The tumors were incident in CD4cre KO animals at a rate on average 4.6-fold greater than in CD4$^+$ WT mice during the course of experiments (negative binominal regression analysis:incidence rate ratio (IRR) = 4.6; 95% confidence interval (CI) 1.97, 10.79).

Despite CD4cre KO mice were developing skin tumors significantly faster and in greater numbers than the other strains, the papillomas in the CD4cre KO mice did not continue to grow in size (Fig. S2A). Instead, a large number of small papillomas visibly disappeared and some converted into chronic ulcers (Fig. S2B). We could not detect similar ulcers in CD4$^+$ WT, LysMcre KO, and LysM WT strains (Fig. 1C and Fig. S2). The histological analysis revealed that the ulcers in CD4cre KO mice had papilloma formations, but also ruptured epidermis and clusters of neutrophils as a sign of compromised physical integrity of the skin (Fig. S2F). In conclusion, the lack of FURIN in T cells, but not in LysM$^+$ myeloid cells, promotes tumor induction and formation in the DMBA/TPA-induced skin cancer. This prompted us to further characterize the role of T-cell-expressed FURIN in squamous skin cancer immunosurveillance.

The susceptibility to tumor formation in CD4cre FURIN KO mice is associated with enhanced cell proliferation, but not with vascularization

To understand the mechanism of the skin tumor-inhibiting function of T-cell-expressed FURIN, we first performed histological analyses to determine the epidermal and dermal thicknesses, cell proliferation (Ki67) and apoptosis (TUNEL) frequency in the back skin of DMBA/TPA treated and untreated mice.

In untreated mice, loss of FURIN from T cells resulted in a significant thickening of the epidermis (Fig. S3A). Treatment with DMBA/TPA induced a substantial increase ($p < 0.001$) in the epidermal thickness in both genotypes (both epidermises $p < 0.0001$ over untreated skin), and approximately a 30% increase in the thickness of the epidermis persisted in the CD4cre KO mice over the CD4$^+$ WT mice ($p < 0.0001$, Fig. S3A). In contrast, significant

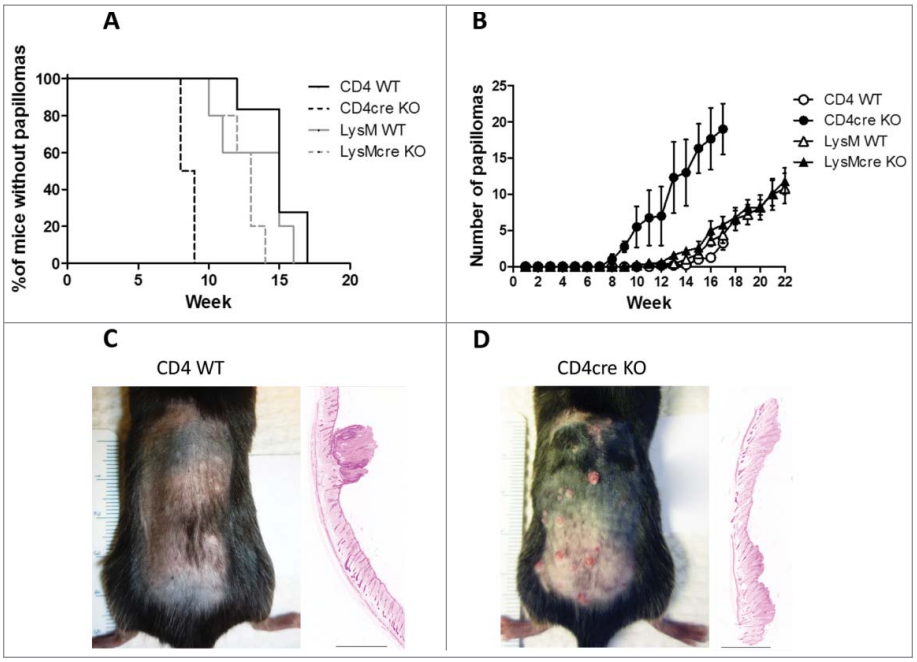

Figure 1. T-cell-specific deletion of FURIN accelerates skin tumor formation. Wild-type (LysM WT and $CD4^+$ WT), T-cell (CD4cre) and macrophage and neutrophil-specific (LysMcre) knockout mice were subjected to DMBA/TPA-induced skin carcinogenesis. (A) The percentage of tumor-free animals at each time point is shown. Survival plot was generated and analyzed via log-rank (Mantel-Cox) test. (B) The mean number of tumors per mouse at each time point is shown ± standard error of the mean. The data were analyzed using STATA 13.0 software. A non-linear regression model was used to compare the slopes of the data. (C) Representative photograph of a $CD4^+$ WT mouse at week 13 of the DMBA/TPA treatment trial, alongside a hematoxylin-eosin stained section of skin at week 17 (the black bar represents 2 mm). (D) Representative photograph of a CD4cre KO mouse at week 13 of the DMBA/TPA treatment trial, alongside a hematoxylin-eosin stained section of skin at week 17 (the black bar represents 2 mm). $CD4^+$ WT n = 6, CD4cre KO n = 4, LysM WT n=5, and LysMcre KO n = 5.

differences were not detected in the dermal thicknesses of untreated or treated animals (Fig. S3B).

CD4cre KO mice had also significantly more proliferating cells (as determined by Ki67-positivity) than WT mice in the epidermis of the untreated skin ($p < 0.0001$, Fig. S3C). After 17 weeks of DMBA/TPA treatment, the CD4cre KO mice had more proliferating cells in both epidermal and dermal parts of the skin ($p < 0.001$ and $p = 0.0056$, Figs. S3C and D). In contrast, there was no difference in the cell proliferation rate in the papilloma tissue (or the dermis beneath it) (Figs. S3C and D). TUNEL staining demonstrated that CD4cre KO mice had less apoptotic cells in untreated dermis ($p < 0.05$, Fig. S4A), but a prolonged DMBA/TPA-treatment (17 weeks) reduced significantly the numbers of dying cells in both genotypes (epidermis, $p < 0.0001$, dermis $p < 0.0001$, Fig. S4).

As the availability of vascular supply is a limiting factor for tumor growth and FURIN has a substantial influence on angiogenesis,[6,7] we also examined the vasculature in the skin. Angiogenesis is turned on already in the earliest stages of papilloma formation and in late stages the blood vessels start to increase in size.[21] There were slightly more blood vessels in CD4cre KO mice in the untreated skin compared to WT as determined by an immunohistological analysis of endothelial cell marker CD31 (Fig. S5A). A 17-week treatment with DMBA/TPA induced a 2-fold increase in the vascular density of the skin only in $CD4^+$ WT mice ($p < 0.0001$), whereas in CD4cre KO the vascular density was significantly smaller ($p < 0.0001$, Fig. S5A). Despite the fact that non-tumorous WT skin had significantly more blood vessels than in the CD4cre KO mice after the DMBA/TPA treatment, the tumor formation required vascular supply in both genotypes as evidenced by the increased number of blood vessels beneath the tumors ($p < 0.0001$, Fig. S5). In conclusion, the accelerated tumorigenesis in CD4cre KO mice was not found to be associated with increased angiogenesis.

Mice lacking FURIN in T cells have an attenuated macrophage extravasation or differentiation response to DMBA/TPA treatment

Previous work has shown that tumorigenesis in the DMBA/TPA model is promoted upon the induction of acute inflammation in the skin at the sites of chemical application.[22,23] To investigate if the CD4cre KO mice had an enhanced skin inflammatory response, we first quantified skin $CD3^+$ T cells as well as the numbers of infiltrating F4/80+ macrophages and elastase positive neutrophils. There were significantly more $CD3^+$ T cells in the CD4cre KO mice than in littermate controls in both untreated skin ($p < 0.0001$) and at 43 h post DMBA/TPA treatment ($p < 0.05$), but after 17 weeks T cell numbers were similar (Figs. 2A and B). In contrast, the numbers of macrophages were more readily induced in KO mice at 43-h time-point, but interestingly this was reverted at later stages; after a 17-week treatment CD4cre KO mice showed significantly reduced macrophage counts in non-tumorous skin ($p < 0.001$, Figs. 2C and D). The significantly lower number of macrophages in the CD4cre KO skin was especially evident underneath the papillomas, where the KO mice had only ca. twenty-five percent of the macrophages seen in the WT dermis (Figs. 2C and D). No significant differences were

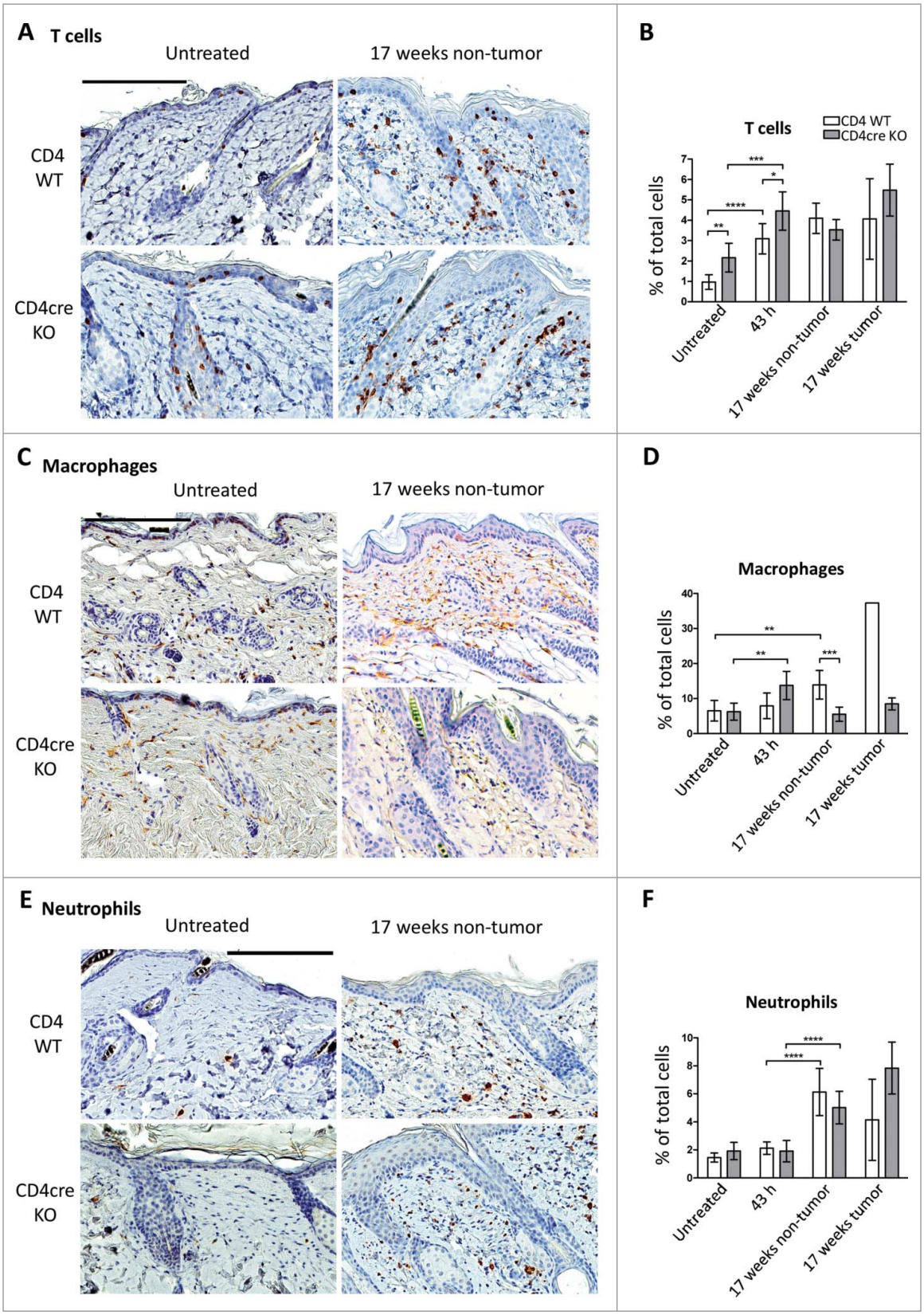

Figure 2. Immune cell numbers in the skin of WT and CD4cre FURIN KO mice. $CD4^+$ WT and CD4cre KO mice were subjected to DMBA/TPA-induced skin carcinogenesis as described in methods. Skin samples were collected from untreated animals and from mice sacrificed at 43 h after the second TPA application, and after 17 weeks of treatment (twice weekly). The skin samples were processed for IHC as described in methods. Skin sections were IHC stained for markers for T cells (CD3), macrophages (F4/80), and neutrophils (neutrophil elastase). Results are shown as mean ± 95% confidence intervals. Data were analyzed by normality tests and unpaired two-tailed Student's *t*-tests (GraphPad Prism 6). The black bar in images represents 200 μm. Nuclei are stained blue, and immune cells brown. (A) Representative photographs of T cell staining in $CD4^+$ WT and CD4cre KO skin. (B) Quantitative analyses of scanned slides were performed as described in supplementary methods. Data is expressed as % of total nuclei. (C) Representative photographs of macrophage staining and (D) results of quantitative analyses of scanned slides. (E) Representative photographs of neutrophil staining and (F) results of quantitative analyses of scanned slides. The information of animal numbers and analyzed tissue regions is shown in Table S1. ($^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$).

observed in neutrophils between the genotypes (Figs. 2E and F). Collectively these findings suggest that CD4cre KO mice may have accelerated skin immune responses at the early stage of cancer development, but this is later followed by reduced macrophage presence in cancerous skin.

The skin-draining lymph node T cells lacking FURIN display an activated phenotype

To gain specific information on the T-lymphocyte populations, the skin draining lymph node (dLN) cells from CD4cre KO mice and their WT littermate controls were subjected to flow cytometric analysis. There were no significant differences in the total $CD4^+$ T cell percentages (of total live cells) between the untreated CD4cre KO and WT mice or between CD4cre KO and WT mice treated with DMBA/TPA for 17 weeks (Fig. 3). In untreated CD4cre KO mice, there were fewer dLN $CD8^+$ T cells (percent of the live cells) than in the untreated WT controls, but the difference was lost after the 17-week DMBA/TPA treatment. However, the dLN T cells from the CD4cre KO animals displayed a more active phenotype, as significantly higher proportion of both $CD4^+$ and $CD8^+$ T cells from KO animals was positive for CD69, a marker for recently activated cells (Fig. 3).

The analysis of T-cell memory phenotypes using CD44 and CD62L antibodies demonstrated a significantly higher percentage of central memory $CD62L^{high}CD44^{high}$ $CD8^+$ T cells (of total $CD8^+$ T cells) in both untreated and 17-week-treated CD4cre KO animals (Fig. 3). This is in parallel with a recent report showing that blocking endogenous TGF-β1 signaling in $CD8^+$ T cells enhances their conversion into central memory cells.[24] Since FURIN processes pro-TGF-β1, the autocrine TGF-β1 signaling is likely to be defective in FURIN-deficient $CD8^+$ T cells. There were also significantly more $CD4^+CD25^+Foxp3^+$ Treg cells in the skin dLNs of CD4cre KO mice compared to those of WT littermates, both from untreated mice and mice treated with DMBA/TPA for 17 weeks (Fig. 3).

In conclusion, FURIN expression did not affect the numbers of T cells in the skin dLN after a 17-week DMBA/TPA-treatment, but T cells from the dLNs of the $CD4^+$ KO mice were inherently more active and constituted more of cells with a Treg phenotype, irrespective of the treatment. Therefore, the increased susceptibility of CD4cre KO mice to develop papillomas is not due to reduced dLN T-cell numbers or lack of T-cell activation.

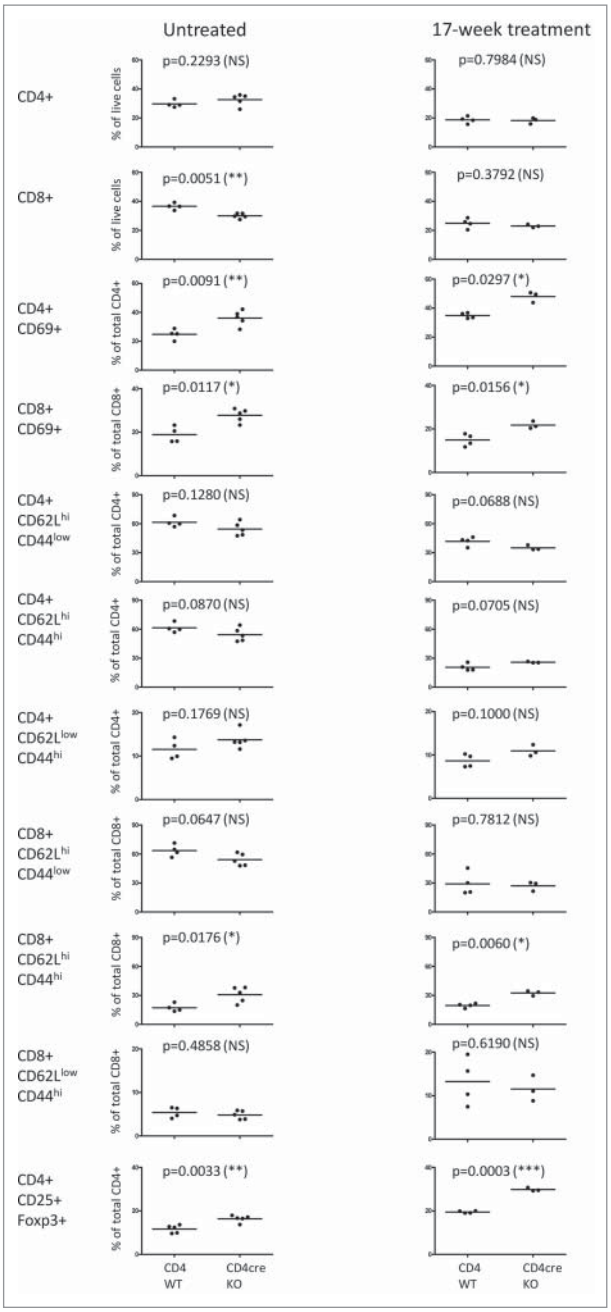

Figure 3. Profiling of the skin draining lymph node T-cell populations in WT and CD4cre KO mice. Cells were isolated from untreated mice and from animals treated with a single application of DMBA and twice-weekly doses of TPA for 17 weeks, and surface markers were analyzed by flow cytometry. Frequency of cells among total live cells or either $CD4^+$ or $CD8^+$ cell populations is shown, each symbol representing an individual mouse, lines indicating the mean. Statistics were calculated with unpaired two-tailed Student's *t*-test (with Welch correction). Untreated mice: n = 5 (for both genotypes), 17-week-treated CD4cre KO animals: n = 3, WT littermate controls n = 4.

T cells lacking FURIN produce more interferon gamma in the early phase of tumor development

To investigate the cytokine production of T cells in the early phase of tumor development, we analyzed dLN T cells at 48 h post the second TPA treatment by intracellular staining and flow cytometry. Both $CD4^+$ and $CD8^+$ effector T cells from the CD4cre KO mice produced more pro-inflammatory interferon gamma (IFNγ) (Figs. 4B and C). On the other hand, there was no difference in the production of another pro-inflammatory cytokine, interleukin (IL) 17A (IL-17A), or in the $CD4^+$ T-cell-produced IL-10, which is considered anti-inflammatory (Figs. 4B and C). In order to gain information on the predominant type of immune response in the CD4cre KO mice and their WT controls, we analyzed the systemic production of various chemokines, by performing a multiplex assay from the sera 43 h post second TPA treatment. There were no obvious differences in the levels of CCL2/MCP1, CXCL-1/GROα, CCL11/Eotaxin, CXCL2/MIP-2, CCL7/MCP-3, CCL5/RANTES, CCL3/MIP-1α, or CCL4/MIP-1β (data not shown), but the level of CXCL10/IP-10 was elevated by 2-folds in CD4cre KO sera compared to those of WT mice ($p = 0.0711$, Fig. 4E). Collectively, these findings imply that in the early

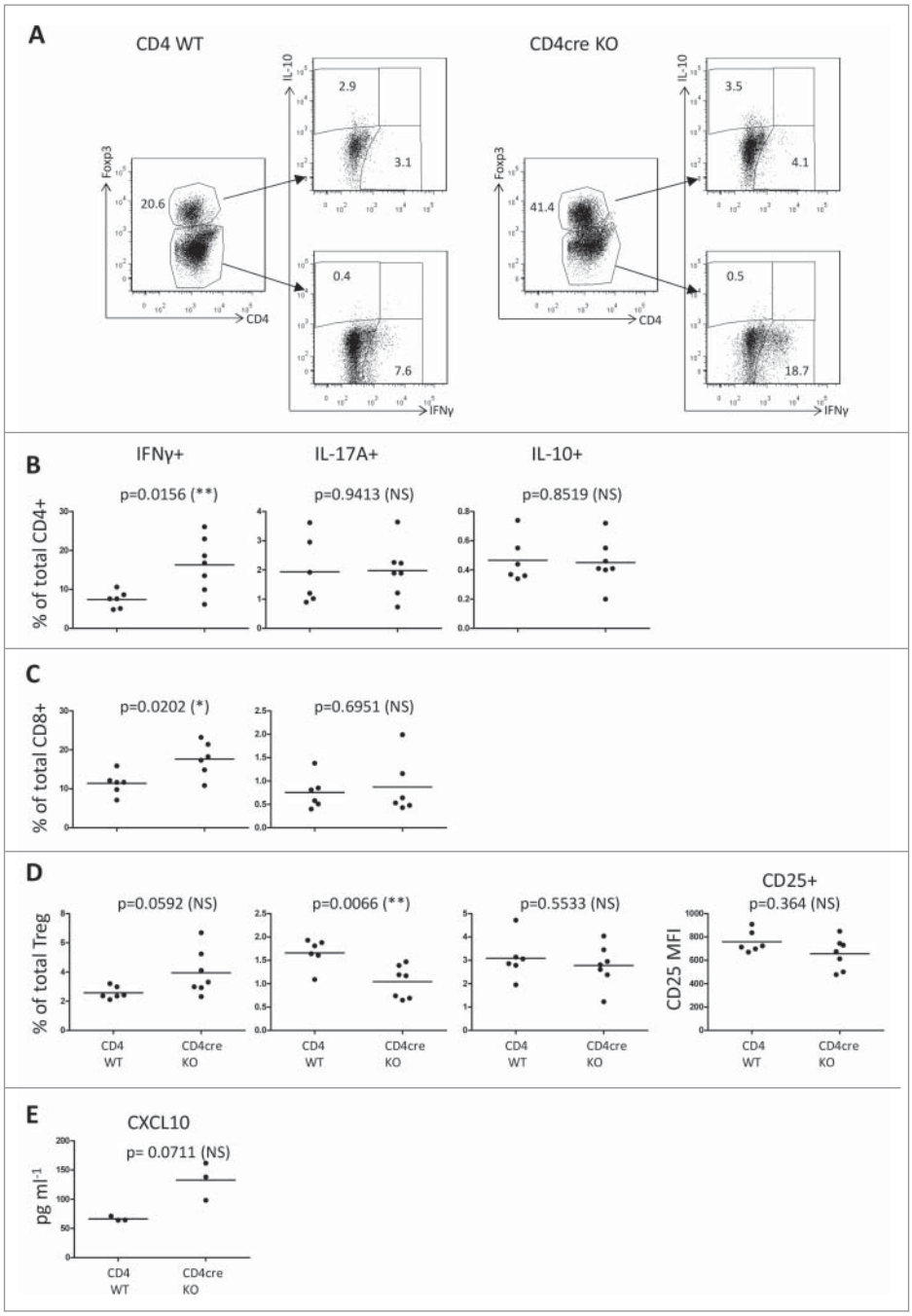

Figure 4. T cells from the skin draining lymph nodes of CD4cre KO mice show elevated IFNγ production in the early phase of tumor development. Intracellular flow cytometry from skin draining lymph node cells. Mice were treated with one dose of DMBA, two doses of TPA on the back skin, then sacrificed after 48 h from the 2nd TPA dose, and the isolated dLN cells were stimulated with TPA + Ca-ionomycin for 4 h *in vitro*. (A) Representative plots from $CD4^+$ WT and CD4cre KO mice, showing live-gated cell populations positive for $CD4^+$, Foxp3, IFNγ, and IL-10. (B) Percentages of $CD4^+$ cells positive for IFNγ, IL-17A, and IL-10. (C) $CD8^+$ cells positive for IFNγ and IL-17A. (D) $CD4^+Foxp3^+$ Treg positive for IFNγ, IL-17A, and IL-10, and the mean fluorescence intensity (MFI) of CD25 from the Treg population. (E) CXCL10 levels from sera from mice treated with TPA for 43 h, determined by the ProCartaPlex Mouse Cytokine & Chemokine 26-plex assay and Bio-Plex 200 instrument. B, C, D: $CD4^+$ WT mice n = 6, CD4cre KO mice n = 7; E: $CD4^+$ WT mice n = 3, CD4cre KO mice n = 3. Each symbol represents an individual mouse, lines indicate the mean. Statistics: Unpaired two-tailed Student's *t*-test with Welch correction.

phase of tumor development the FURIN T-cell-specific knockout mice have a Th1-type-skewed immune response.[25]

We also studied the production of IFNγ, IL-17A, and IL-10 by $CD4^+Foxp3^+$ Treg cells isolated from the dLNs of CD4cre KO and WT mice. There was a trend toward a higher production of IFNγ in the Treg cells from the CD4cre KO animals ($p = 0.0592$), and a significant reduction in the percentage of CD4cre KO Treg cells positive for IL-17A ($p = 0.0066$, Fig. 4D). However, the percentage of dLN Treg cells producing IL-10 was not different between the CD4cre KO and WT. Also the mean fluorescence intensity of the CD25 surface staining was similar in Treg cells isolated from dLNs of both CD4cre KO and WT mice indicating normal Treg activation status.

Collectively, the flow cytometric analyses suggest that the tendency of CD4cre KO animals to develop more papillomas could be related to accelerated Th1-type immune responses.[26] In contrast, despite CD4cre KO animals showing an increased number of regulatory $CD4^+CD25^+Foxp3^+$ cells, these cells are

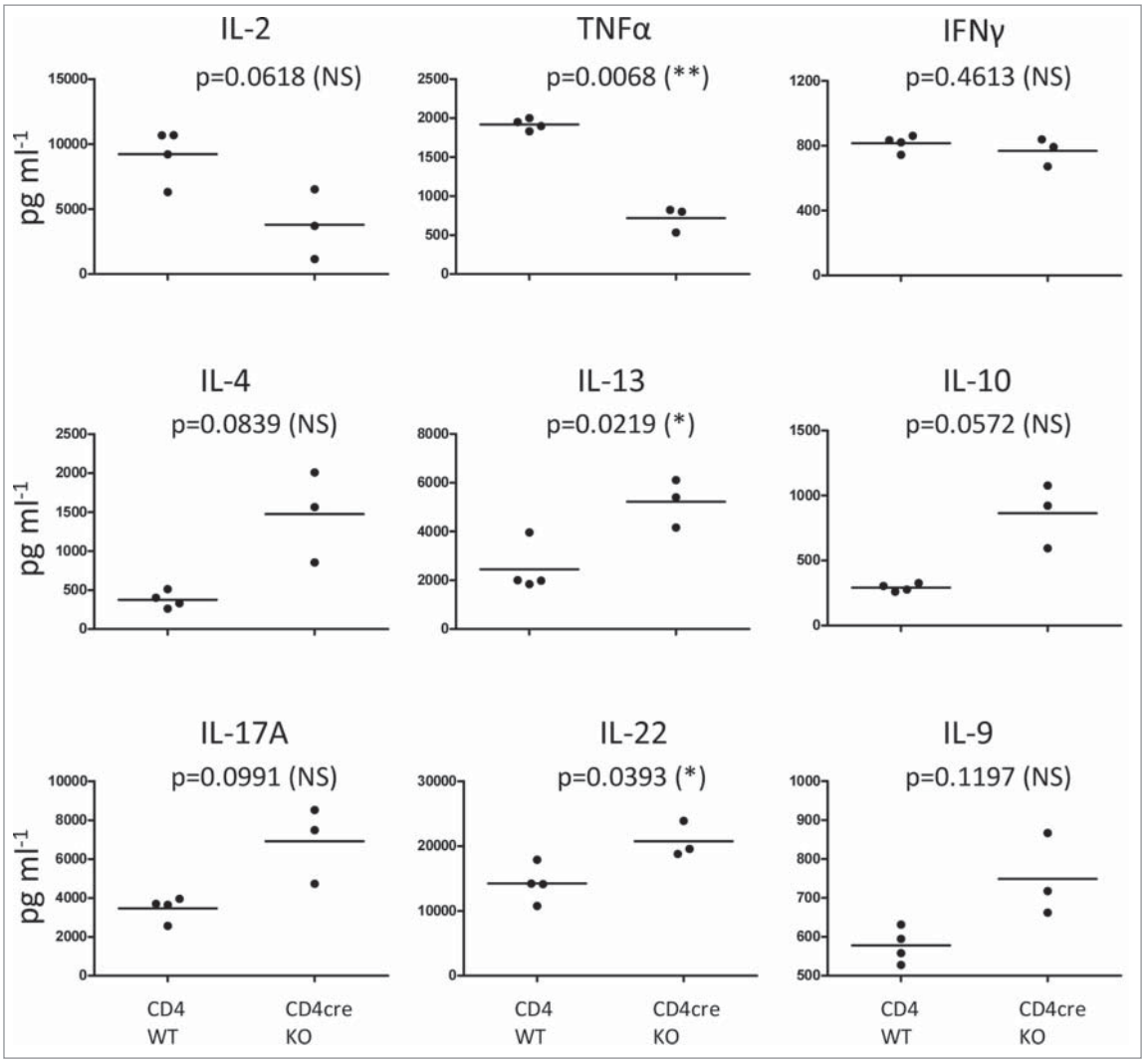

Figure 5. FURIN-deficient T cells show a Th2- and Th17-type cytokine profile of in the late phase of tumor progression. CD4cre KO and WT mice were treated once with DMBA, then with TPA for 17 weeks (twice a week) on the back skin as in Fig. 1. At 17 weeks, animals were sacrificed and skin draining lymph node cells were cultured for 48 h in the presence of plate-bound anti-CD3 and soluble anti-CD28 antibodies. Cytokine levels were determined from the culture supernatants with ProCartaPlex Mouse Cytokine & Chemokine 26-plex assay and Bio-Plex 200 instrument, or for IFNγ, with ELISA. Statistics: Unpaired two-tailed Student's *t*-test with Welch correction. $CD4^+$ WT n = 4, CD4cre KO n = 3. Each symbol represents an individual mouse, lines indicate the mean.

likely defective in their suppressive activity as suggested by upregulated CD69 expression and IFNγ production in $CD4^+$, $CD8^+$ effector T cells, and Tregs. This conclusion is also supported by our previous data demonstrating impaired peripheral Treg-dependent immune tolerance in CD4cre KO mice.[14]

In the later stage of carcinogenesis T-cell responses in the FURIN deficient T cells switch toward Th2 and Th17 type

In addition to T-cell activation, FURIN regulates the T helper cell balance of the immune system.[27,28] We profiled the levels of multiple cytokines and chemokines from the cells isolated from skin dLNs of the mice that had been treated with DMBA/TPA for 17 weeks. Surprisingly, the cells from CD4cre KO mice no longer produced elevated IFNγ levels (like in the early phase), but the production of Th2-type cytokine IL-13 and Th17-type cytokine IL-22 was higher ($p = 0.0219$ and $p = 0.0393$, respectively, Fig. 5). Also, the levels of other Th2/Th17 cytokines, IL-4, IL-17A, IL-9, or IL-10 showed a trend toward upregulated production in the CD4cre KO animals (Fig. 5). QRT-PCR performed from the back skins confirmed the switch in Th responses, showing an upregulation of *IFNγ* in the KO animals in the early time points and of *IL-17a* in the late time point (Fig. S6). The levels of IL-2 and tumor necrosis factor α (TNFα) were lower in the dLN cells isolated from CD4cre KO mice compared to those isolated from WT animals, as we have previously shown in the untreated animals.[14] Altogether, the results suggest that during the course of carcinogenesis progression, the early Th1-biased immune responses of the T-cell-specific FURIN deficient mice switch increasingly toward Th2 and Th17 type responses. Thus, FURIN has an inherent role in modulating T helper cell balance in a chronic immune insult.

Discussion

Various studies have shown that FURIN overexpression is associated with accelerated carcinogenesis. Thus, its inhibition could be a viable cancer treatment. However, the function of

FURIN in different cell types that contribute to tumor formation is poorly characterized. To study the role of FURIN in immune cells in the context of squamous skin cancer development, we utilized two mouse strains that lack FURIN specifically either in macrophages and granulocytes or in T cells.

As our previous results have shown that FURIN-deficient T cells and macrophages display an overtly activated phenotype,[14,19,40] we assumed that the FURIN-deficient immune cells might be more effective in anticancer immune responses. However, after being subjected to the DMBA/TPA treatment, mice with the T-cell-specific knockdown of FURIN (CD4cre KO) developed more papillomas and they appeared faster in the knockout than their WT littermates. The CD4cre KO mice presented with a thicker epidermis, with more epidermal cell proliferation than the WT mice, and this difference between the genotypes was maintained after 17 weeks of DMBA/TPA treatment. Under normal conditions, the CD4cre KO skin harbored more $CD3^+$ T cells than the WT skin, but upon treatment that difference was lost. Analysis of skin dLN cells showed no major FURIN-dependent effects of the DMBA/TPA treatment on the numbers of $CD4^+$ or $CD8^+$ T-cell populations. The higher number of $CD4^+CD25^+Foxp3^+$ Tregs present in CD4cre KO dLNs in the steady state persisted after the treatment, as did the number of activated $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells. In the early phase of tumor promotion, the CD4cre KO mice had more pro-inflammatory IFNγ-producing T cells, whereas CD4cre KO dLN cells secreted more Th2- and Th17-type cytokines after 17 weeks of DMBA/TPA treatment (Fig. S7).

The fact that CD4cre KO, but not LysMcre KO, mice had an increased susceptibility for papilloma development is in line with several studies showing a tumor-enhancing role for $\alpha\beta$ T cells.[29,30] Previous work has demonstrated that IFNγ promotes tumor development primarily in the early stage of papilloma development.[26] IL-17A, in turn, has a role in the promotion process in both human non-melanoma skin cancer and mouse models of skin cancer, suggesting that Th2- and Th17-type cytokine profile of the T cells lacking FURIN could be driving the carcinogenesis process at later stages.[31-33] LysMcre KO mice, in contrast, display accelerated innate immunity responses, and a heterozygous, inactivating mutation in the *FurinA* gene results in enhanced innate responses in *Mycobacterium marinum* –infected zebrafish.[19,40] However, the pro-inflammatory phenotype due to lack of FURIN in myeloid cells does not seem to play a major role in the papilloma development in the DMBA/TPA-model.

FURIN and other PCSK family members play an important role in carcinogenesis and metastasis, and there has been considerable interest to develop pharmaceutical inhibitors of their activity for cancer treatment. Inhibiting FURIN/PCSK is reasoned to directly block the processing of factors that are associated with tumor invasion and metastatic activity, including matrix metalloproteinases, growth factors, and others. Furthermore, FURIN is critical for the activation of anti-inflammatory cytokine TGF-β1. Thus, specific blocking of FURIN is thought to support antitumor host-responses by promoting cancer immunosurveillance. The assessment of a bifunctional GM-CSF-FURINshRNA construct (FANG^{TM}, Vigil^{TM}, Gradalis) has already entered the phase II clinical trials for treatment of melanoma, ovarian cancer, and colorectal cancer with liver metastasis.[17,34] Recently, the shRNA-mediated inhibition of FURIN together with dendritic cell supporting GM-CSF expression was also found to be efficacious in metastatic, advanced Ewing's sarcoma.[35] In contrast, in liver cancers FURIN overexpression suppresses tumor growth and predicts better postoperative survival.[36] The beneficial effect of FURIN in hepatocellular carcinoma was also reported in mice where FURIN was deleted using liver specific Albumin CRE.[37] It seems therefore plausible that FURIN has tumor type-specific effects, and thus FURIN inhibition systematically may not always be beneficial for oncology patients.

The role of FURIN in T-cell-dependent immunity is clearly multifaceted. A major function of FURIN is to control the bioavailability of anti-inflammatory TGF-β1, and Treg-dependent peripheral immune tolerance.[14] TGF-β1 is a multifunctional growth factor that has roles in both promoting and suppressing tumorigenesis.[38] Deleting TGF-β1 specifically from activated $CD4^+$ T cells and Treg cells reduced metastatic B16-OVA tumor cell spread to the mouse lung, indicating that activated $CD4^+$ T-cell-derived TGF-β1 inhibits tumor immunosurveillance.[39] Importantly, we have also shown that FURIN is an important factor in modulating the T helper cell balance. Mice that were chronically infected with intracellular *Toxoplasma gondii* parasite had less pathogen-specific Th1-type immune cells, and naïve, OVA-specific FURIN KO $CD4^+$ T cells showed an increased tendency to polarize into the IL-4-producing Th2 cells.[27] The switch from Th1 into Th2/Th17-type responses was also seen here in CD4cre KO mice after a 17-week DMBA/TPA treatment. Thus, inhibiting FURIN does not just promote T-cell-driven adaptive immunity, but it also modulates the type of T helper cell responses. It is also noteworthy that the regulatory role of FURIN in $CD8^+$ cytotoxic lymphocytes remains incompletely understood. Our data showed that FURIN-deficient CTLs produced more IFNγ and showed an upregulation of CD69 activation marker, but a careful analysis of for example their cytotoxic potential, granzyme B, and perforin expressions needs further studies.

In conclusion, our data demonstrate that FURIN expression in T cells clearly modulates adaptive immune responses in both untreated mice and in animals suffering from DMBA/TPA-induced skin papillomas. This leads to accelerated tumor development accompanied with aberrant T-cell cytokine production in T-cell-specific FURIN KO mice. Our findings suggest that inhibiting FURIN systematically, or specifically in T cells, may promote the development of cancer types wherein a chronic immune insult has a cancer provoking role. This is an important aspect when considering FURIN inhibitors' therapeutic potential in human cancers.

Materials and methods

Mice

T-cell-specific FURIN conditional knockout (CD4cre KO) mice on a C57BL/6 background have been described previously.[14,27] Macrophage-specific FURIN conditional knockout mice (LysMcre KO) were generated using a LysMcre C57BL/6 background.[19,40] Mice were fed with standard laboratory pellets and

water *ad libitum*. All animal experiments were performed in accordance with protocols approved by the National Animal Ethics Committee of Finland.

Skin tumor induction

Both FURIN KO strains, LysMcre and CD4cre, as well as their respective littermate control C57BL/6 WT, LysM WT and $CD4^+$ WT mice were treated with DMBA and TPA to induce skin tumors as previously described.[22] In brief, the backs of 8–14-week-old mice were shaved and 24 h later 50 μg DMBA (7,12-Dimethylbenz[a]anthracene) (Sigma, Dorset, UK) in 200 μL acetone was applied topically on the shaved area of the dorsal skin. After a week, the back skin of the mice was treated twice a week with 5 μg TPA (12-*O*-tetradecanoylphorbol-13-acetate) (Sigma) in 200 μL acetone for 16 or 21 weeks. The fur excluding tumors was carefully shaved every 2 weeks. Tumors (1 mm in diameter or larger) were counted twice a week and changes in tumor development were recorded for each individual tumor.

Immunohistochemical (IHC) and TUNEL staining

Samples of back skin from sacrificed, shaved control mice or mice at 43 h or week 17 of the tumor induction experiment were collected and fixed with 4% paraformaldehyde and embedded in paraffin according to standard protocols. Hematoxylin/eosin staining and DAB immunohistochemical staining (IHC) was performed on 6-μm thick paraffin sections as previously described.[22] The following primary antibodies were used for IHC: A0452 rabbit anti-CD3 (DakoCytomation, Glostrup, Denmark), MF48000 BM8 rat anti-F4/80 (Life Technologies Ltd., Paisley, UK), and 68672 rabbit anti-neutrophil elastase (AbCam, Cambridge, UK). More detailed list of reagents, imaging, and quantitation are described in the Supplementary methods.

Flow cytometry

For surface markers, the skin dLN cells were stained with antibodies against mouse CD4, CD8, CD44, CD62L, and CD69 (all from eBioscience, San Diego, California, USA). For intracellular staining, isolated dLN cells were stimulated with PMA and Caionomycin for 4 h, and Brefeldin A and Monensin were applied for the last 2 h of the stimulation. The cells were stained with surface markers and subsequently fixed overnight with Fixation/Permeabilization solution (from Foxp3/Transcription Factor Staining Buffer Set, eBioscience), permeabilized with Permeabilization Buffer (eBioscience) and stained with intracellular antibodies (IL-10, IFNγ, IL-17A, Foxp3; all from eBioscience), according to the manufacturer's instructions. All cells were analyzed with FACSCanto II (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA), data analysis performed with FlowJo software (Flowjo LLC, Ashland, Oregon, USA).

Luminex and ELISA assays

Skin dLN cells from mice treated with DMBA/TPA for 17 weeks were cultured for 48 h in the presence of plate-bound anti-CD3 antibody (10 μg/mL, clone 17A2, eBioscience) and soluble anti-CD28 Ab (2 μg/mL, clone 37.51, eBioscience). Multiplex cytokine/chemokine measurement was done from the culture supernatants using ProCartaPlex assay (Mouse Cytokine & Chemokine 26-plex, eBioscience) according to the manufacturer's instructions, and with Bio-Plex 200 instrument (Bio-Rad). IFNγ levels were determined from the cell culture supernatants with a Ready-Set-Go! ELISA kit (eBioscience), according to the manufacturer's instructions.

Statistical analysis

Mean averages are shown with 95% confidence intervals, in the case of Fig. 1B with SEM. Immunohistochemistry data were analyzed to determine if it was normally distributed (D'Agostino & Pearson omnibus and Shapiro–Wilk normality tests). Significance at a given time point was calculated by two-tailed Student's *t*-test for normally distributed data. An α level less than 0.05 was considered significant. Tumor-free survival plot data were analyzed by log-rank (Mantel-Cox) test and non-normally distributed time course data were analyzed by non-linear regression. Prism 6 (GraphPad Software, La Jolla California, USA) was used for a majority of the analyses and STATA 13.0 (StataCorp LP, College Station, Texas, USA:) statistical analysis software was used for non-linear negative binomial regression analysis, as indicated.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

T.J., M.V., M.P., S.A., H.U.-J., and Z.M.C. designed the research. M.V., S. A., Z.M.C., and U.M. performed the research. M.V., S.P., S.A., Z.M.C., and U.M. analyzed the data. T.J., M.V., U.M., S.A., and M.P. wrote the manuscript. M.V., S.A., and U.M. made the figures. M.V., S.P., U.M., S.A., H.U.-J., M.P., and T.J. reviewed and edited the paper.

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Supplementary Information

Supplementary Materials and Methods

Immunohistochemical (IHC) and TUNEL staining

Samples of back skin from sacrificed, shaved control mice or mice at 43 hours or week 17 of the tumor induction experiment were collected and fixed with 4% paraformaldehyde and embedded in paraffin according to standard protocols. Hematoxylin/eosin staining and DAB immunohistochemical staining (IHC) was performed on 6 µm thick paraffin sections as previously described (1). The following primary antibodies were used for IHC (according to the manufacturer's instructions): M7249 TEC-3 rat anti-Ki67 and A0452 rabbit anti-CD3 (DakoCytomation, Glostrup, Denmark), 550274 rat anti-CD31 (BD Pharmingen, Oxford, UK), 68672 rabbit anti-neutrophil elastase (AbCam, Cambridge, UK), MF48000 BM8 rat anti-F4/80 (Life Technologies Ltd., Paisley, UK) and H-220 rabbit anti-FURIN (Santa Cruz, Dallas, TX, USA). The blocking reagents used for IHC were S2O23 REAL and S0809 Antibody Diluent (Dako). In the case of blocking prior to CD3 or neutrophil elastase staining, G9023 goat serum or A4503 BSA (Sigma) were used respectively, at 5% in PBS. The horseradish peroxidase (HRP) conjugated secondary antibody reagents used were: PO448 goat anti-rabbit (Dako), 414311F anti-rat Histofine (Nichirei Bio, Tokyo, Japan) and for neutrophil elastase staining RMR622 Rabbit on Rodent (Biocare Medical, Concord, CA, USA). XMF963 XM-Factor (Biocare) was used to block before secondary staining with Rabbit on Rodent. Peroxidase reactive chromogens used were K3465 DAB (DAKO) and RAEC810 Romulin AEC (BioCare). Immunohistochemical TUNEL staining for apoptotic nuclei was performed using the K403-50 TUNEL IHC Kit (Biovision, Milpitas, CA, USA) with Methyl Green nuclear counter stain as previously described (1).

Quantitative analysis of immunostaining and histochemical staining

All slides were scanned using the Aperio ScanScope® CS and XT systems (Aperio Technologies Inc., California, USA) as previously described (1-2). Slides were viewed and analyzed remotely using desktop personal computers employing the web-based ImageScope™ viewer. The Spectrum digital pathology system analysis algorithm package and Image Scope analysis software (version 9; Aperio Technologies Inc.) were applied to quantify immunohistochemical signal. These algorithms calculate the area of positive staining, the average positive intensity (optical density), as well as the percentage of weak (1+), medium (2+), and strong (3+) positive staining. All quantified histochemical analyses (Ki-67, CD31, F4/80, CD3, elastase, TUNEL) were performed according to the protocols used to establish these algorithms for each respective staining. Approximately 5 000 cells were counted for each measurement from each sample.

For FURIN expression analysis, the slides were imaged via Olympus light microscope and Cell Sens Dimensions software and analyzed with ImageJ 1.44p software using thresholding method to measure the percentage of FURIN positive area in dermis from the total dermal area.

Extraction of RNA and quantitative PCR (qPCR) analysis

Samples of back skin were harvested into RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) and the mRNA was extracted using Invitrogen Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Homogenization and RNA extraction was done as previously described (1). RNA was converted to cDNA by reverse transcription using the Thermo Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific) according to the manufacturer's instructions. 40 ng cDNA (for IFNɣ, reference genes HPRT and TBP), or 80 ng cDNA (for IL-17A, HPRT and TBP) and Thermo Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Fisher Scientific) were used for qPCR analysis, performed in a 7500 Real-Time PCR System (Thermo Fisher Scientific). The thermal cycler profile for all primer sets was: 2min 50°C, 10 min 95°C, 40 x (15 sec 95°C, 30 sec 59°C, 32 sec 72°C). Raw fluorescence data were analyzed with the LinRegPCR program (version 2014.8, http://LinRegPCR.HFRC.nl), based on the methods and procedures described by Ruijter et al., 2009 (3).

Primer sequences were as follows: IFNɣ, 5'-CCAAGTTTGAGGTCAACAACC-3' (forward) and 5'-GCTTCCTGAGGCTGGATTC-3' (reverse). IL-17A, 5'-GACTCTCCACCGCAATGAA-3' (forward) and 5'-GACCAGGATCTCTTGCTGGA-3' (reverse). HPRT, 5'-AGGGATTTGAATCACGTTTGTGT-3' (forward) and 5'-GGCCACAGGACTAGAACACC-3' (reverse). TBP, 5'-CCCACCAGCAGTTCAGTAGC-3' (forward) and 5'-TCTGCTCTAACTTTAGCACCTGTT-3' (reverse). FURIN, 5'-CAG AAG CAT GGC TTC CAC AAC-3' (forward) and 5'-TGT CAC TGC TCT GTG CCA GAA-3' (reverse). 18S, 5'-GTG ATC CCT GAG AAG TTC CAG-3' (forward) and 5'-TCG ATG TCT GCT TTC CTC AAC-3' (reverse).

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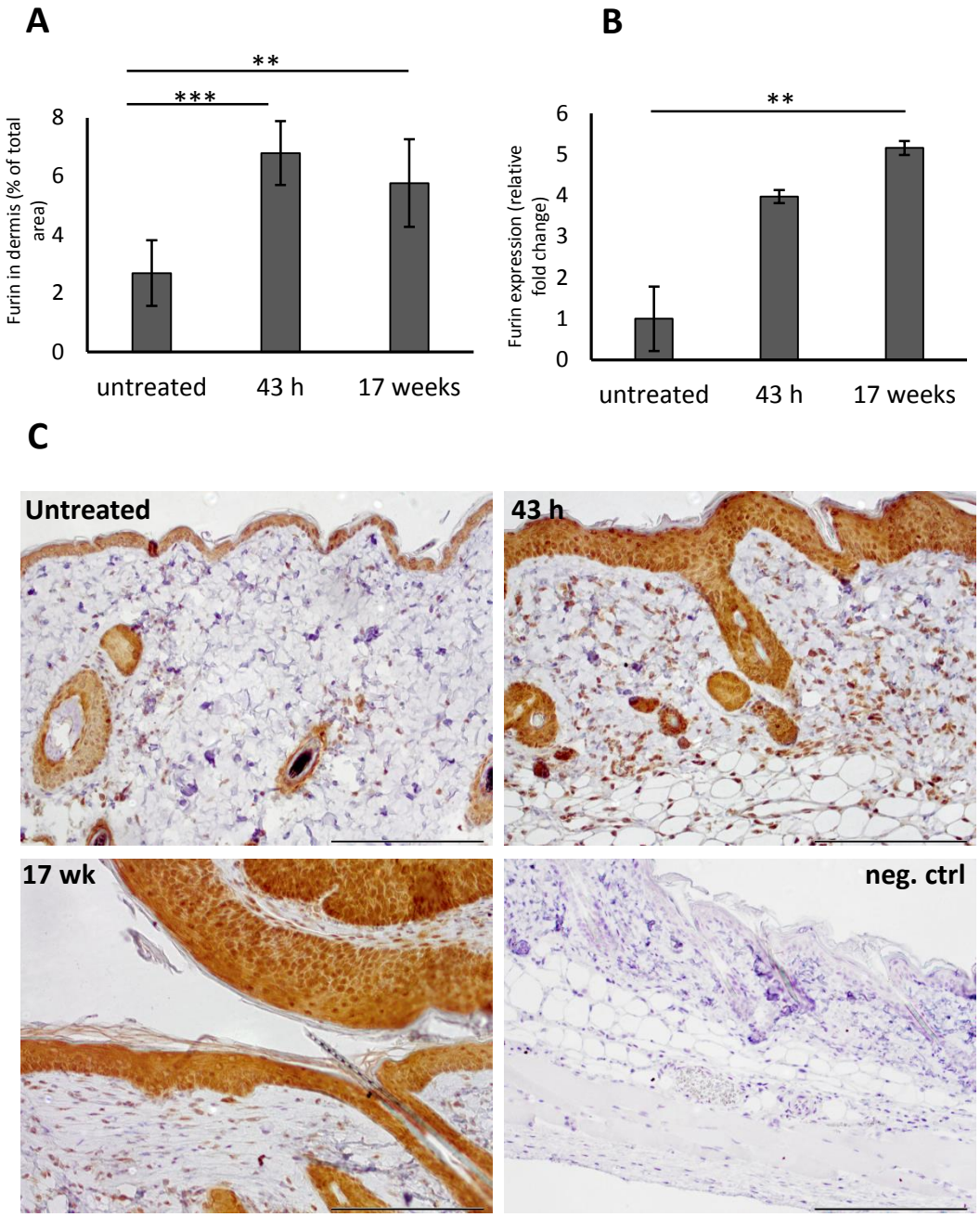
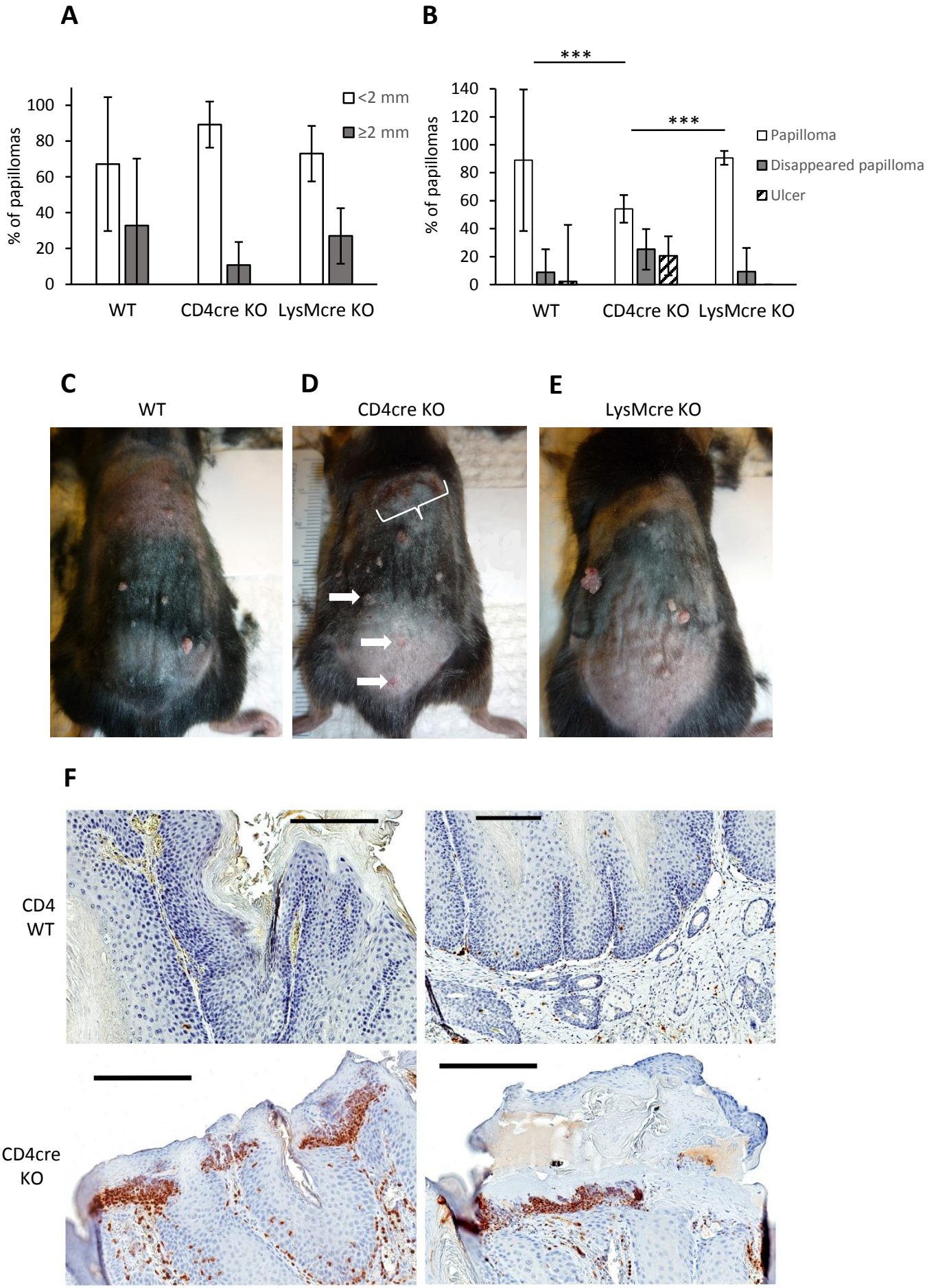
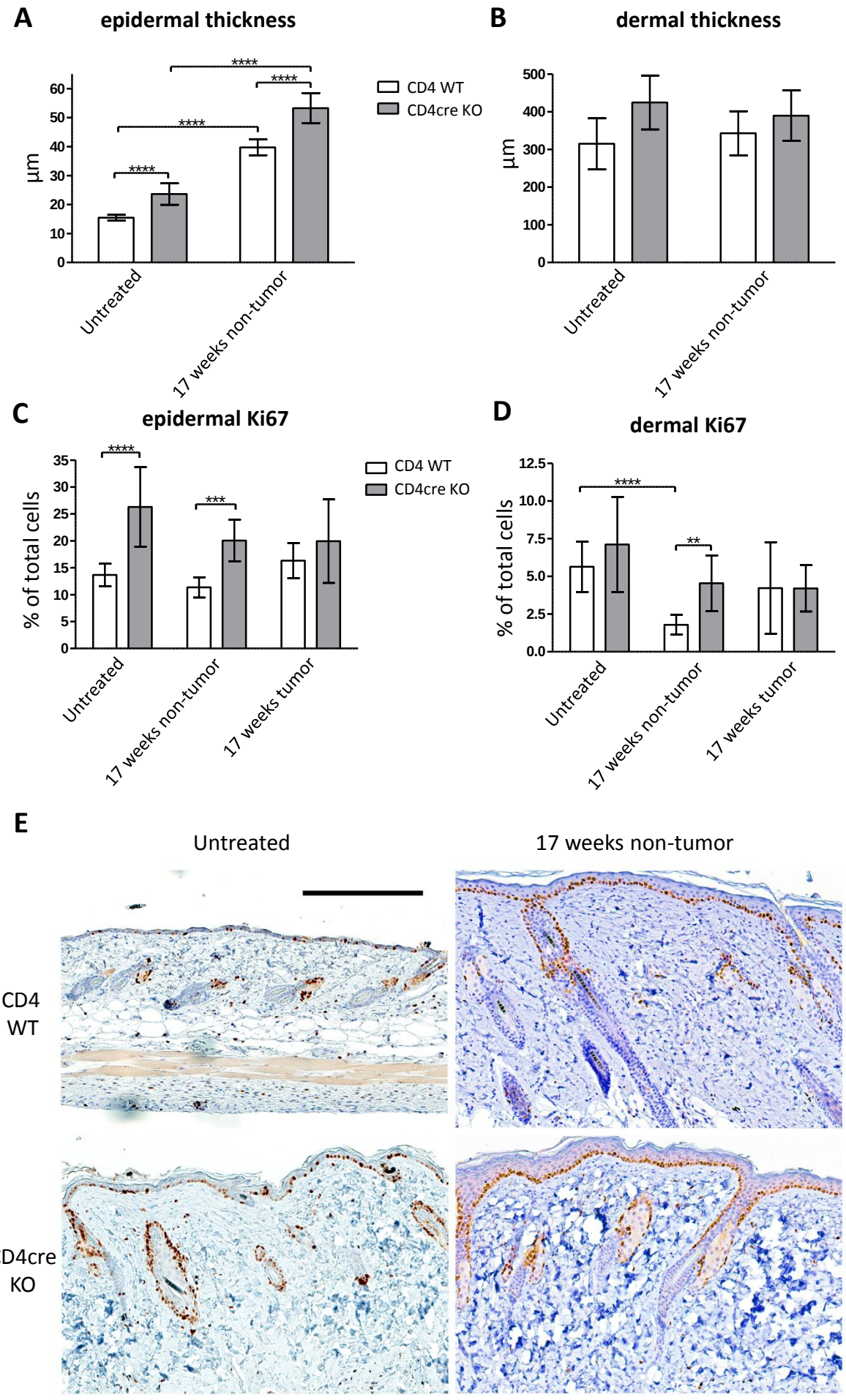


Supplementary Figure S1. FURIN expression is induced in wild-type mouse skin after DMBA/TPA treatment. The back skins from untreated and DMBA/TPA-treated (for 43 h or 17 weeks) wild-type animals were collected for FURIN expression analyses. (**A**) FURIN positive cells in dermis were quantified using immunohistochemistry (IHC). Results are presented as mean ± 95% confidence intervals. The information of animal numbers and analyzed tissue regions is shown in Supplementary Table 1. (**B**) The FURIN and 18S (housekeeping gene) expressions in skin were determined by quantitative RT-PCR. The normalized FURIN expression in untreated skin was arbitrarily set to 1. Results are presented as mean ± SEM. The data were analyzed with one-way ANOVA and Tukey HSD test. Animal numbers; untreated n=4; 43 h n=3; 17 weeks n=4. (**C**) Representative photographs of the FURIN expression in the DMBA/TPA-model are shown. The black bars represent 200 µm. ($P<0.001$, ***; $P<0.01$, **)



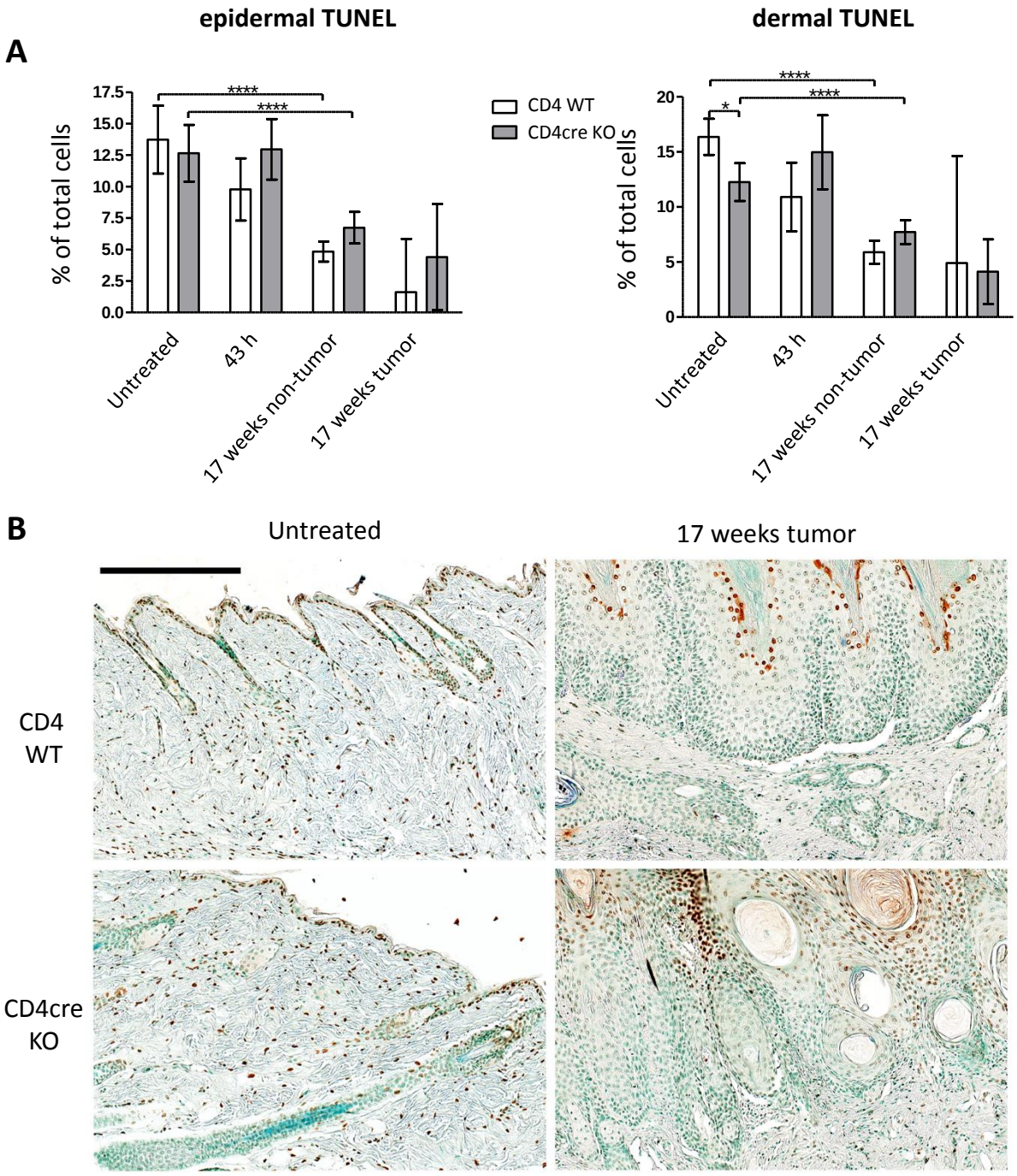
Supplementary Figure S2.

Supplementary Figure S2. Skin tumors disappear and turn into skin ulcers in mice deficient of FURIN in T cells. WT, (CD4 WT and LysM WT combined) CD4cre and LysMcre KO mice were subjected to DMBA/TPA-induced skin carcinogenesis as described in methods. Changes in the tumor development were recorded for each individual tumor. (**A**) Distribution of <2 mm and ≥2 mm papillomas in each group of animals is shown as a percentage of total papillomas. The average percentage of tumors grouped by their size during the first three weeks after a papilloma had reached 1 mm in diameter is shown. Error bars represent standard deviations. (**B**) The distribution of events in tumor development is shown in percentages in WT (CD4cre + LysMcre), CD4cre KO and LysMcre KO animals. (P<0,001, ***, Pearson Chi-Square test). Representative images of LysM WT (**C**), CD4cre KO (**D**) and LysMcre KO (**E**) animals. Arrows point disappeared papillomas and the bracket points to chronic ulcerations. (**F**) Large clusters of neutrophils were seen in the epidermis of small papillomas (lower left corner) or ulcers (lower right corner) in CD4cre KO mice but not in CD4 WT mice (upper row). Scale bar represents 200 µm. The information of animal numbers and analyzed tissue regions is shown in Supplementary Table 1.

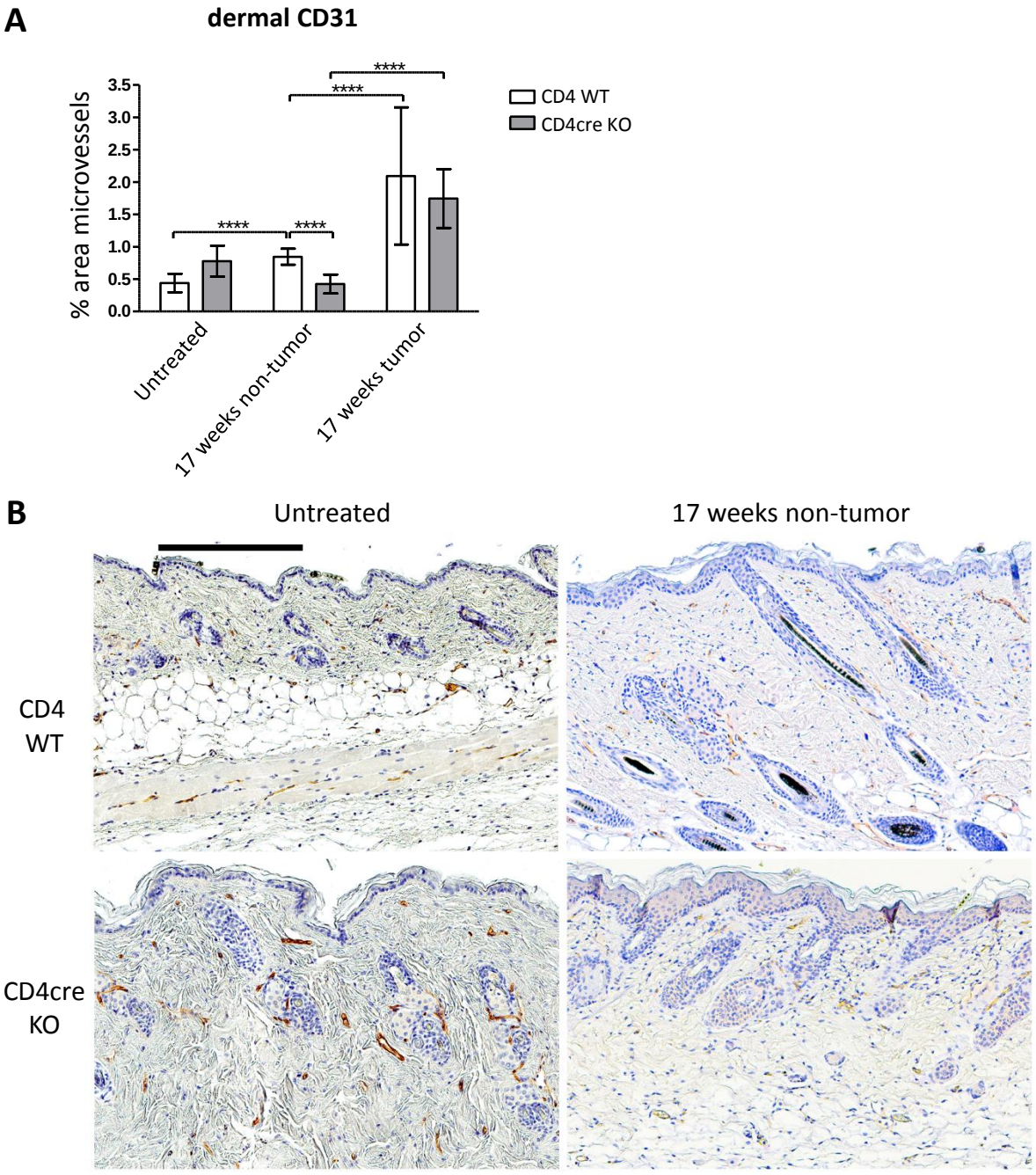


Supplementary Figure S3.

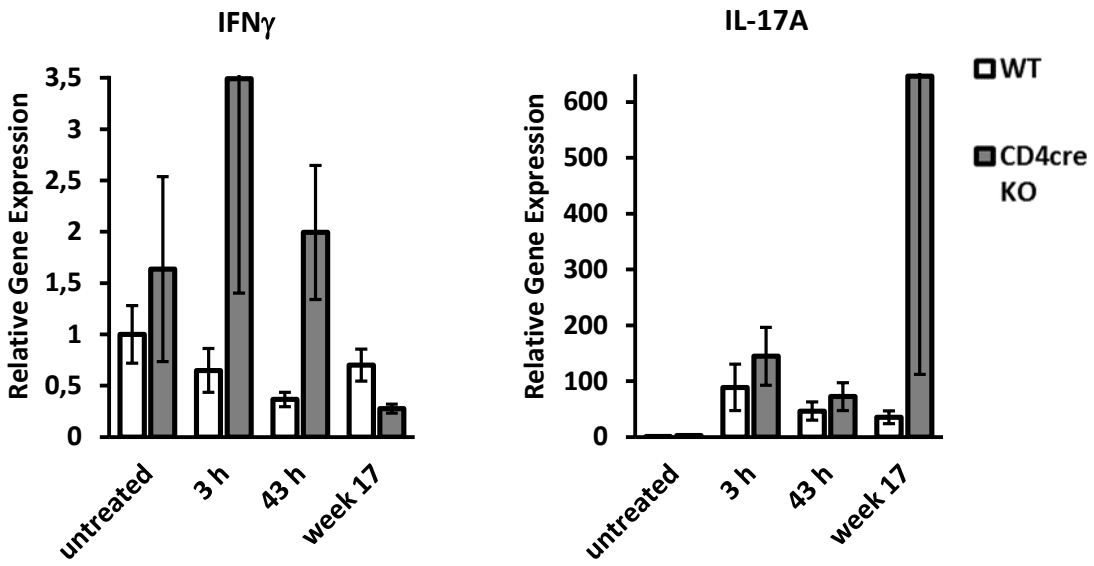
Supplementary Figure S3. Thicknesses of epidermis and dermis and skin cell proliferation. CD4 WT and FURIN CD4cre KO mice were subjected to DMBA/TPA-induced skin carcinogenesis as described in methods. Skin samples were collected, fixed and processed for IHC staining of proliferating nuclei. Quantitative digital pathology analyses of scanned slides were performed. Statistical analyses were performed with GraphPad Prism 6 software. Results are shown as mean ± 95% confidence intervals. The data were analyzed by standard unpaired two-tailed Student's t-tests. (**A**) Measurements of epidermal thickness of non-tumor regions of skin from paraffin sections in triplicates from CD4 WT and T-cell-specific FURIN KO mice (CD4cre KO). (**B**) Measurements of dermal thickness were taken from CD4 WT and CD4cre KO mice. The results are expressed as mean ± 95 % confidence intervals. Proliferating nuclei were stained by IHC with rat anti-Ki67 antibody, and the % of proliferating nuclei determined (**C**) in epidermis and (**D**) in dermis. (**E**) Representative Ki67 staining for proliferating cells in untreated and DMBA/TPA-treated skin is shown for the CD4 WT and FURIN CD4cre KO animals. Black bar in images represents 300 μm. The information of animal numbers and analyzed tissue regions is shown in Supplementary Table 1. ($P<0.0001$, ****; $P<0.001$, ***; $P<0.01$, **)



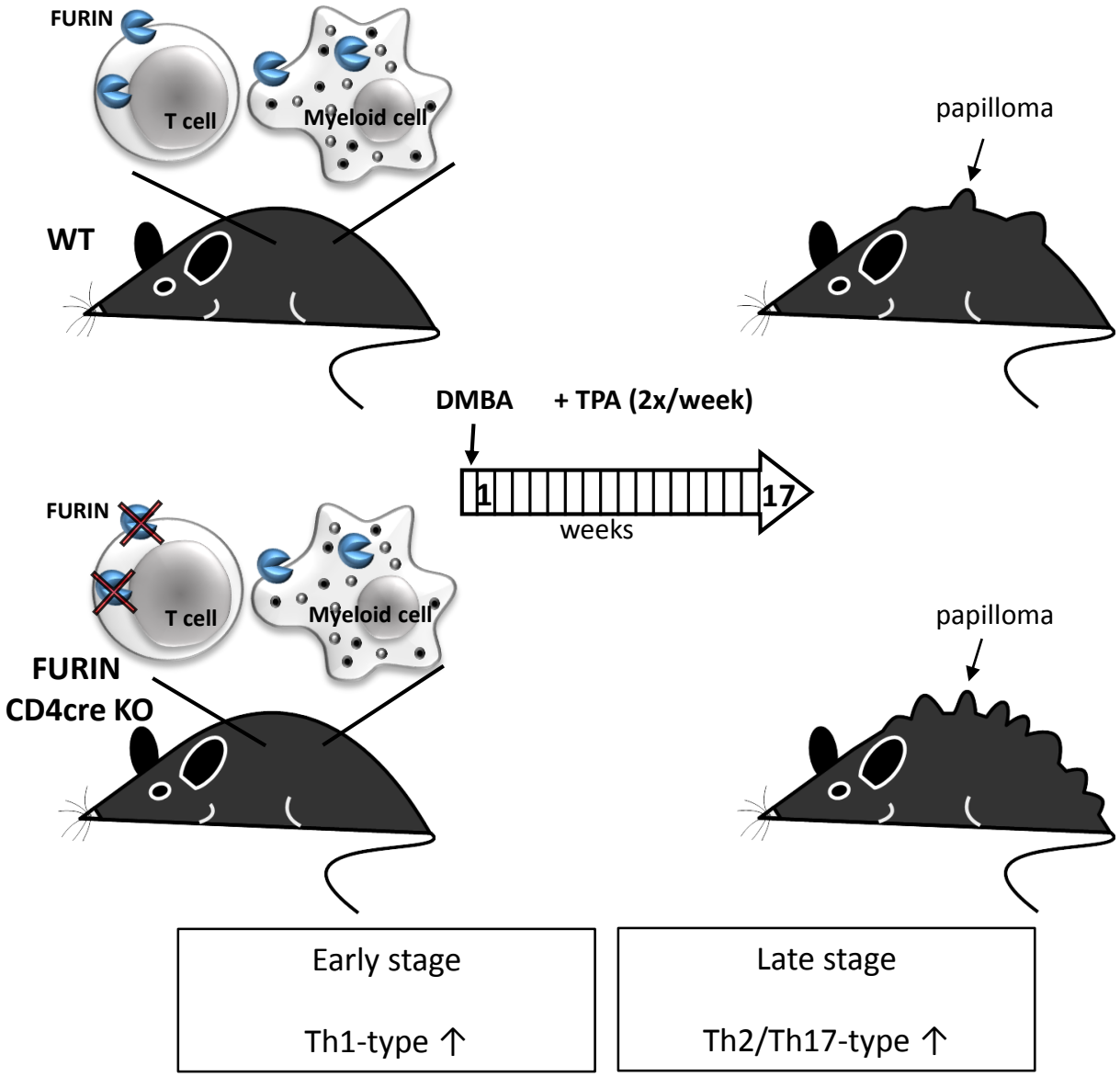
Supplementary Figure S4. Quantification of apoptotic cells in DMBA/TPA treated WT and T-cell-specific FURIN KO mice. CD4 WT and FURIN CD4cre KO mice were subjected to DMBA/TPA-induced skin carcinogenesis. Skin samples were collected, fixed and processed for IHC staining of proliferating and apoptotic nuclei as described in supplementary methods. Quantitative digital pathology analyses of scanned slides were performed. Statistical analyses were performed with GraphPad Prism 6 software. Results are shown as mean ± 95% confidence intervals. The data were analyzed using standard unpaired two-tailed Student's t-tests. (**A**) Apoptotic cells were stained by immunohistochemical TUNEL staining, and the % of apoptotic nuclei was determined. ($P<0.0001$, ****; $P<0.05$,*) (**B**) Representative photographs of apoptotic cells in untreated and DMBA/TPA treated CD4 WT and CD4cre KO skin. Nuclei are stained turquoise, and apoptotic nuclei brown/black. The black bar in images represents 300 µm. The information of animal numbers and analyzed tissue regions is shown in Supplementary Table 1.



Supplementary Figure S5. Microvessel density in the dermis in DMBA/TPA treated WT and T-cell-specific FURIN KO mice. FURIN CD4cre KO and CD4 WT littermates were subjected to DMBA/TPA-induced skin carcinogenesis as described in methods. Skin samples were collected, fixed and processed for IHC. (**A**) The percentage area of dermis with microvasculature was determined by immunohistochemical staining for CD31 of 4% paraformaldehyde fixed, paraffin embedded sections of back skin. Quantitative analysis of blood vessel density in dermis was performed by Spectrum digital pathology system/Image Scope analysis software as described in supplementary methods. The values are shown as mean ± 95% confidence interval. The data were analyzed using standard unpaired two-tailed Student's t-tests ($P<0.0001$, ****). **B**) Representative CD31 staining for blood vessels in untreated and DMBA/TPA-treated skin is shown for the CD4 WT and FURIN CD4cre KO animals. Black bar in images represents 300 µm. The information of animal numbers and analyzed tissue regions is shown in Supplementary Table 1.



Supplementary Figure S6. QRT-PCR analysis of *Ifng* and *Il17a* gene expression in the skin of the DMBA/TPA treated WT and T-cell-specific FURIN KO mice. The animals were subjected to DMBA/TPA-induced skin carcinogenesis as described in methods. Skin samples were collected from untreated mice and from mice sacrificed at 3 h and 43 h after the 2nd TPA application, and after 17 weeks of treatment (twice weekly) from non-tumorous skin. The skin samples were processed for qRT-PCR analysis as described in supplementary methods. Results are shown as mean ± standard error of the mean. Numbers of mice: untreated n=4 (both genotypes); 3 h and 43 h n=3 (both genotypes); week 17 CD4 WT n=4; week 17 CD4cre KO n=3.



Supplementary Figure S7. Schematic representation summarizing the outcome of T-cell specific deletion of FURIN in DMBA/TPA-induced skin cancer development.

Supplementary Table 1.
N numbers of immunohistological stainings. Shown are n numbers of animals and analyzed tissue regions (n mouse / n tissue regions). Each tissue region contained mainly between 1000 and 5000 nuclei.

	Ki67 and epidermal thickness	**TUNEL**	**CD31 and dermal thickness**	**F4/80**	**Neutrophil Elastase**	**CD3**	**Furin**
untreated WT	5 / 27	4 / 27 for epidermis, 25 for dermis	5 / 27	5 / 26	4 / 23	4 / 21	4/15
untreated KO	Ki67: 6 / 18; epid. thick.: 5 / 15	3 / 18	5 / 27	5 / 26	4 / 23	4 / 23	not done
43 h treated WT	not done	3 / 18	not done	1 / 6	3 / 18	3 / 18	3/16
43 h treated KO	not done	3 / 17	not done	3 / 18	3 / 18	3 / 18	not done
17 weeks treated WT non-tumor	6 / 21	4 / 15 for epidermis, 14 for dermis	6 / 21	6 / 21	6 / 24	5 / 21	3/14
17 weeks treated KO non-tumor	4 / 21	4 / 13	4 / 20	4 / 21	4 / 22	3 / 17	not done
17 weeks treated WT tumor	4 / 6	2 / 3	4 / 5	1 / 1	4 / 4	4/ 8	not done
17 weeks treated KO tumor	4 / 17	2 / 5	4 / 13	3 / 14	4 / 12	4 / 10	not done