ANNIKA RAITALA

Clinical Implications and Genetic Regulation of Indoleamine 2,3-dioxygenase Serum Activity

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ACADEMIC DISSERTATION
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University of Tampere, Medical School
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Supervised by
Professor Mikko Hurme
University of Tampere
Finland

Reviewed by
Docent Petteri Arstila
University of Helsinki
Finland
Professor emeritus Pekka Saikku
University of Oulu
Finland

Distribution
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 3 3551 6055
Fax +358 3 3551 7685
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To my family

Contents

List of original communications

This dissertation is based on the following original communications, which are referred to in the text by their Roman numerals:

I Raitala A, Pertovaara M, Karjalainen J, Oja SS, Hurme M (2005): Association of interferon-γ +874 (T/A) single nucleotide polymorphism with the rate of tryptophan catabolism in healthy individuals. Scand J Immunol 61:387-390.

II Raitala A, Karjalainen J, Oja SS, Kosunen TU, Hurme M (2006): Indoleamine 2,3-dioxygenase (IDO) activity is lower in atopic than in non-atopic individuals and is enhanced by environmental factors protecting from atopy. Mol Immunol 43:1054-1056.

III Raitala A, Karjalainen J, Oja SS, Kosunen TU, Hurme M (2007): *Helicobacter pylori* -induced indoleamine 2,3-dioxygenase activity *in vivo* is regulated by TGFB1 and CTLA4 polymorphisms. Mol Immunol 44:1011-1014.

IV Pertovaara M, Raitala A, Juonala M, Lehtimäki T, Huhtala H, Oja SS, Jokinen E, Viikari J, Raitakari O, Hurme M (2007): Indoleamine 2,3-dioxygenase enzyme activity correlates with risk factors for atherosclerosis. The Cardiovascular Risk in Young Finns Study. Clin Exp Immunol 148:106-111.

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In addition, this dissertation contains unpublished data.

Abbreviations

APC	antigen presenting cell
BMI	body mass index
bp	base pair
CCA	common carotid artery
CRP	C-reactive protein
CTLA-4	cytotoxic T lymphocyte associated protein 4
DC	dendritic cell
GCN2	general control non-derepressible 2
3-HAA	3-hydroxyanthranilic acid
HDL	high-density lipoprotein
HIV	human immunodeficiency virus
3-HK	3-hydroxykynurenine
IDO / INDO	indoleamine 2,3-dioxygenase
IDO2 / INDOL1	indoleamine 2,3-dioxygenase-like protein 1
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMT	intima-media thickness
IRF-1	interferon-regulatory factor-1
ISRE	interferon-stimulated response elements
Kyn	kynurenine
Kyn / trp	kynurenine to tryptophan ratio
LD	linkage disequilibrium
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MHC	major histocompatibility complex
mRNA	messenger RNA
1MT	1-methyl-tryptophan
NF-κB	nuclear factor kappa B
NK	natural killer cell
NO	nitric oxide
NOS	nitric oxide synthase
PCR	polymerase chain reaction
PGE2	prostaglandin E2
ROS	reactive oxygen species
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
STAT1	signal transducer and activator of transcription 1

TCR	T cell receptor
TDO	tryptophan 2,3-dioxygenase
TGF-β	transforming growth factor beta
Th	T helper cell
TLR	Toll-like receptor
TNF-α	tumor-necrosis factor alpha
TPH	tryptophan 5-hydroxylase
T-reg	regulatory T cell
tRNA	transfer ribonucleic acid
Trp	tryptophan
WARS	tryptophan-tRNA ligase

Abbreviations are defined at first mention in the abstract and the review of the literature and used only for concepts that occur more than twice.

Abstract

Mechanisms induced by the catabolism of the amino acid tryptophan (trp) are important in the regulation of both normal and pathogenetic immune responses. Trp is catabolized by two distinct biochemical pathways. In central nervous system trp 5-hydroxylase (TPH) converts trp into serotonin. Secondly, it is transformed into kynurenine (kyn) and subsequent metabolites by two enzymes; trp 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO).

The basal blood level of trp is mainly controlled by the homeostatic enzyme TDO, expressed in the liver. Unlike TDO, IDO is highly responsive to signals from the immune system. IDO is mainly expressed in antigen presenting cells (APC) and responsible for the immunomodulatory effects. The activity is mainly upregulated by typical T helper (Th) 1 differentiating signals such as interferon gamma (IFN-γ) and bacterial lipopolysaccharide (LPS). Activation of IDO during the inflammatory response leads to a decrease in local trp levels. These decreased levels have an inhibitory effect on proliferation of T lymphocytes. The concentration ratio between kyn and trp (kyn/trp) allows an estimate of IDO activity and can be used as an indicator for trp degradation and therefore an activated immune system.

In the studies constituting this dissertation we investigated the regulation of IDO activity and its role in certain clinical conditions by means of epidemiology and genetic epidemiology. Trp and kyn concentrations were measured from a total of 2608 individuals; healthy Finnish blood donors, control participants in an adult asthma study, young adults from the Cardiovascular Risk in Young Finns Study and participants of the Health 2000 Study.

IDO enzyme activity was increased in carriers of *IFNG* genotype associated with high IFN-γ production. The finding confirms the role of IFN-γ produced by Th1 cells in the regulation of IDO activity. Enzyme activity was significantly lower in a Th2-associated disease, atopy, and certain environmental factors, i.e. *Helicobacter pylori,* increased the activity. We also observed that high expression or producer genotype of the genes *CTLA4* and *TGFB1* was required for the *H. pylori* induced elevation of IDO activity.

The inflammatory nature of atherosclerosis is well recognized. The atherosclerotic plaque contains T lymphocytes, most of which are of the IFN-γ secreting Th1 phenotype, i.e. proinflammatory cells. In this study we wanted to ascertain the possible effect of IDO in the development of atherosclerosis. In two population cohorts of adults of various ages we observed that IDO activity correlated significantly with several risk factors for atherosclerosis and with intima-media thickness (IMT), which is often used as a presymptomatic predictor of the disease.

Tiivistelmä

Aminohappo tryptofaanin (trp) katabolian on havaittu olevan merkittävässä asemassa niin normaalissa immuunivasteessa kuin myös useissa sairauksissa. Trp hajotetaan kahta eri metaboliareittiä; trp 5-hydroksylaasi (TPH) muuttaa sen keskushermostossa serotoniiniksi, ja toisella reitillä toimivat kaksi entsyymiä, trp 2,3-dioksigenaasi (TDO) ja indoliamiini 2,3-dioksigenaasi (IDO), hajottavat aminohapon kynureniiniksi (kyn) ja edelleen muiksi metaboliiteiksi.

Toisin kuin maksassa esiintyvä ja veren homeostaattisista tryptofaanipitoisuuksista vastaava TDO, IDO reagoi immuunijärjestelmän signaaleihin. Sen katsotaan olevan keskeinen immuunisäätelyssä myös koska sitä tuottavat pääasiassa immuunijärjestelmän antigeenia esittelevät solut (APC). Merkittävimmät IDO:a aktivoivat tekijät ovat interferoni gamma (IFN-γ) ja bakteerien lipopolysakkaridit (LPS). Aktivaatio johtaa paikalliseen trp-tason laskuun, mikä inhiboi lähellä olevien T-solujen lisääntymistä ja toimintaa. Verestä mitattujen kyn ja trp -konsentraatioiden suhdetta (kyn/trp) voidaan käyttää trp:n hajoamisen ja aktivoituneen immuunisysteemin indikaattorina.

Väitöskirjatyössä tutkittiin IDO-entsyymin aktiivisuuden merkitystä ja säätelyä käyttäen epidemiologista ja geneettis-epidemiologista lähestymistapaa. Trp ja kyn -pitoisuudet määritettiin yhteensä 2608 henkilöltä; terveiltä verenluovuttajilta, aikuisiän astmaa käsittelevään tutkimukseen osallistuneilta verrokkihenkilöiltä, Lasten ja nuorten aikuisten sepelvaltimotaudin riskitekijät (LASERI) –tutkimukseen osallistuneilta nuorilta aikuisilta, sekä Terveys 2000 –tutkimukseen osallistuneilta henkilöiltä.

Terveiden aikuisten IDO-aktiviteetti todettiin korkeammaksi henkilöillä, joilla oli korkeaan IFN-γ -tuotantoon liittyvä *IFNG*–genotyyppi. Löydös vahvistaa Th1–solujen (tyypin 1 auttaja T-solu) tuottaman IFN-γ:n merkityksen IDO:n säätelijänä. Entsyymiaktiivisuus havaittiin normaalia matalammaksi atopiaa eli tyypillistä Th2–tyypin vasteen tautia sairastavilla, ja tiettyjen atopialta suojaavien ympäristötekijöiden, mm. *Helicobacter pylorin*, huomattiin kohottavan IDO-aktiivisuutta. *H. pylorin* kohottaman aktiivisuuden todettiin liittyvän *CTLA4*- ja *TGFB1*-geenien korkean ekspression tai tuotannon genotyyppiin.

Immuuni- ja tulehdusreaktioilla tiedetään olevan merkitystä ateroskleroosin kehittymisessä. Plakin sisältämät T-solut ovat pääasiassa IFN-γ:a tuottavaa proinflammatorista Th1-tyyppiä, joten väitöskirjatyössä haluttiin selvittää IDO:n mahdollista vaikutusta ateroskleroosin synnyssä. IDO-aktiivisuuden havaittiin korreloivan varhaisena ateroskleroosin merkkinä pidettävään kaulavaltimon intiman paksuuntumiseen sekä useisiin muihin sydän- ja verisuonitautien riskitekijöihin kahdessa eri-ikäisistä aikuisista koostuneessa väestöotoksessa.

Introduction

IDO is one of the two enzymes that degrade the essential and the rarest amino acid trp to kyn, and promote the formation of kyn pathway metabolites. Trp degradation was previously thought to be merely a mechanism of the innate immune system, since IDO suppresses microbial infections by reducing trp availability in infected tissues (Taylor and Feng 1991). Since the discovery of the involvement of IDO in maintaining maternal tolerance toward the fetus in the late 1990's (Munn et al. 1998), interest in the enzyme has focused on its role in the generation of immune tolerance to foreign antigens. Currently the importance of the IDO enzyme is well recognized both in immunity and pathogenesis of many diseases. The recent discovery of a novel enzymatic IDO isoform (INDOL1, IDO2) also suggests that the kyn pathway of trp metabolism may be involved in more biological processes than previously thought (Ball et al. 2009).

The removal of trp from the cell environment not only enables the host to inhibit the growth of various infectious pathogens, but it also leads to "starvation" of T lymphocytes, thereby inducing arrest of cell cycling and activation. In addition, the degradation products such as kyn itself may send anti-proliferative signals to T cells. IDO is mainly expressed by APCs, especially by dendritic cells (DC) and macrophages, which are able to take up antigens and present them to T lymphocytes in a recognizable form. IDO expression increases when inflammation occurs as a consequence of normal tissue functions, or when inflammation is induced by wounding, infection or tumor growth (Mellor and Munn 2004, King and Thomas 2007). The most competent inducer of IDO enzyme is the multifunctional cytokine IFN-γ, which is essential in the development of T cells, in the defence against various pathogens and in the induction of immune-mediated inflammatory responses. The rate of trp degradation reflects the IDO enzyme activity, and the concentration ratio between kyn and trp (kyn/trp) can be used as an indicator for trp degradation and therefore an activated immune system (Schröcksnadel et al. 2006b).

The present dissertation focuses on the function and genetic regulation of the IDO-dependent immunoregulation. The dissertation is based on the results from five different studies, representing kyn/trp ratios and enzyme activity levels from altogether 2608 study subjects. In the first study, the association of a single nucleotide polymorphism in the *IFNG* gene with IDO enzyme activity was investigated in healthy Finnish blood donors. In the second study, the effect of the atopic phenotype and different risk-modifying environmental factors on IDO activity was studied in a group of non-asthmatic healthy adults, who were control subjects in a Finnish population-based case-control study aimed at identifying risk factors and predictors of the outcome of adult asthma. In light of the results

from the second study, the aim in the next one was to study the genetic regulation of IDO activity in *Helicobacter pylori* seropositive individuals. This study consisted of the same subjects as the second one. In the last two studies the possible effect of IDO enzyme in atherosclerosis and on its risk factors was examined in two population-based adult cohorts. The subjects in the fourth study were young and middle-aged participants in the ongoing Cardiovascular Risk in Young Finns Study. In the fifth study the subjects were participants of the Finnish Health 2000 Study. They were older than the subjects in the previous study, with more advanced atherosclerotic changes expected.

Review of the literature

1. Tryptophan

L-tryptophan (trp) is a large neutral amino acid essential to human metabolism, because it is the metabolic precursor of neurotransmitter serotonin, neurohormone melatonin and vitamin B3, niacin. Unlike plants and microorganisms, which commonly synthesize trp by themselves, humans are not able to synthesize it, and therefore trp must be supplied in the diet. As a component of dietary protein, trp is particularly plentiful, for example, in chocolate, oats, bananas, peanuts, milk, meat and fish. The mean daily uptake of trp for people in developed countries is about 1 g, and the minimum requirement is about 200 mg. Though trp is the rarest of all 20 amino acids, accounting only for 1 % to 1.5 % of total amino acids in cellular proteins, it is required in several physiological processes in addition to protein synthesis (Peters 1991).

Trp present in the plasma is not only derived from dietary trp, but also from trp released by protein turnover (Brown 1996). Dietary trp enters the liver through the hepatic portal system, where it has been demonstrated to induce protein synthesis (Sidransky 1976). Excess trp can be delivered to the bloodstream, where it is taken up by tissues and used in protein synthesis or in the synthesis of serotonin and melatonin. Even though serotonin synthesis is the most investigated aspect of trp catabolism, only about 1 % of trp from food is converted to serotonin in this pathway (Takikawa 2005). The dietary intake of trp correlates with the ratio of trp to large neutral amino acids in the plasma and with brain serotonin levels (Fernstrom and Wurtman 1972). Thus acute depletion of trp affects mood and aggression due low serotonin levels (Young and Leyton 2002).

Besides the general protein synthesis and serotonin biosynthesis, the third fate of trp in the body is catabolism through the kynurenine (kyn) pathway (Figure 1).

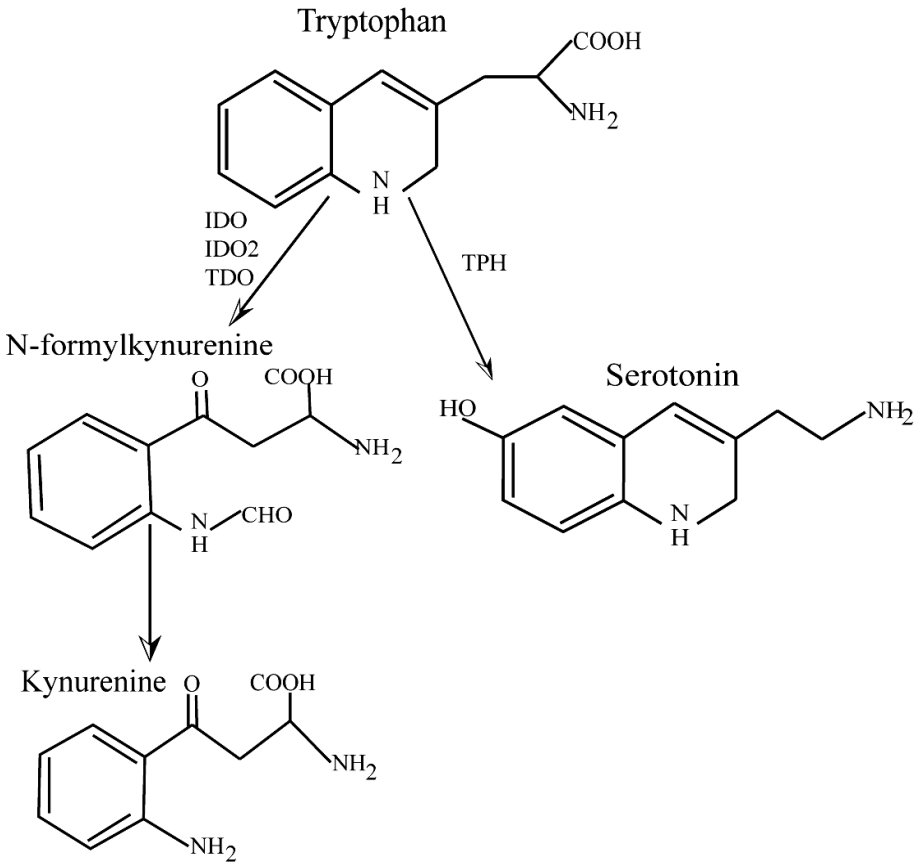

Figure 1. *Catabolic pathways of trp: trp 5-hydroxylase (TPH) initiates the production of serotonin, whereas trp 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) catalyze the formation of kyn.*

1.1. The kynurenine pathway of tryptophan metabolism

The major catabolic route of L-trp in mammals is the kyn pathway (Figure 2), through which approximately 95 % of trp is metabolized (Takikawa 2005). This pathway ultimately produces significant amounts of nicotinamide ribonucleotide, a precursor of the coenzyme nicotinamide adenine dinucleotide (NAD), thus decreasing the requirement for dietary niacin. The kyn pathway is the only source of endogenous NAD, and therefore trp is an important pro-vitamin (MacKenzie et al. 2007). The first and rate-limiting step in the pathway is the oxidative cleavage of the pyrrole ring of trp catalyzed by two unrelated enzymes; hepatic trp 2,3-dioxgenase (TDO, EC 1.13.11.11) or ubiquitous and extrahepatic indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.52).

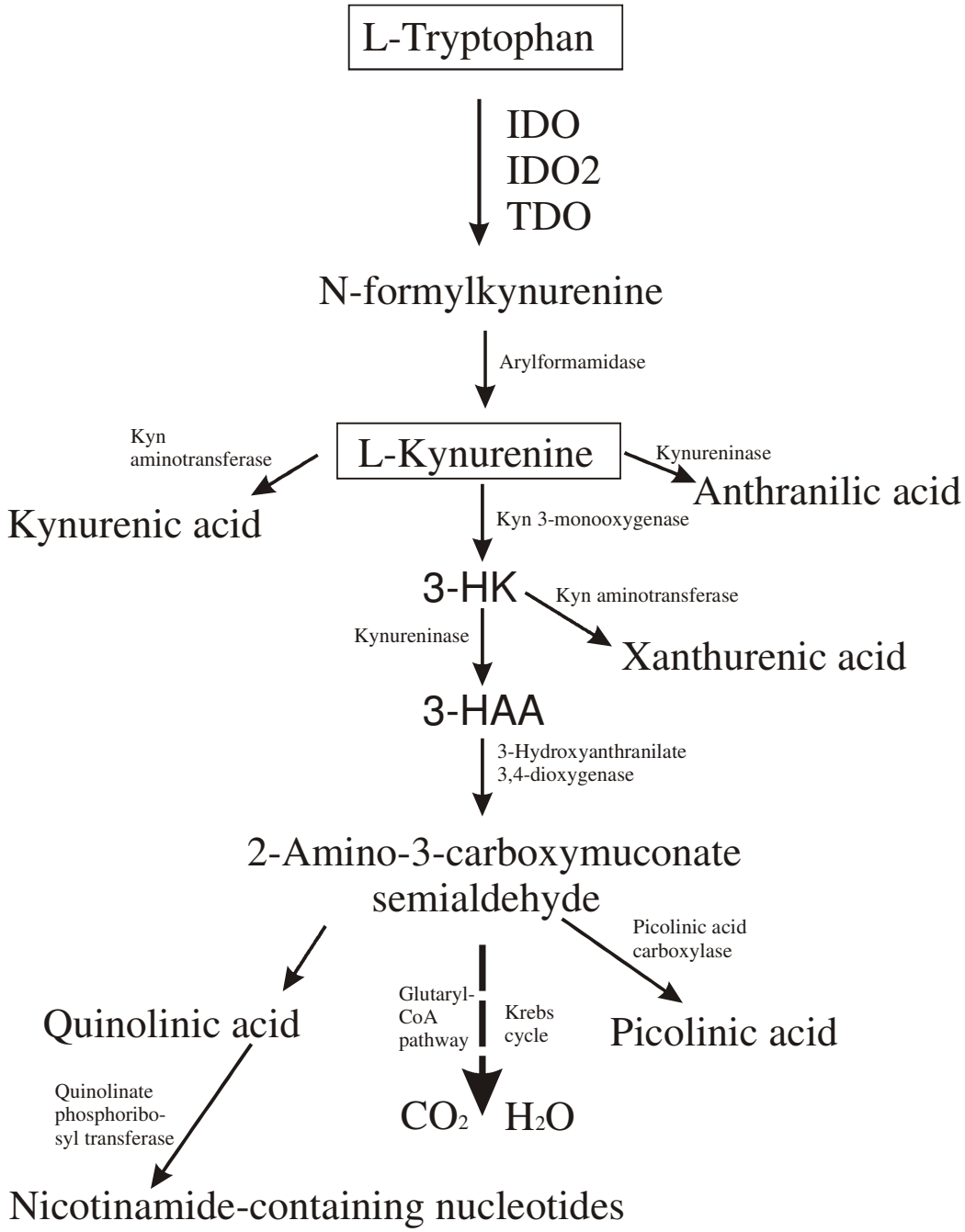

Figure 2. *The kyn pathway of trp metabolism.*

In the first reaction of the kyn pathway trp is converted into N-formyl-kyn. In the next reaction, catalyzed by arylformamidase (EC 3.5.1.9) or occurring spontaneously under acidic conditions, the more stable L-kyn is formed with the concomitant release of formate. Kyn can be hydroxylated by the flavin-dependent kyn 3-monooxygenase (EC 1.14.13.9) to form the aminophenol, 3-hydroxy-kyn (3-HK). Kyn and 3-HK are converted to kynurenic acid and

xanthurenic acid respectively via a reaction catalyzed by kyn aminotransferase (EC 2.6.1.7). Alternatively, kyn or 3-HK may be converted into anthranilic acid or 3-hydroxyanthranilic acid (3-HAA) respectively via pyridoxal-phosphate (vitamin B6) requiring kynureninase enzyme (EC 3.7.1.3). 3-HAA can be cleaved by 3-hydroxyanthranilate 3,4-dioxygenase (EC 1.13.11.6) to 2-amino-3-carboxymuconic semialdehyde. This unstable intermediate either spontaneously undergoes ring closure to quinolic acid, serves as a substrate for picolinic acid carboxylase to form picolinic acid, or is catabolized via the glutaryl-CoA pathway and the Krebs cycle to CO_2 and H_2O. Quinolinate phosphoribosyltransferase (EC 2.4.2.19) initiates the ultimate conversion of quinolinic acid to nicotinamide-containing nucleotides (Thomas and Stocker 1999).

The regulation of the kyn pathway is quantitatively and qualitatively dependent on the type of tissue or cell in which it is expressed (Stone 1993). For example, kyn production in the brain in comparison to the peripheral organs is low and, furthermore, astrocytes preferentially degrade kyn to kynurenic acid, which is a dead-end side arm of the kyn pathway. Microglial cells, in contrast, metabolize trp almost completely to quinolinic acid (MacKenzie et al. 2007).

In single-cell organisms, nutrient depletion is a common biological strategy to control proliferation of competing cells. Mammalian cells grow in a nutrient rich environment and even the slightest deviation from growth controlling concentrations alters not only their function, but can possibly cause cell arrest. In particular, different types of immune cells use amino acid metabolism as a tool either of regulating their activity or affecting adjacent cells. Lymphocytes, particularly T cells, are very sensitive to the presence and concentration of particular amino acids in their immediate environment (Edinger and Thompson 2002) and their usage or degradation can cause altered immune functions (Zamanakou et al. 2007).

Not only the trp degradation but also the downstream metabolites of the kyn pathway may have physiological or patho-physiological functions. It is not always clear whether the induction of this pathway exerts its effects through trp depletion, by production of kyn and/or kyn-derived metabolites or a combination of these two processes (Ball et al. 2007). For example, the proliferation of T lymphocytes is inhibited both by depletion of trp and by downstream catabolites of the kyn pathway (Frumento et al. 2002). A number of compounds in kyn pathway are neurotoxic, and some have been associated with diseases of the central nervous system (Smith et al. 2007). Despite the low level of kyn production in the brain, the most important physiological effects of kynurenines have been described by analysing brain cells (MacKenzie et al. 2007).

In normal conditions, kyn concentration is related to trp level. Decreased dietary intake of trp lowers the endogenous trp level, and under these circumstances lower kyn concentrations are also observed. The kyn to trp ratio (kyn/trp), i.e. the ratio of the concentration of the first product of TDO and IDO versus the concentration of their substrate is a suitable indicator of trp degradation (Schröcksnadel et al. 2006b). The ratio provides a normalized and

better measurement than absolute trp or kyn concentration, and is commonly used to reflect IDO enzyme activity.

2. Indoleamine 2,3-dioxygenase

TDO was the first enzyme discovered that catalyzed the conversion of L-trp to N-formyl-kyn (Kotake 1936). IDO was discovered later, in 1967, as a D-trp degrading enzyme from rabbit intestine by Osamu Hayaishi's group (Yamamoto and Hayaishi 1967). In contrast to TDO, which has been isolated not only in mammals but also in different insects, scallop and yeast, IDO has to date been found only in mammals and yeast (Yuasa et al. 2007).

2.1. The INDO gene and the induction of gene expression

IDO enzyme is encoded by the gene *INDO*, which is present as a single copy and located in the short arm of human chromosome 8 (8p12-p11) (Najfeld et al. 1993). The cDNA clone was originally isolated, its nucleotide sequence determined and the protein product identified as IDO in 1990 (Dai and Gupta 1990, Tone et al. 1990). The *INDO* gene spans 15 kilobases (kb) with 10 exons, and the 5' terminus of the messenger RNA (mRNA) is 33 nucleotides upstream of the translation initiation codon ATG. The gene has been well conserved (Suzuki et al. 2003), although IDO effector functions may have been adapted for various applications during evolution. The regulation of *INDO* transcription is complex, cell-type specific, and the complete mechanisms are still unknown. Transcription is under tight immunological control, responding to specific inflammatory mediators and confined to a limited range of cell types (Mellor and Munn 2004). Several factors, e.g. cytokines, hormones and certain drugs are known to induce and inhibit *INDO* gene expression either directly, or indirectly via an interferon gamma (IFN-γ)-dependent mechanism (Table 1.).

Table 1. *Known direct and indirect (acting via an IFN-γ-dependent mechanism) inducers and inhibitors of INDO gene expression.*

	Inducers	Inhibitors
Direct	IFN-α IFN-β IFN-γ Bacterial lipopolysaccharide (LPS) Cytotoxic T lymphocyte associated antigen 4 (CTLA-4) Interleukin (IL)-12 IL-18	Transforming growth factor-β (TGF-β)
Indirect	Tumor necrosis factor (TNF)-α IL-1 IL-2 IL-4 IL-13 Corticosteroids Prostaglandin E2 (PGE2) Estrogen Prolactin Bacteria, viruses	IL-6 Atorvastatin Acetylsalicylic acid Beer Wine and grape juice Brassinin Reservatrol Vitamin C and E Sodium sulfate Sorbic acid Anti-inflammatory plant extracts

2.1.1. Induction by IFN-γ

Type II interferon, IFN-γ, is a pleiotropic cytokine, involved in hematopoiesis, T cell differentiation, in the induction of immune-mediated inflammatory responses and has anti-proliferative, anti-tumor and antimicrobial functions. IDO is a cytokine-inducible enzyme and the expression of IDO increases when inflammation is induced by wounding, infection or tumor growth (Jung et al. 2007). Various cells, including certain myeloid-lineage cells (monocyte-derived macrophages and DCs), fibroblasts, endothelial cells and some tumor cell lines express IDO after exposure to IFN-γ. During infection, IFN-γ pathway is required for the normal upregulation of IDO expression

(Mellor and Munn 2004). There are two well-known polymorphisms in the *IFNG* gene that control the production of IFN-γ. The 12 CA repeat microsatellite allele in the noncoding region of the first intron is associated with a higher level of *in vitro* cytokine production, and there is complete linkage disequilibrium (LD) between this repeat allele and the presence of the T allele at the +874 position from the translation start site (Pravica et al. 1999, 2000).

As *INDO* is mainly induced *in vitro* and *in vivo* by IFN-γ, the 1254 base pairs (bp) long promoter region of *INDO* contains multiple sequence elements that confer responsiveness. Namely, two IFN-stimulated response elements (ISRE1 and ISRE2) homolog-binding and gamma-activated sequence (GAS) binding elements in the promoter recognize IFN regulatory factor-1 (IRF-1) and activated signal transducer and activator of transcription-1 (STAT-1) respectively (King and Thomas 2007). STAT-1 and IRF-1 act cooperatively to mediate the induction of *INDO* expression by IFN-γ (Chon et al. 1996), and mice lacking either IFN-γ or IRF-1 are known to be deficient in expression during infection (Silva et al. 2002). Data from the deletion and point mutation analyses have revealed that ISRE1 and ISRE2 are two elements critical for full *INDO* promoter induction (Konan and Taylor 1996).

Type I interferons, IFN alpha (IFN-α) and beta (IFN-β), are also able to induce *INDO*, although markedly less than IFN-γ (King and Thomas 2007). All three have been shown to induce *INDO* in human monocytes, IFN-γ being the most potent. Maturation of monocytes to macrophages increases their responsiveness to IFN-γ-induced IDO activity (Carlin et al. 1989). In human astroglioma cells IFN-α and IFN-β did not increase the expression and activity of IDO (Cano et al. 2008). In other cells than monocytes, IFN-α and IFN-β are poor inducers compared to IFN-γ despite the presence of a sequence highly homologous to ISRE in the *INDO* promoter. However, the ISRE control region distinguishes between IFN-γ and IFN-α (Hassanain et al. 1993). Minor differences between the nucleotide sequence of the ISRE homolog present in the *INDO* gene promoter and the ISRE consensus sequence characteristic of IFN-α-inducible genes may be responsible for this (Thomas and Stocker 1999).

2.1.2. LPS, TNF-α and interleukins in INDO induction

Despite the indisputable correlation between inflammation, IFN-γ and induced IDO expression, IFN-γ is not essential for *INDO* induction. Several inflammatory agents and extracellular signals, such as bacterial lipopolysaccharide (LPS), the inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor alpha (TNF-α), phorbol esters and muramyl tripeptide act synergistically with IFN-γ to enhance *INDO* expression *in vitro* (Edelstein et al. 1989, Hissong et al. 1995, Babcock and Carlin 2000, Robinson et al. 2003). These agents can increase the enzyme activity induced in IFN-γ-treated macrophages in the TPH-1 cell line (Hissong et al. 1995, Hu et al. 1995). The mechanism for synergy may include an increase in cytokine receptor expression. The study by Shirey et al. (2006) indicated that TNF-α and IL-1β are able to

cross-regulate cytokine receptor expression, resulting in increased signaling by IFN-γ and enhanced activation of *INDO* gene.

Inducing the expression may be indirect as in the case of IL-2 and IL-12. Both of these increase the expression of *INDO* via an IFN-γ-dependent mechanism, by stimulating production of IFN-γ (Carlin et al. 1987, Yu et al. 1996). However, it has been demonstrated that IL-12 and IL-18, and especially in combination, induce *INDO* expression besides the known pathway via IFN-γ in human osteosarcoma cell lines (Liebau et al. 2002). Since the expression could still be shown after complete blocking of IFN-γ, Liebau et al. concluded that at least a second pathway is responsible for inducing IDO activity.

LPS may also induce *INDO* expression via an IFN-γ-independent mechanism, which may be mediated by Toll-like receptors (TLRs) (Hayashi et al. 2004) and related to the activities of the p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB (Fujigaki et al. 2006a, Jung et al. 2007). Studies with THP-1 cells indicate that the effect of LPS on *INDO* is independent of TNF-α and IL-1 (Currier et al. 2000). However, responsiveness to LPS *in vivo* depends essentially on TNF, but does not require IFN-γ (Fujigaki et al. 2001), also suggesting the existence of an IFN-γ-independent pathway for the induction of *INDO* expression (Mellor and Munn 2004). Jung et al. (2007) showed that the LPS-induced expression in murine bone marrow derived DCs (BMDCs) is mediated by PI3 kinase and c-Jun N-terminal kinase (JNK), whereas IFN-γ-induced expression is regulated by Janus kinase (JAK). Their results suggest that LPS and IFN-γ belong to different signaling pathway responses of *INDO* induction.

In the case of TNF-α the increase in IFN-γ-mediated signaling is directly responsible for the synergistic transcriptional activation and is independent of TNF-α-specific activator recruitment to the *INDO* regulatory region (Robinson et al. 2005). The synergistic upregulation in response to IFN-γ and TNF-α is dependent upon the activation of three transcription factors; STAT-1, IRF-1 and NF-κB, and the availability of NF-κB and its increased nuclear translocation is the rate-limiting and essential event (Robinson et al. 2006).

The anti-inflammatory cytokines IL-4 and IL-13 have been shown in several studies to control *INDO* expression by antagonizing the effects of IFN-γ in different cell types (Musso et al. 1994, Yuan et al. 1998, O'Keefe et al. 1999, Chaves et al. 2001). When Yadav et al. (2007) investigated the effects of these cytokines in microglia, which are the main IDO producing cells besides macrophages in brain, they observed that both IL-4 and IL-13 greatly enhanced IFN-γ-induced *INDO* expression However, in the same study trp-transfer-RNA (tRNA) ligase (WARS, EC 6.1.1.2), was downregulated by IL-4 and IL-13. WARS is the only aminoacyl synthetase that responds to inflammatory mediators, and it is coinduced with *INDO* by IFN-γ (Frolova et al. 1993). WARS catalyzes the attachment of trp to its cognate transfer RNA (tRNA) molecule, with the resulting trp-tRNA complex used for protein synthesis (Fleckner et al. 1995). Thus, it has been suggested that WARS provides a reservoir of trp available for protein synthesis thereby protecting the cells from IDO-induced trp depletion by allowing them to use limiting amounts of trp during protein

synthesis (Boasso et al. 2005). The results of Yadav et al. (2007) showed that at least in microglia, IL-4 and IL-13 modulate the expression of these enzymes in a distinct fashion.

2.1.3. Induction by CTLA4

Regulatory T cells (T-regs) constitutively express a costimulatory molecule cytotoxic T lymphocyte associated antigen 4 (CTLA-4), which is a vital negative regulator of T cell activation and proliferation (Sun et al. 2008). Soluble CTLA-4-immunoglobulin (CTLA-4-Ig) fusion protein has been found to induce *INDO* expression through the ligation of cell-surface CD80/CD86 molecules (Grohmann et al. 2002). This role for CD80/CD86 molecules has subsequently been confirmed in both mouse and human DCs in several model systems (Fallarino et al. 2003, Mellor et al. 2003, Mellor et al. 2004, Munn et al. 2004b). In some, IFN-γ was required for the induction, but in others, ligation of CD80/CD86 induced functional expression by IFN-γ-receptor-deficient DCs, showing that IFN-γ signals are not essential for CD80/CD86-induced upregulation of *INDO* expression by these DCs (Mellor et al. 2004). The signal-transduction pathway connecting CD80/CD86 ligation with the induction of *INDO* expression has not been completely ascertained, but this pathway could have important implications for the mechanisms by which ligands for CD80/CD86 suppress T-cell-mediated immunity (Finger and Bluestone 2002, Grohmann et al. 2003).

2.1.4. Other inducers

TDO is known to be a substrate- and hormone-inducible enzyme (Lapin and Oxenkrug 1969, Oxenkrug 2007), but the direct hormonal regulation of *INDO* is not very clear. Xiao et al. (2004) showed that estrogen can regulate T cell functions by increasing the expression of IDO mRNA in DCs. Hormones might also amplify the cytokine-induced activation of IDO. Kawaguchi et al. (2008) showed that prolactin sensitizes monocytes to induce *INDO* expression in response to low doses of IFN-γ without affecting the typical IFN-γ signaling events, such as STAT-1 phosphorylation and IRF-1 induction. This indicates a priming effect of prolactin on monocytes occurring before IFN-γ signaling and increases their sensitivity to IFN-γ for *INDO* induction, rather than a synergistic effect of prolactin and IFN-γ. Also, the expression of the IFN-γ gene (*IFNG*) may be subject to direct hormonal control, since prolactin and IFN-γ receptors share the same structure and signal transduction pathway. 17-β-estradiol has been shown to increase *IFNG* promoter activity in lymphoid cells (Fox et al. 1991). According to the results of Verthelyi and Klinman (2000), sex hormones may modulate cytokine production *in vivo* and contribute to gender-related differences in normal and pathological immune responses.

Cyclooxygenase 2 (prostaglandin-endoperoxide synthase 2, EC 1.14.99.1), which is induced in activated macrophages and other cells at sites of inflammation, is implicated as being an inducer of *INDO*. Induction occurs through the production of the proinflammatory molecule prostaglandin E2 (PGE2), which is consistent with a role for cyclooxygenase 2 in promoting immune suppression (Katz et al. 2008). PGE2 has been found to induce mRNA expression of *INDO*, although a second signal through TNF receptor or TLR was necessary to activate the enzyme (Braun et al. 2005). This finding was extended when von-Bergwelt-Baildon et al. (2006) demonstrated a concomitant induction of *INDO* and secretion of soluble CD25 after DC maturation in the presence of PGE2. While maturation of DCs induced *INDO* expression on transcriptional level, only integration of PGE2 signaling led to upregulation of functional IDO protein as well as significant expression of cell-surface and soluble CD25 protein. They also showed that different carcinoma entities associated with elevated levels of PGE2 coexpress CD25 and IDO in peritumoral DCs, suggesting that PGE2 might influence IDO expression in human DCs in the tumor environment. Thus PGE2 could be a mediator of early events during induction of immune tolerance in cancer.

Applying hydrocortisone or the synthetic glucocorticoid dexamethasone in both human astrocytoma cells and native human astrocytes potentiated the effect of IFN-γ and resulted in enhanced induction of *INDO* (Ozaki et al. 1987, Türck et al. 2005). Dexamethasone administered *in vivo* activates IDO through the symmetric induction of the glucocorticoid-inducible TNF receptor (GITR) in CD4 T cells and its natural ligand (GITRL) in plasmacytoid DCs. Induction of *INDO* could be an important mechanism underlying the anti-inflammatory action of corticosteroids (Grohmann et al. 2007). However, the data from animal experiments have also yielded contrary results. Dexamethasone administered *in vivo* inhibited IDO activity in mouse brain and lung after systemic pokeweed mitogen administration (Saito et al. 1994), and in mouse brain in animals suffering from malaria (Sanni et al. 1998).

Finally, cellular infection with microbial agents, e.g. some viruses and other intracellular pathogens, can induce *INDO* in certain cell types (Thomas and Stocker 1999). Besides the TLR4 ligand LPS, bacterial DNA and its synthetic immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) analogs (TLR9 ligands) induce *INDO* expression *in vitro* and *in vivo* (Grohmann et al. 2003, Mellor and Munn 2004). Membrane protein TLR9, expressed in B cells, macrophages and DCs, also binds unmethylated cytidyl guanosyl (CpG)-ODNs present in the genome of bacteria and viruses, but not in the human genome. This leads to the production of IFN-γ and other proinflammatory cytokines (Krieg 2006). In addition, the TLR3 ligand polyinosinic:polycytidylic acid (polyI:C, PIC), a synthetic double-stranded RNA that is often used experimentally to model viral infections *in vivo*, has been shown to induce *INDO* in human astrocytes (Suh et al. 2007).

2.2. Inhibitors

Transforming growth factor-β (TGF-β) antagonizes many cellular responses to IFN-γ, and the interaction of these two cytokines plays an important role in maintaining homeostasis during inflammation and repair. TGF-β displays complex interactions with physiologically relevant cytokines and growth factors, for example it inhibits the production of IFN-γ induced by LPS. TGF-β is also a potent and selective inhibitor of the expression of the *INDO* gene, which has been demonstrated in IFN-γ-treated human fibroblasts *in vitro* (Yuan et al. 1998). The repression of *INDO* expression by TGF-β is mediated at both transcriptional and posttranscriptional levels. In contrast, IL-10, which also both antagonizes the effects of IFN-γ on the expression of various genes and inhibits IFN-γ synthesis, has not been shown to have an effect on IFN-γ-induced *INDO* expression (Yuan et al. 1998).

IDO activity has been shown to be responsive to IL-6. It has been reported that the activity of IL-6 includes downregulation of IFN-γ receptor expression in the mouse $CD8^+$ DC subset and correlates with a reduced ability of these cells to metabolize trp and initiate T cell apoptosis *in vitro* (Grohmann et al. 2001). In another study blocking the effects of IL-6 in mouse DCs also resulted in enhanced trp catabolism (Orabona et al. 2004).

In vitro studies have revealed a large number of substances that inhibit trp degradation. Cholesterol medicine atorvastatin was shown to decrease the degradation in mitogen- and IFN-γ-stimulated peripheral blood mononuclear cells (PBMC) and also in human monocytic cell lines (Neurauter et al. 2003). Acetylsalicylic acid (aspirin) had the same effect (Schröcksnadel et al. 2005b), but instead of a direct inhibitory effect of these drugs on *INDO*, they rather inhibit the production of IFN-γ.

Several extracts of plants and seeds with supposed anti-inflammatory properties also suppress the trp degradation by inhibiting the IFN-γ production, among them St. John's wort (*Hypericum perforatum*) (Winkler et al. 2004a), Cat's claw (*Uncaria tomentosa*) (Winkler et al. 2004b), seeds of summer squash (*Cucurbita sepo*) (Winkler et al. 2005a) and beetroot (*Beta vulgaris*) (Winkler et al. 2005b). Common beverages like red and white wine, or beer, including their alcohol free analogues like grape juice and alcohol-free beer, suppress trp degradation in a dose-dependent way (Neurauter et al. 2004, Winkler et al. 2006b). In addition, plant derived phytoalexin brassinin (Gaspari et al. 2006), and pure antioxidant compounds resveratrol (Wirleitner et al. 2005), vitamin C (ascorbic acid) and vitamin E (α-tocopherol) (Winkler et al. 2007) are effective in suppressing trp degradation. Likewise, food preservatives sodium sulfate (E221) and sorbic acid (E200) have a significant suppressive effect on trp degradation and also on the release of IFN-γ (Winkler et al. 2006a). These results are best summarized with the conclusion that compounds and extracts suppressed T helper (Th) 1-type immune response which includes the production of IFN-γ. Since the substances studied yielded results similar to those of pure antioxidant compounds, the data suggest that antioxidant contents are also relevant for their suppressive effect. The process by which antioxidants suppress

stimulated T cells and IFN-γ production is most likely due to their capacity to detoxify reactive oxygen species (ROS) (Schröcksnadel et al. 2007).

2.3. Protein structure and expression of IDO

Human *INDO* cDNA encodes a protein of 403 amino acids with a molecular weight of ~45 kDa. IDO is an intracellular enzyme; there is no known secreted or extracellular form. While TDO is highly specific for L-trp, IDO has much broader substrate specificity for compounds containing an indole structure (Malachowski et al. 2005). In IDO substrates include e.g. L-trp, D-trp, 5-hydroxy-trp, tryptamine and serotonin (Shimizu et al. 1978).

The tertiary structure of recombinant human IDO has been defined by X-ray crystallography (Sugimoto et al. 2006). The protein is folded into two distinct alpha-helical domains of which one is small and the other large. Monomeric protein contains heme as its sole prosthetic group and it is positioned between the alpha-helical domains (King and Thomas 2007). Different from TDO, whose catalytic activity is not dependent on heme or superoxide, the heme of IDO is essential for its enzyme activity (Katz et al. 2008). Heme is co-ordinated to the active site of the protein by a histidine (His) imidazole as the proximal fifth ligand (King and Thomas 2007). Data obtained from site-directed mutagenesis showed that His^{346} is the proximal ligand for human IDO (Littlejohn et al. 2003). The heme moiety of IDO provides a superoxide anion to donate oxygen to trp. To achieve full *in vitro* activity, methylene blue and ascorbic acid are required as reductants in the reaction, suggesting a related requirement *in vivo*. The co-factor binding site is separate from the substrate-binding site (Sono 1989) indicating the potential for allosteric regulation and possibly opportunities for developing non-competitive as well as competitive enzyme inhibitors for IDO (Katz et al. 2008).

The major difference between IDO and TDO is the expression patterns of the two enzymes. TDO is predominantly expressed in hepatic cells. It is not inducible, and regulates homeostatic trp concentrations (Mellor and Munn 2003). Catabolizing excess dietary trp to maintain serum trp concentrations below threshold levels is considered the main function of TDO, although TDO transcripts have been found also to be present in the uterus during gestation (Suzuki et al. 2001). IDO is detected in various tissues under normal conditions, though the expression is not necessarily constitutive in all tissues. IDO is expressed constitutively in immune privileged environments and in tissues with large areas of mucosal surface, such as the gut and placenta. In these environments, a constitutive shift of immune responses towards the induction of tolerance limits potentially harmful inflammatory responses. At the human maternal-fetal interface IDO is expressed constitutively by extravillous trophoblast cells (Kudo and Boyd 2000, Hönig et al. 2004). It has been shown by immunohistochemistry, that at a corresponding interface in mice the primary trophoblast giant cells of fetal origin express IDO (Baban et al. 2004). However, these results differ from those of another study, where a different IDO antibody

was used, and cells in the maternal metrial glands were reported to express IDO (Mackler et al. 2003).

Outside the placenta, functional IDO expression has been reported to be highest in mouse epididymis (Britan et al. 2006), gut (distal ileum and colon), lymph nodes, spleen, thymus and lungs (Takikawa et al. 1986). LPS treatment *in vivo* significantly increased the enzyme activity in these locations (Yoshida et al. 1980, Yoshida et al. 1981b). IDO might serve in the epididymis as an antioxidant enzyme in order to protect the epithelia from the damaging effect of ROS (Britan et al. 2006). The other tissues all have large mucosal surfaces and/or lymphoid compartments. The presence of constitutive and inducible IDO expression in these tissues might function as an anti-inflammatory and immunosuppressive mechanism (Mellor and Munn 2004). In humans, IDO has been detected in lung, stomach, spleen, liver, kidney, thymus, epididymis and brain.

Most commentaries on the immunoregulatory role of IDO focus on DCs and macrophages, i.e. antigen presenting cells. Terness et al. (2006) divided the human IDO-producing DCs into three subtypes; DCs that express IDO that does not break down trp (Munn et al. 2004b), DCs that express IDO that breaks down trp but does not suppress the T cell response (Terness et al. 2005), and DCs that express IDO that breaks down trp and suppresses the T cell response (Munn et al. 2002, Munn et al. 2004b). However, besides the APCs, functional IDO activity can also be expressed by endothelial and epithelial cells, eosinophils, fibroblasts, keratinocytes, lung cells, vascular smooth muscle cells, bone marrow stromal cells, microglial cells in the central nervous system and, and also certain tumor cell lines. Thus IDO is by no means a molecule confined to the immune system (Terness et al. 2006, King and Thomas 2007, Opitz et al. 2007) .

2.4. Regulation of enzyme activity

While IDO expression is widespread, the protein can also be expressed without functional enzymatic activity (Mellor and Munn 2004). As IDO consumes the essential nutrient trp, it would probably be toxic if it were constitutively active. Thus the activity must be strictly regulated. The post-translational regulation is not entirely clear, but possible mechanisms include controlling the supply of enzyme co-factors and substrates, post-translational modification of the enzyme, alternative splicing, regulation of protein stability and the presence of inhibitors of the enzyme active site (Mellor and Munn 2004). The heme prosthetic group in the active site is required for IDO activity, and inhibitors of heme biosynthesis inhibit functional activity without affecting protein levels. Antioxidants and the cellular redox potential also affect IDO activity (Thomas et al. 2001).

In mature IDO^+ DCs the protein is constitutively expressed, but the enzymatic activity appears only in response to specific triggering signals (Munn et al. 2002). Isolated mouse splenic $CD8\alpha^+$ DCs were found to catabolize trp when exposed to IFN-γ, whereas other ($CD8\alpha^-$) DCs did not, even though both subsets expressed comparable amounts of IDO protein (Fallarino et al. 2002b). However, the most potent trigger for functional activity was found to be exposure to

activated T cells during antigen presentation (Munn et al. 2002). This was shown not to be due to T cell-derived IFN-γ, since treating isolated DCs with it did not recapitulate the IDO-inducing effect of activated T cells and, in fact, produced the opposite effect; marked downregulation of IDO protein, mRNA and enzymatic activity (Munn et al. 2002). Thus, activated T cells supply an additional signal over IFN-γ, which is obligately required to trigger functional IDO. The signal seems to be the ligation of B7-1/B7-2 molecules on human IDO^+ DCs by their counter-receptors CTLA-4 and CD28 on T cells. In the absence of this signal the DCs failed to upregulate functional IDO activity, and lost the ability to inhibit T cells (Munn et al. 2004b).

Orabona et al. (2006) examined the expression of a series of relevant genes in IDO^+ compared with IDO^- DCs, and found an association of the IDO-competent phenotype with downmodulation of the *TYROBP* gene, encoding the membrane protein adapter DAP12, which typically associates with activating receptors. Their studies showed that IFN-γ induces in $CD8^+$ DCs the expression of the transcription factor IFN regulatory factor (IRF)-8, which induces IDO expression and downregulates DAP12. The downregulation of DAP12 seems to be necessary for full IDO enzyme activity, suggesting that the loss of DAP12 expression could be a distinctive mark of the IDO-competent DCs.

Various studies have shown that nitric oxide (NO) can inhibit IDO enzyme activity, mainly by blocking its heme prosthetic group and thus inactivating the active site, or by breaking the Fe-N bond and inducing conformational changes (Thomas et al. 1994, Alberati-Giani et al. 1997, Aitken et al. 2004). It has also been shown to promote proteasome-mediated degradation of the IDO protein (Hucke et al. 2004). NO is produced from L-arginine by the enzyme nitric-oxide synthase (EC 1.14.13.39, NOS), and is a highly reactive free radical and one of the most versatile molecules in the immune system (Bogdan 2001). IFN-γ induces the expression on NOS2 (iNOS), which is the inducible form of the three different NOS enzymes. It has also been shown that IDO protein can be nitrated by peroxynitrate, which is formed when NO reacts with superoxide anion. The nitration of the residue Tyr^{15} was particularly associated with decreased IDO activity (Fujigaki et al. 2006b).

The evidence suggests that NOS2 and IDO are reciprocally controlled, i.e. where the product of one pathway regulates the enzyme of the other pathway; e.g. NO inhibits IDO (Thomas and Stocker 1999). Although NOS2 and IDO may be induced in the same cell, it is unclear how one gains precedence over the other. Whether it is determined by the pervading immunological milieu or directly influenced by the infectious organism is as yet unclear (King and Thomas 2007). One of the properties of NO, due to its high reactivity, is that it even has opposite effects depending on the concentration, the molecular target and the type of reaction produced (Bogdan 2001). Low micromolar concentrations of NO were shown to stimulate IDO activity, while there was also a nitration of the IDO protein. Higher concentrations of NO, on the contrary, decreased IDO activity (Lopez et al. 2006).

2.5. Gene polymorphisms

According to the NCBI single nucleotide polymorphism (SNP) database the coding sequence of *INDO* contains seven SNPs, of which five are nonsynonymous substitutions, one synonymous and one causes a frame shift. The *INDO* gene region including the areas ~2000 bp upstream and ~1000 bp downstream contains 73 SNPs overall, though some of these may simply be artefacts (only one genotype exists) or have very low minor allele frequency.

At the moment there are no published studies on the association of *INDO* polymorphisms and diseases, but recent studies of the human *INDO* gene have revealed genetic variability that may contribute to differences in IDO activity between individuals. To identify such genetic variants Arefayene et al. (2006) resequenced the exons and intron-exon borders of the *INDO* gene in 96 DNA samples. They found 15 variants, of which eight SNPs were novel findings and seven previously identified SNPs. The novel findings included a non-synonymous SNP located in exon 3; however, the exact location of the SNP was not reported. Expression of the variant protein in COS-7 and human embryonic kidney 293 (HEK293) cells showed that this SNP causes almost complete loss of enzyme activity compared to the wild type variant, demonstrating the presence of a nonfunctional allele of the *INDO* gene in human population. A subsequent study of the same group revealed in exon 7 a nine bp deletion, which also causes a nonfunctional IDO protein (Arefayene et al. 2007). However, this deletion seems to be very rare; it was detected only in one of the 48 Afro-American samples.

2.6. IDO and host defence

The literature on IDO published before 1997 suggested that the biological role of the enzyme was to suppress microbial infections by reducing trp availability in infected tissues, thus representing an innate immune effector mechanism (Taylor and Feng 1991). Although most microorganisms can synthesize their own trp, some depend on exogenous trp (auxotrophs). It has been shown *in vitro* in several human cell lines that IDO inhibits the growth of a wide range of viral and microbial pathogens, both intracellular and extracellular. These data are supported by the *in vivo* finding that trp degradation is enhanced in humans during infectious diseases (Murray 2003). In most cases of infection with viruses and parasites, the induction of IDO is limited to the tissues infected with the pathogens (Takikawa 2005). However, the systemic induction of IDO occurs during endotoxin shock (Takikawa et al. 1986), and this may thus cause a systemic trp deprivation in the body.

Among the pathogens sensitive to trp depletion in human cells *in vitro* are toxoplasma, several strains of *Chlamydia*, human immunodeficiency virus (HIV), cytomegalovirus, herpes group viruses, measles virus, vaccinia virus, *Staphylococcus aureus*, enterococci, mycobacteria and group B streptococci (Mellor and Munn 2004, MacKenzie et al. 2007, Zamanakou et al. 2007). The

anti-viral, anti-parasitic and anti-bacterial function of IDO in non-professional immune cells has an essential role in the defence against invasive pathogens and this function is more than likely a system in which the activity of each individual cell or cell group acts in concert with other cells to ensure a maximal effect of IDO activity (MacKenzie et al. 2007).

Like most other innate elements of the immune response, IDO is clearly modulated by the adaptive immune system. Secretion of the most potent IDO inducer IFN-γ by cells that promote inflammation and initiate a cascade of tissue protection against any type of pathogen appears to play a key role in the expression of IDO at sites of inflammation. This is in agreement with the idea that constitutive IDO expression can be observed by cells of many tissues and organs in putative sites of immune tolerance or privilege, where it would be expected to participate in the host defence mechanisms against pathogens such as the case in lung, mucosa of the gut, epididymis, placenta and the eye (Mellor and Munn 1999, 2004).

2.7. IDO-mediated immunoregulatory effects

2.7.1. T lymphocytes

In the late 1990s, Mellor and Munn (1999) proposed an additional role for IDO, suggesting that IDO-dependent suppression of T cell responses might function as a natural immunoregulatory mechanism. This was based on their previously reported evidence that IDO activity was crucial to prevent allogeneic fetal rejection due to maternal T cell immunity in mice (Munn et al. 1998). Subsequent studies have broadly extended knowledge of the immunosuppressive role of IDO, and the importance of the enzyme has been increasingly recognized both in immunity and pathogenesis of many diseases.

T cells can be divided into two major classes that have different effector functions. These two classes are distinguished by the expression of the cell-surface proteins CD4 and CD8, and they differ in the class of major histocompatibility complex (MHC) molecule they recognize. During antigen recognition CD4 or CD8 associate on the T cell surface with the T cell receptor (TCR) and bind to the MHC portion of the composite MHC:peptide ligand, which is required for the T cell to make an effective response. CD4 binds to the MHC class II and CD8 to the MHC class I molecule. Naïve CD8 cells differentiate into cytotoxic T cells and are essential in host defense especially against cytosolic pathogens, the commonest of which are viruses. Cytotoxic T cells kill their targets by programming them to undergo apoptosis. They also produce cytokines IFN-γ, TNF-α, and TNF-β, which contribute to host defense in several ways (Janeway Jr et al. 2005).

CD4 T cells play a central role in immune protection through their capacity to help B cells make antibodies, to induce macrophages to develop enhanced

microbicidal activity, to recruit granulocytes to sites of infection and inflammation, and, through the production of cytokines and chemokines. CD4 cells are not a unitary set of cells but represent a series of distinct cell populations with different functions. While some of these populations are actually distinct lineages of cells already distinguished from one another when they emerge from the thymus, such as natural killer (NK) T cells, several represent alternative patterns of differentiation of naïve CD4 cells. Naïve conventional CD4 T cells have open to them at least four distinct fates that are determined by the pattern of signals they receive during their initial interaction with antigen; Th1, Th2, Th17 (effector T cells) and induced T-regs (Zhu and Paul 2008).

The existence of two distinct effector Th populations, Th1 and Th2, was first demonstrated over two decades ago (Mosmann et al. 1986). These populations differ in the cytokines they produce and thus in their function. The decision on which fate the progeny of a naive CD4 T cell will follow is made during the clonal expansion that takes place after the first encounter with antigen. The selective production of Th1 cells leads to cell-mediated immunity and the production of opsonizing antibody classes (mainly IgG), whereas the production of predominantly Th2 cells provides humoral immunity, especially IgM, IgA and IgE (Janeway Jr et al. 2005). Th1 cells mediate immune responses against viruses and other intracellular pathogens, and they are also responsible for the induction of some autoimmune diseases. Their principal cytokines products are IFN-γ, IL-2 and lymphotoxin α. IFN-γ produced by Th1 cells is important in activating macrophages to increase their microbicidal activity. Th2 mediate host defence against extracellular parasites, and they are important in the induction and persistence of asthma and other allergic diseases. Th2 cell produce IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and epidermal growth factor amphiregulin. Th1 and Th2 responses are mutually regulated. In this process, which is known as immune deviation or re-direction, the cytokine products of Th1 and Th2 cells are inhibitory for the differentiation and effector function of the opposite subset (Romagnani 2006, Zhu and Paul 2008). The immunosuppressive effects of IDO are mostly described in models with Th1 dominant responses, and the roles of IDO in Th2 cell regulation are still poorly understood. The role of IDO in Th2 cell regulation may be different from that in Th1 immune responses. Xu et al. (2008b) reviewed the current evidence on the regulatory function of IDO and trp metabolites in Th1/Th2 differentiation, and suggested that IDO-pathway may serve as a negative feedback loop for Th1 cells, but it can play a distinct role in upregulating Th2 dominant immune responses.

It became clear only recently that IL-17 producing T cells constitute a separate T cell subset, termed Th17, distinct from Th1 and Th2 cells. Th17 cells do not produce the “classical” Th1/Th2 cytokines, and the Th1/Th2 signature cytokines IFN-γ and IL-4 suppress Th17 cell differentiation (Harrington et al. 2005). IL-6, IL-1β, TGF-β, and IL-23 are considered to promote the differentiation of Th17 subset from naïve CD4 T cells. However, human Th17 cells seem to exhibit different features from murine Th17 cells, and the differentiation process still needs to be further studied. Besides IL-17, Th17 cells

produce IL-21 and IL-22, and mediate immune responses against extracellular bacteria and fungi. Th17 are also responsible for, or participate in, the induction of many organ-specific autoimmune diseases (Zhu and Paul 2008).

T-regs play an important role in maintaining self-tolerance and in regulating immune responses. Induced T-regs are now well established as an inducible population that phenotypically resembles the "natural" T-regs, which emerge from the thymus as fully functional suppressor T cells. Currently, it is not clear whether T-regs of direct thymic origin differ in function from those of peripheral origin. TGF-β plays a major role in differentiation and development of T-regs, and also IL-2 is required for the survival and function of T-regs even after they have differentiated. In 2003, the forkhead box protein 3 (FoxP3) transcription factor, which is induced by TGF-β, was identified as both a marker and a lineage commitment factor for CD4 T-regs. Overexpression of FoxP3 in conventional T cells converts them to a T-reg phenotype, and continuous expression of the protein is critical for maintaining the suppressive activity of T-regs. T-regs exert their suppressive functions through several mechanisms, some of which require cell-cell contact. Possible mediators of suppression include cytokines, like TGF-β, IL-10 and IL-35, which may be secreted or membrane bound, IDO induced in target cells, or adenosine. Induction of apoptosis by a variety of mechanisms may also contribute to their suppressive activity. (Allan et al. 2008, Zhu and Paul 2008).

2.7.2. Tryptophan depletion

In addition to inhibiting pathogen proliferation, trp deficiency has been shown to arrest lymphocyte cell cycle progression. Mellor and Munn (1999) developed a model called trp depletion hypothesis to explain T cell unresponsiveness. A basic feature of the model is that IDO activity in APCs does not affect T cell entry into the cell cycle, but reduced access to free trp blocks cell cycle progression. Naïve (primary) human and murine T cells activated in chemically defined trp-free media entered the cell cycle, but cell cycle progression arrested at the approximate mid-point of G_0-S phase transition (Munn et al. 1999). Cell cycle arrest prevents the clonal expansion of T cells and may promote their death by apoptosis, induce T cell ignorance, anergy and deviation, or generate T-regs (Mellor and Munn 2003).

It seems that T cells may be specially sensitive to low levels of trp during the G_0-S phase transition, rather than generally susceptible to amino acid deprivation. T cells entered S-phase before succumbing to the effects of amino acid deprivation when activated in media containing no leucine or isoleucine, even though the frequency of these two amino acids is ~10 % while trp accounts for only ~1 % of amino acids (Mellor and Munn 2003). A critical trp concentration was defined by Munn et al. (1999) who showed that reduction of the local concentration of trp to approximately 0.5 μM results in half maximal T cell proliferation. However, most of the results suggesting trp depletion as the mechanism of IDO-induced immunosuppression have been obtained *in vitro*, and

the question whether it is responsible for the immunosuppressive effects of IDO also *in vivo* is open to debate. *In vivo* a local decrease of trp is rapidly compensated by diffusion from surrounding tissues and plasma, where the concentration is between 50 μM and 100 μM. In addition, at the site of inflammation, dead cells provide an additional source of trp by releasing their intracellular stocks (Terness et al. 2006), and plasma levels do not reach such low concentrations as 0.5 μM. Thus the trp depletion hypothesis may not be the only explanation for the immunomodulatory properties elicited by IDO.

The inhibition of T cell response by trp depletion mediated by IDO positive cells is not simply due to an inhibition of protein synthesis by lack of trp. Munn and colleagues (2005) identified the general control non-derepressible-2 (GCN2) kinase as a downstream mediator for several key effects of IDO-mediated trp depletion. GCN2 is a stress response kinase, which is activated by the accumulation of uncharged tRNA in response to insufficient amino acid supply (Dong et al. 2000). This initiates an integrated stress response which can trigger apoptosis or cell cycle arrest. GCN2 kinase is required for CD8 T cells to sense and respond to trp depletion created by IDO. T cells lacking GCN2 proliferate normally in the presence of IDO^+ DCs and are not susceptible to IDO-induced anergy (Munn et al. 2005). Thus, the response to amino acid withdrawal is not just metabolic shutdown, but an active, regulated and specific signaling pathway (Mellor and Munn 2003). Forouzandeh et al. (2008) showed recently that even though both subsets of human T cells are sensitive to IDO induced low trp environment, it is more so for CD8 relative to that of CD4 cells. They also found that this differential response is due, at least in part, to the difference in the level of GCN2 kinase activation between these two subsets of T cells.

From an evolutionary standpoint the downregulation of the immune system by trp metabolism, i.e. the suppression of T cell proliferation, appears to be in direct conflict with the antimicrobial function of IDO. To efficiently combat an aggressor, a strong immune response is required at the site of infection. On the one hand this IFN-γ-induced system protects against invading pathogens, while on the other inhibiting the production of the same IFN-γ by T cells. These contradictory effects may be explained if one considers the local concentrations of trp products and the threshold for trp concentration of both T cells and for micro-organisms. MacKenzie et al. (2007) investigated the threshold for staphylococci and T cells and found that this concentration is two to three times higher for the bacteria than for the T cells. They suggest that IDO-mediated trp depletion in the first instance leads to an inhibition in the growth of the pathogen and then, at lower concentrations, act as negative feedback loop by downregulating the T cell proliferation and hence IFN-γ production. This would prevent an overshoot of the immune response and thus protect the host tissue from possibly damaging trp starvation. Thus IDO would be a “late contributor” in the immune reaction, having a role of downregulating a successful response. However, optimal conditions for induction of IDO activity – such as bacterial components, superoxide anion and activatory cytokines – are clearly present at the beginning of an inflammatory process. If IDO is induced early, it might either lack inhibitory action, not reach suppressive concentrations or exist in a

functionally inactive form (Terness et al. 2006). The latter can be achieved by coexpression of IDO inactivators, such as NO (Thomas et al. 1994).

2.7.3. Immune modulation by tryptophan catabolites

According to another hypothesis, toxic metabolites of trp seem to have a prominent role in mediating the immunosuppressive effects of IDO. Although the exact mechanisms of the action of these molecules are largely unknown, trp catabolites are known to differentially regulate immune responses. Most studies have focused on the effect of trp catabolites on T cell proliferation. Frumento et al. (2002) showed that both trp depletion and kyn excess are required for the full antiproliferative effects of IDO. They found that trp depletion alone was not sufficient to inhibit peripheral blood leukocyte proliferation, and that higher concentrations of kynurenines were needed to inhibit proliferation in conditions of trp repleteness. 3-HK and 3-HAA, and to a lesser extent kyn and picolinic acid, have been shown to inhibit T cell proliferation. This can be enhanced by combining different trp catabolites, indicating that they share common signaling pathways to suppress T cells. Reduced trp concentrations also augment the T cell suppression by trp catabolites (Frumento et al. 2002, Terness et al. 2002).

Trp catabolites inhibit the proliferation of CD4 and CD8 T cells stimulated by a variety of stimuli such as allogeneic cells and phytohemagglutinin. Suppression appears to be specific for activated T cells, as kyn-suppressed T cells cannot be restimulated by the same stimulus, but resting T cells respond normally to a different stimulus (Frumento et al. 2002, Terness et al. 2002). Besides the T cells, trp catabolites also suppress NK cells (Frumento et al. 2002, Terness et al. 2002), whereas B cells are not affected (Frumento et al. 2002). Fallarino et al. (2002a) exposed T cells, macrophages and DCs to the trp catabolites, 3-HAA and quinolinate, and demonstrated increased apoptosis. They also demonstrated a selective sensitivity of Th1 cells over Th2 cells to these molecules, suggesting a potential role for IDO in Th1 vs. Th2 differentiation.

In the study by Fallarino et al. (2002a) the trp catabolite concentrations required to affect macrophages and DCs were 10-fold those required for lymphocytes, thus IDO-producing cells seem to display resistance to the effects of IDO. Macrophages have been described as possessing a high affinity trp transport system, which may allow them to access the scant trp available during increased IDO activity (Seymour et al. 2006). It also may be partly explained by the findings that some IDO competent cells also express the IFN-γ-inducible enzyme WARS, which generates an IDO-resistant source of trp for protein synthesis. However, these findings do not explain DC and macrophage resistance to trp catabolites. It may be that specific kynurenines have a particular affinity for certain cell types owing to cell-specific uptake mechanisms or the differential expression of enzymatic breakdown pathways (Frumento et al. 2002). While DCs and macrophages seem to be resistant to the apoptotic effects of IDO, it is not known whether the phenotype of these cells is altered by trp deficiency or excess catabolites. It also remains to be ascertained whether each trp catabolite

has a specific role, singularly or in combination, in regulating the various cell types involved in immune responses in addition to their antipathogen effects (Mulley and Nikolic-Paterson 2008).

In conclusion, current evidence suggests a synergistic role of both trp depletion and downstream trp catabolites in regulating adaptive immunity. They are not necessarily mutually exclusive and each could be active in different degrees in various tissues or cells.

2.7.4. Animal models in IDO studies

Mouse models have a significant role in studies concerning IDO, and the evidence for a role of IDO-producing DCs *in vivo* comes almost exclusively from experiments on mice. In mouse, trp is mainly metabolized in the kyn pathway, and it is therefore an ideal model for studying the effects of IDO (Allegri et al. 2003b). However, great differences in the activity of kyn pathway enzymes have been reported among organs derived from rabbits, rats, mice and guinea pigs (Allegri et al. 2003a). These data indicate that the concentration of trp metabolites in tissues, which is decisive for their immunoregulatory function, depends on the species.

Austin et al. (2009) investigated the similarities and differences between mouse and human recombinant IDO, and observed significant differences in the conversion of substrates and pH stability, and in inhibitor potency and thermal stability. Also, in contrast to the data in human cells, IDO has not been detectable as an anti-parasitic effector mechanism in murine cells (MacKenzie et al. 2007). Though many physical and kinetic properties of recombinant human and mouse IDO show similarities, the differences must be recognized and all the findings in mouse systems carefully interpreted before application to human models (Austin et al. 2009). According to the study by Drenzek et al. (2008), nonhuman primates such as rhesus monkey and common marmoset could provide better experimental animal models for exploring the basic biological function of IDO and its feasibility as a therapeutic target.

3. Indoleamine 2,3-dioxygenase-like protein 1

Since the 1970s it has been thought that the first and the rate-limiting step in the kyn pathway is performed by either of two enzymes, TDO or IDO, depending on the tissue and cell type. This belief was recently overturned when a gene with homology to *INDO* was reported (Murray 2007) and soon after that demonstrated by three other groups to encode a trp catabolizing enzyme (Ball et al. 2007, Metz et al. 2007, Yuasa et al. 2007). This new enzyme has been referred to as IDO-like protein (INDOL1), IDO2 or proto-IDO due to its structural similarity to IDO and its enzymatic activity. The discovery of IDO2

suggests that the kyn pathway of trp metabolism may be involved in more biological processes than previously thought (Ball et al. 2009).

The discovery of IDO2 enzyme is so recent that its exact biological role is yet to be defined. However, its currently known expression pattern suggests that it has a role in kidney function, fertility and immunomodulation (Ball et al. 2009). The trp-catabolizing activity of IDO2 expressed in intact mammalian cells has been shown (Ball et al. 2007, Metz et al. 2007). It seems that the enzyme can catabolize a similar range of substrates to IDO but with much lower efficiency (Ball et al. 2009).

3.1. INDOL1 genetics

INDOL1 gene is located on chromosome 8p12 just downstream of the gene *INDO*, suggesting that these genes arose via gene duplication (Ball et al. 2009). The human and mouse *INDOL1* genes span 11 exons in a ~74 kb genomic region. The complete coding region of *INDOL1* is 1260 bp long. Alignments between IDO and IDO2 sequence have revealed highly conserved features that mediate heme and substrate binding, although the overall level of sequence conservation in human is not particularly high (43 % identical) (Metz et al. 2007). At least two different transcripts are expressed with alternate 5' exons, suggesting that the genes of both these enzymes have multiple promoter regions (Ball et al. 2009). The mouse and human *INDOL1* genes also have several alternatively spliced transcripts missing various exons from within the coding sequence, and the human gene has an alternate transcript with more a restricted tissue expression pattern (Metz et al. 2007).

Like *INDO, INDOL1* gene is also polymorphic. According to the NCBI SNP database there are nine SNPs in the coding sequence of *INDOL1* gene; five nonsynonymous, two synonymous, one nonsense and one causing a frame shift. When the regions ~2000 bp upstream and ~1000 bp downstream are included, there are 797 SNPs in the gene region altogether. However, the database may contain several SNPs with only one genotype, being very likely artefacts and SNPs with very low minor allele frequency. To date there is only one published study focusing on the association of *INDOL1* SNPs with IDO2 enzyme activity. Metz et al. (2007) identified two SNPs that abolished the enzyme activity; R248W and Y359stop. The nonsynonymous substitution R248W in exon 9 reduced catabolic activity by about 90 % in human 293-T-REx™ cells expressing Tet repressor protein (T-REX). The other SNP affecting Y359 generates a premature stop codon which completely abolished the activity. According to their data these SNPs have an extensive effect on IDO2 activity in humans, since both SNPs are common especially in individuals of European and Asian descent.

3.2 Structure and expression of IDO2

Only mammals possess both IDO and TDO enzymes, and it seems that vertebrate species and sea urchins (Echinodermata) also potentially have the IDO2 (Yuasa et al. 2007). According to Yuasa et al. (2007), IDO2-like proteins also exist in lower vertebrates such as frog and fish, based on BLAST searches of sequence databases using the sequences of mammalian IDOs. However, a search of the chicken and zebrafish genomes revealed only one IDO protein similar to mammalian IDO2. This suggests that the ancestor IDO gene was probably more similar to IDO2 and a gene duplication generating IDO occurred in mammals (Ball et al. 2009). The duplication very likely occurred before the divergence of marsupial and eutherian (placental) mammals in the mammal lineage. The amino acid sequences of vertebrate IDO2s are well conserved, suggesting that it has some essential function in vertebrates (Yuasa et al. 2007).

Human and mouse IDO2 proteins consist of 420 and 405 amino acids respectively, and are more conserved (72 % identical) than IDO proteins (62 % identical). Human IDO and IDO2 proteins are 43 % identical at the amino acid level, but neither of them is structurally related to TDO protein. Significantly, certain catalytically important residues determined by IDO mutagenesis and crystallographic analysis, are highly conserved in IDO2 (Metz et al. 2007, Ball et al. 2009).

Metz et al. (2007) found *INDOL1* expression by RT-PCR analysis in a subset of tissues expressing IDO. Primers spanning the complete human coding region detected full-length mRNAs only in placenta and brain, whereas primers specific to exon 10 (found in all human *INDOL1* cDNAs) detected *INDOL1* mRNAs in liver, small intestine, spleen, placenta, thymus, lung, brain, kidney and colon. A more narrow range of expression was detected in mouse, revealing *INDOL1* transcripts only in liver and kidney. According to Ball et al. (2007), IDO2 protein in mice is predominantly expressed in the kidney followed by epididymis, testis and liver, as well as the ovary, uterus and placenta. Although both IDO and IDO2 are expressed in some tissues, it seems that they are expressed in distinct cell types, suggesting they are not functionally redundant. Moreover, the expression of the proteins in response to stimuli differs (Ball et al. 2009).

Löb et al. (2008) showed that human DCs express both IDO and IDO2, but IDO2 is functionally inactive, and the trp-degrading function of these cells is contributed entirely by IDO. The same group investigated the function of competitive IDO-inhibitor 1-methyl-trp (1MT), a novel therapeutic approach in cancer. It is known that 1MT isomer 1-methyl-D-trp (D-1MT) is selective for inhibition of IDO2, whilst its stereoisomer 1-methyl-L-trp (L-1MT) is more selective for IDO (Metz et al. 2007). Löb et al. (2009) analysed IDO2 expression in human cancer cells and the impact of two 1MT isoforms on IDO activity. They observed that primary gastric, colon and renal carcinomas constitutively expressed both IDO and IDO2, whereas cancer cell lines had to be induced by IFN-γ. Treatment of Hela cells with IDO-specific small interfering RNA (siRNA) resulted in complete abrogation of trp degradation. Only L-1MT, not D-

1MT, was able to block the IDO activity in IFN-γ treated Hela cells as well as in protein isolates of primary human colon cancer. They concluded that although IDO2 is expressed in human tumors, trp degradation is provided entirely by IDO, and D-1MT does not inhibit IDO activity in malignant cells. If ongoing clinical studies with D-1MT show a therapeutic effect, it cannot be attributed to the inhibition of IDO in tumor cells, and alternative immunological or non-immunological mechanisms have to be considered for its action (Löb et al. 2009).

4. Tryptophan catabolism in diseases

Decreased trp concentration and increased concentrations of kyn and other trp catabolites have been described in various diseases and clinical conditions (Table 2.). To confirm that trp degradation is due to activation of IDO rather than TDO, the concomitant immune system activation has been demonstrated in some of the studies. Thus, activated IDO is indicated when kyn/trp correlates with an immune activation parameter and endogenous IFN-γ production (Schröcksnadel et al. 2006b). For example, the determination of neopterin concentration in body fluids or cell culture supernatant is a sensitive laboratory diagnostics tool to detect and monitor Th1-type immune activation in humans and primates (Murr et al. 2001). Increased neopterin concentration has been detected during viral infection, in autoimmune disease, allograft rejection and in malignant disease, and in these clinical conditions a close association between neopterin concentration and accelerated trp degradation has been found (Schröcksnadel et al. 2006b).

Table 2. Examples of clinical conditions associated with trp degradation.

Disease		Reference
Atopic diseases	Asthma	Warraki et al. 1970, Collipp et al. 1975, Hayashi et al. 2004, Maneechotesuwan et al. 2008
	Asymptomatic atopy	von Bubnoff et al. 2004
Autoimmune syndromes	Rheumatoid arthritis	Forrest et al. 2003, Schröcksnadel et al. 2003
	Sjögren's syndrome	Pertovaara et al. 2005
	Systemic lupus erythematosus	Widner et al. 2000b, Pertovaara et al. 2007
	Inflammatory bowel disease	Wolf et al. 2004
Infection	*Chlamydia* spp.	Summersgill et al. 1995, Byrne et al. 1986, Leonhardt et al. 2007
	Cytomegalovirus	Bodaghi et al. 1999
	Epstein-Barr virus	Bellmann-Weiler et al. 2008
	Hepatitis C virus	Larrea et al. 2007
	HIV	Fuchs et al. 1990
	Listeria monocytogenes	Popov et al. 2006
	Neuroborreliosis	Gasse et al. 1994
	Sepsis	Pellegrin et al. 2005
	Streptococcus pyogenes	Murr et al. 2001
	Toxoplasma gondii	Pfefferkorn 1984
Malignancy	Acute myeloid leukemia	Corm et al. 2009
	Adult T cell leukemia	Giusti et al. 1996, Hoshi et al. 2009
	Bladder cancer	Boyland and Williams 1955
	Breast cancer	Rose 1967
	Colorectal cancer	Huang et al. 2002, Brandacher et al. 2006
	Gynecological cancer	Schröcksnadel et al. 2005a
	Hematological neoplasias	Denz et al. 1993
	Hodgkin's lymphoma	Ambanelli and Rubino 1962
	Melanoma	Weinlich et al. 2007
	Prostate cancer	Wolf et al. 1968, Feder-Mengus et al. 2008
Mental and neurological disorders	Alzheimer's disease	Widner et al. 2000a
	Bipolar mania	Myint et al. 2007
	Huntington's disease	Leblhuber et al. 1998, Stoy et al. 2005
	Major depression	Maes et al. 1994
	Parkinson's disease	Widner et al. 2002
	Schizophrenia	Barry et al. 2009
Other conditions	Cardiovascular disease	Wirleitner et al. 2003
	Irritable bowel syndrome	Fitzgerald et al. 2008

4.1. Infection

Infection provides ideal conditions for the induction of IDO activity, because it leads to the generation of strong inducers of IDO, such as cytokines, LPS and superoxide. Terness et al. (2006) suggested that the largest proportion of IDO might be produced not by the thinly scattered DCs, but by the bulk of other cells at the site of inflammation. In that case an important question is whether IDO expressed by non-immune cells can suppress the immune response. If suppression is mediated by trp deficiency, this would be the case, because most IDO-expressing cells can break down trp (Moffett and Namboodiri 2003). If, by contrast, downstream metabolites are operational, suppression would depend on whether a certain cell type produces the toxic metabolites. However, it has been shown that various non-immune organs are able to generate immunosuppressive metabolites, and even if organ-specific cells do not synthesize these compounds, many organs harbor immune cells, and during infections great numbers of immunocytes infiltrate the affected tissue. This suggests that during inflammation, non-immune tissues produce immunosuppressive IDO (Terness et al. 2006).

In 1981, when IFN-γ was found to be one of the primary inducers of IDO activity (Yoshida et al. 1981a), investigations focused on the role of IDO in those infections in which IFN-γ plays a dominant role, namely, *Chlamydia* spp. and *Toxoplasma gondii* infections (Zelante et al. 2009). Restriction of *Chlamydia* spp. intracellular growth was linked to trp deprivation, since the addition of trp reversed the effects of IFN-γ (Byrne et al. 1986). However, in the presence of suboptimal IFN-γ, trp starvation could induce chlamydiae to enter a persistent state (Beatty et al. 1994). Only conditions of severe IDO-induced trp degradation would counteract bacterial persistence and concomitantly reduce the rate of reactivation (Leonhardt et al. 2007). Pfefferkorn (1984) observed that IFN-γ blocked the growth of *T. gondii* in human fibroblasts in an IDO-dependent way, and favored the hypothesis that the parasites were starved of the essential amino acid trp.

It has been shown in a small study that decreased plasma trp levels and lymphopenia typically occur after major trauma. Trp degradation was found to be associated with the development of sepsis, and also with neopterin and kyn concentrations, suggesting that trp deficiency was caused by IDO-mediated trp degradation (Pellegrin et al. 2005). An extremely high rate of trp degradation was observed in patients infected by *Streptococcus pyogenes*, which may cause tonsillitis, scarlet fever and so called streptococcal toxic shock-like syndrome (Murr et al. 2001). There is also data that indicate a role of the immune system and particularly of endogenously formed cytokines, like TNF-α and IFN-γ, effecting trp and neopterin metabolism in patients with acute Lyme neuroborreliosis, caused by the bacteria *Borrelia burgdorferi* (Gasse et al. 1994). Popov et al. (2006) found that infection of immature human DCs with a virulent strain of *Listeria monocytogenes* results in significant upregulation of *INDO* and other transcripts coding for the downstream enzymes of the trp pathway,

followed by significant protein expression and trp depletion with massive kyn accumulation.

Trp degradation has been found to be at work in special viral infections as well. Inhibition of cytomegalovirus replication in epithelial cells could be reverted by supplemental trp (Bodaghi et al. 1999). It has been shown recently that Epstein Barr virus viremia is associated with cell-mediated immune activation and increased trp degradation, which may partly account for the symptoms found in this disorder. Patients suffering from more severe symptoms tended to have aggravated trp degradation (Bellmann-Weiler et al. 2008). Upregulation of IDO expression in the liver and an increased serum kyn/trp ratio was found from patients with chronic hepatitis C, suggesting that induction of IDO may dampen T cell reactivity to viral antigens in chronic hepatitis C virus infection (Larrea et al. 2007).

Patients with HIV infection have decreased trp and increased kyn concentration, indicating accelerated trp degradation and a role of IDO in the disease. A strong association between kyn/trp ratio and concentrations of kyn and neopterin, and IFN-γ (Fuchs et al. 1990) and other markers of immune activation have been observed (Huengsberg et al. 1998, Look et al. 2000). Antiretroviral therapy is able to significantly reduce trp degradation (Huengsberg et al. 1998). It is likely that this happens via reduction of immune activation, particularly IFN-γ production. HIV production eliciting T cell activation and release of IFN-γ may represent the main stimulus for activating IDO in monocyte-derived macrophages, DCs and various other cells (Schröcksnadel et al. 2006a). Boasso et al. (2007) found that IDO activity appears to have different effects on CD4 and CD8 T cells, and that although both subsets are impaired in their proliferative activity during HIV infection, this can be explained by an IDO-mediated mechanism only in case of CD4 T cells.

4.2. Cardiovascular disease

Inflammation and immune activation are important in the pathogenesis of atherosclerosis and cardiovascular disease. Several different cell types are involved in the generation of atherosclerotic lesions; endothelial and smooth muscle cells (SMC) of the artery wall and circulating blood cells, namely monocytes, platelets and T lymphocytes interact with each other in many ways. Lesions, which occur principally in large and medium-sized elastic and muscular arteries, are asymmetric focal thickenings of the innermost layer of the artery, the intima. They may be present throughout a person's lifetime, and can lead to ischemia of the heart, brain or extremities, resulting in infarction (Ross 1999, Hansson 2005).

The presence of immunocompetent T cells and mononuclear phagocytes marks one of the earliest detectable atherosclerotic events that persist throughout atherogenesis. In the initiation phase of atherosclerotic lesion formation these cells are recruited in response to exposure to modified low-density lipoprotein (LDL). In response to the enhanced formation of endothelial adhesion molecules

induced e.g. by oxidized lipids, free radicals and oxidative stress, monocytes migrate into the intima, where they proliferate and differentiate into macrophages. Oxidized LDL is internalized by macrophages by means of the scavenger receptors on the surfaces of these cells. Macrophages then change their morphology and differentiate gradually to lipid loaded foam cells, i.e. activated macrophages (Ross 1999). The earliest lesions in atherogenesis, fatty streaks, which are frequently found very early in infants and young children (Napoli et al. 1997), are characterized by these foam cells. Fatty streaks have no clinical relevance and cause no symptoms. They may eventually disappear or progress into more advanced lesions (Hansson 2005).

Inflammatory reactions are important also in the further development of atherosclerosis. Several pathogens, such as *Chlamydia pneumoniae*, *Helicobacter pylori,* the periodontal pathogen *Porphyromonas gingivalis*, hepatitis A virus, influenza virus and various herpes group viruses, notably cytomegalovirus and HSV-1, may contribute to the development of cardiovascular diseases (Leinonen and Saikku 2002, Stassen et al. 2008), possibly by triggering inflammation. Even in young children, infections seem to associate with intimal thickening, predisposing coronary arteries to atherosclerosis (Pesonen et al. 1999). Although infection can affect atherosclerotic process indirectly from distant site, e.g. by inducing cytokines and raised CRP, direct effects of a pathogen inside atherosclerotic lesions are more convincing. In the vessel wall pathogens can promote a proinflammatory, procoagulant and proatherogenic environment (Leinonen and Saikku 2002). The micro-organism most comprehensively studied is *C. pneumoniae*; since the initial seroepidemiological study indicating a possible link between *C. pneumoniae* and atherosclerosis (Saikku et al. 1988), morphological and microbiological evidence for the presence of this bacteria has been found in a large variety of arteries using several different techniques (Stassen et al. 2008). Kalayoglu et al. (2000) showed that macrophages infected with *C. pneumoniae* begin to uptake LDL cholesterol and transform into foam cells, and the molecule responsible for this is chlamydial LPS. IFN-γ was shown to stimulate IDO mRNA expression and dose-dependent enzymatic activity in aortic smooth muscle cells, leading to a marked inhibition of *C. pneumoniae* growth (Pantoja et al. 2000). However, several human clinical trials of antibiotic therapy have produced conflicting results or failed to find association between the treatment and the disease outcome. Thus the role of *C. pneumoniae* in atherosclerosis is still controversial and needs further studies (Watson and Alp 2008).

At the center of atheroma, foam cells and extracellular lipid droplets form a core region, which is surrounded by a cap of SMCs and a collage-rich matrix. T cells, macrophages and mast cells infiltrate the lesion. Many of the immune cells exhibit signs of activation and produce inflammatory cytokines. SMCs release fibrous elements, and consequently, occlusive fibrous plaques develop and grow. Lesions first grow towards the adventitia, while afterwards they expand into the lumen of the vessel. Thrombotic events are mostly due to atheroma disruption. Plaque stability seems to be closely connected with the number of macrophages in plaques; the more inflammatory cells the higher the risk of disruption. Death

of macrophages via apoptosis seems to decisively influence the size of the thrombogenic lipid pool, which determines the plaque instability. The integrity of the fibrous cap overlying the lipid core and its resistance to rupture is heavily dependent on the collagenous extracellular matrix of the cap. Macrophages seem to have even more important role, however, as they are able to trigger collagen breakdown by expressing matrix-degrading enzymes that contribute to the weakening of the fibrous cap (Hansson 2005, Schröcksnadel et al. 2006a).

Not only macrophages, but also activated T cells in unstable plaques seem to have a crucial role, especially in early lesion formation. Both CD4 and CD8 cells, and also NK cells, have been found from atherosclerotic lesions, the major part comprising of CD4 cells. Depending on the surrounding cytokines, CD4 cells can differentiate into the Th1 or Th2 lineage, and the switch towards Th1 type immune response is a key determinant of the disease progression (Hansson 2005).

Th1 type cytokine IFN-γ appears to be a key player in atherogenesis and in the development and progression of cardiovascular disease, though its role remains controversial. IFN-γ induces several important biochemical pathways like ROS formation, neopterin release and trp degradation. Trp degradation may represent an auto-regulatory mechanism to slow down cellular immune response in atheroma formation intended to downregulate the further recruitment of other inflammatory cells (Schröcksnadel et al. 2006a). It has been demonstrated that IFN-γ inhibits the oxidation of LDL in human mononuclear cells (Christen et al. 1994), and trp availability and IDO activity also seem to inhibit the oxidation of LDL directly (Thomas and Stocker 1999). Thus the activation of IDO may be effective in downregulating foam cell formation. On the other hand, IFN-γ arising from the activated T cells in the plaque can halt collagen synthesis by SMCs, limiting its capacity to renew the collagen that reinforces the plaque (Libby 2002). It has been shown that patients with atheromatous plaques have lower serum trp concentration compared to healthy controls (Baldo-Enzi et al. 1996). After an oral load with L-trp lower serum trp and higher kyn concentrations compared with those of healthy controls were reported in patients suffering from myocardial infarction or angina pectoris (Rudzite et al. 1991). There is also evidence from a small study of subjects with angiographically verified coronary heart disease that IDO activity is increased in patients compared with healthy controls (Wirleitner et al. 2003).

4.3. Malignancy

A relationship between cancer and elevated trp catabolism was first recognized in the urine of bladder cancer patients in the 1950's (Boyland and Williams 1955). Further studies revealed similar findings in other cancer types, and it was shown that the effect was reversed upon surgical tumor reductive therapy (Rose 1967). Expression of IDO by cancer cells as well as by APCs has been detected in the tumor infiltrating zone, in the peritumoral stroma and in tumor-draining lymph nodes (Zamanakou et al. 2007). Uyttenhove et al. (2003) discovered that

most human malignant tumor cells express IDO at varying levels. In all cases most normal cells of the stroma were negative, suggesting that IDO expression by tumor cells did not result from *in vivo* exposure to IFN-γ. They showed in a mouse model that tumors expressing IDO at a high level effectively escape the immune surveillance of the host by degrading local trp, which thus inhibits T cell responses. This data suggests that the T cells are more sensitive to the antiproliferative effects of IDO than the tumor cells. Beyond the trp depletion, accumulation of IDO metabolites in the tumor environment seems to increase the suppression of anti-tumor immune responses (Zamanakou et al. 2007). Several studies have also suggested that IDO overexpression is associated with poor prognosis. Lower trp concentration and increased kyn/trp ratio are related to more advanced stages of disease. The extent of expression in tumor cells can thus be used for the prognosis of certain tumors.

One explanation of how IDO may become upregulated during tumor formation is attenuation of the gene *Bin1*, which is known to occur in many human cancers. *Bin1* encodes an adapter protein localized in nucleus, where it has been implicated in transcriptional repression (Prendergast 2008). Muller et al. (2005) observed that its presence strongly supports T cell-mediated immune surveillance. The tumor cells lacking *Bin1* displayed elevated IDO and this elevation was essential to gain the benefits of *Bin1* loss for tumor growth.

IDO elevation and the capacity to inhibit T cells also occurs in a subset of plasmacytoid DCs in tumor-draining lymph nodes (Munn et al. 2004a), where naïve T cells encounter the tumor antigen presented by DCs, and where the tumor response is likely to begin (Spiotto et al. 2002). In this case IDO-expressing DCs would account for an environment depleted of trp resulting in inappropriate tumor antigen-specific T cell responses. IDO may thus help to turn the tumor microenvironment from hostile to supportive for tumor cells, and also elaborate a peripheral mechanism of immune escape that could facilitate progression to invasive status (Prendergast 2008).

4.4. Other diseases

Trp degradation correlates with disease activity and markers of immune activation in several autoimmune diseases. The autoimmune process includes the induction of various cytokine cascades also involving IFN-γ production. High local expression of IDO may represent an anti-inflammatory mechanism aiming to counterbalance the tissue-damaging effects of activated T cells (Wolf et al. 2004). However, in autoimmune syndromes, accelerated trp catabolism is obviously insufficient to counteract the deleterious effects of the immune reaction against targeted structures (Schröcksnadel et al. 2006b).

Immune activation and degradation of trp have also been found in neurodegenerative disorders like Alzheimer's, Huntington's and Parkinson's disease. In the early disease course only minor changes in trp catabolism can be found, but in the very late stages significant acceleration of trp degradation and immune activation is common (Schröcksnadel et al. 2006b). As in certain

cancers, IDO activation also appears to correlate with disease stage in neurodegenerative disorders. Although increased trp degradation and signs of immune activation are common during normal ageing (Frick et al. 2004, Pertovaara et al. 2006), the degree of trp catabolism in patients with neurodegenerative disorders is substantially greater than in normal ageing. It is likely that lowered trp concentration in the elderly as well as younger patients may be related to an increased risk of developing mood disturbances, depression and/or memory loss, because the decreased availability of trp will negatively influence the production of catabolites serotonin and melatonin. Immune-mediated trp degradation by IDO may thus elicit neuropsychiatric symptoms when the availability of trp is insufficient for normal serotonin biosynthesis (Schröcksnadel et al. 2006b).

Aims of the study

The aims of the present study were to

1. Analyse the effect of *IFNG*+874 polymorphism on plasma trp and kyn concentrations and IDO activity

2. Investigate the effect of the atopic phenotype and risk-modifying environmental factors on IDO activity

3. Examine the associations of *CTLA4* and *TGFB1* polymorphisms on IDO-activity in *Helicobacter pylori* seropositive individuals

4. Investigate in two population-based adult cohorts the possible association of IDO activity with the grade of atherosclerosis by using the carotid artery intima-media thickness (IMT) as an indicator, and to study the associations of IDO activity with the risk factors in coronary artery disease

Subjects and methods

1. Subjects

1.1. Study I

Four hundred blood samples (buffy coats) were obtained from the Finnish Red Cross Blood Transfusion Centre, Tampere. The study population consisted of 309 healthy adults, whose age range was 21-45 years. They had no blood-transmitted diseases or any signs of other infections during a 2-week period before the blood donation.

1.2. Studies II and III

The subjects in Studies II (n=392) and III (n=391) were participants in a Finnish population-based case-control study aimed at identifying risk factors and predictors of the outcome of adult asthma (Karjalainen et al. 2002). The study population consisted of non-asthmatic adults, who were initially selected as healthy control subjects through a register covering the entire population of Finland. The mean age of the cohort was 60 years and the age range was 31-89 years.

1.3. Study IV

The subjects in the study were participants of the ongoing Cardiovascular Risk in Young Finns Study, which is a prospective multi-center cohort study being conducted in five university hospital cities in Finland. The study was initiated in 1980, when 3596 participants aged 3, 6, 9, 12, 15 and 18 were randomly selected. The cohort in the current study comprised 986 participants (544 female, 442 male aged 24–39 years) from two of the centers, the Finnish cities of Helsinki and Turku with their rural surroundings.

1.4. Study V

The study population consisted of 921 participants from a large Finnish cross-sectional health examination survey (the Health 2000 Survey). The survey was

carried out 2000–2001, and the overall study cohort was a two-stage stratified cluster sample of 8028 persons representing the entire Finnish population aged 30 years and above. In order to study cardiovascular diseases and diabetes more thoroughly, a supplementary study was carried out 2001-2003. The supplementary study was conducted in the catchment areas of the five Finnish university hospitals (sample size 1867 and participation rate 82 %). 921 subjects in Study V, aged 46-76 years, were included in this Health 2000 sub-study cohort.

2. Methods

2.1. Tryptophan and kynurenine measurements

Trp (μmol/l) and kyn (μmol/l) concentrations in peripheral blood were measured by reverse-phase high-performance liquid chromatography (HPLC), as described previously (Laich et al. 2002). Trp was separated with a Shimadzu liquid chromatograph LC-10AD VP (Shimadzu Co, Kyoto, Japan) using a 50-mm BDS Hypersil C18 5 μm column (Thermo Electron Co, Bellefonte, PA, USA). It was monitored by fluorescence with a Shimadzu RF-10A XL detector at 266 nm excitation and 366 nm emission wavelengths. Kyn was separated with a Hewlett Packard 1100 liquid chromatograph (Palo Alto, CA, USA) using Merck LiChroCart 55–4150 mm cartridge containing a Purospher STAR RP-18 3 μm column (Merck Co, Darmstadt, Germany). It was determined by ultraviolet absorption at 360 nm wavelength with a Hewlett Packard G13144 detector. Kyn/trp (μmol/mmol) was calculated by relating concentrations of kyn (μmol/l) to trp (mmol/l), this allowing an estimate of IDO activity.

2.2. Genotyping

2.2.1. Study I: IFNG+874

Genomic DNA was extracted from buffy coats using the QIAmp DNA blood Mini Kit (QIAGEN Inc., USA). The T>A SNP at the base position +874 of the *IFNG* gene (GenBank SNP Database rs number 2430561) was analysed by the amplification refractory mutational system-polymerase chain reaction method (ARMS-PCR) as described earlier (Pravica et al. 2000). DNA was amplified using Thermoprime^{PLUS} DNA polymerase (Abgene, Surrey, UK) in two different PCR reactions; each reaction used 250 pmol of generic antisense primer and 250 pmol of one of the two allele-specific sense primers. To assess the success of PCR amplification, one internal control of 426 bp was amplified using a pair of primers designed from the nucleotide sequence of the human growth hormone

(6,25 pmol each). The PCR was performed in a 25 µl reaction volume. The PCR conditions were as follows: 95°C for 1 min, then 10 cycles of 95°C for 15 sec, 62°C for 50 sec and 72°C for 40 sec, followed by 20 cycles of 95°C for 20 sec, 56° for 50 sec and 72°C for 50 sec. The amplified products were separated by electrophoresis in ethidium bromide stained 2 % agarose gel, and visualized under UV light.

2.2.2. Study III: TGFB1-509 and CTLA4+49

Genomic DNA was extracted from citrated whole blood samples using the QIAmp DNA blood Mini Kit (QIAGEN Inc., USA).

The promoter region polymorphism C>T at the position -509 of the *TGFB1* gene (rs1800469) was genotyped in Study III by restriction fragment length polymorphism (RFLP) analysis as previously described (Silverman et al. 2004). The PCR was performed in a 50 µl reaction volume, and the conditions were as follows: 95°C for 3 min, then 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 50 sec, and finally 72°C for 7 min. After the amplification the PCR products were digested with *Sau*I restriction enzyme (Fermentas, International Inc., Burlington, Canada) at 37°C for 3 hours. The fragments were separated by electrophoresis in 2 % agarose gel, and visualized with ethidium bromide staining under UV light. DNA from subjects with the homozygous C genotype (CC) produced two bands; one at 223 bp and one at 183 bp. The homozygous T genotype (TT) produced one band at 406 bp, and the heterozygous genotype CT produced all three bands.

Genotyping of the +49 A>G SNP in exon 1 of the *CTLA4* gene (rs231775) was performed with 5'nuclease assay for PCR and allelic discrimination using the ABI Prism 7000 Sequence Detection System (ABI, CA, USA). A commercial kit from Applied Biosystems was used (Assay ID C___2415786_20). The universal PCR thermal conditions were followed in 25 µl reaction volume using a 96-well plate: first 50°C for 2 min and 95°C for 10 min, then 40 cycles at 95°C for 15 sec and 60°C for 1 min. PCR reaction contained 1x TaqMan® Universal PCR Master Mix with AmpErase® UNG (ABI), 1x Assay Mix (ABI) and 10-100 ng of template DNA. The genotypes were selected manually from the allelic discrimination tab.

2.2.3. INDO and INDOL1 polymorphisms

We also investigated the possible role of *INDO* and *INDOL1* polymorphisms in the regulation of IDO activity using the same study populations as in other studies of this thesis; healthy Finnish blood donors (n=309), control participants of an adult asthma study (n=392) and the Cardiovascular Risk in Young Finns Study participants (n=986). In addition to these we genotyped a group of 284 nonagenarians (Goebeler et al. 2003) (unpublished data). Altogether 14 SNPs were detected from the *INDO* gene. Four of these were from the promoter area,

and 10 from the coding sequence. From the *INDOL1* gene six SNPs were selected; one from the promoter area and the remaining five from the coding sequence, four of them causing an amino acid change. The SNPs studied were selected from the public NCBI SNP database. Detailed information on the studied SNPs and the genotyping methods used is presented in Table 3a. (*INDO*) and Table 3b. (*INDOL1*).

Genotyping was performed with 5'nuclease assay for PCR and allelic discrimination using either the ABI Prism 7000 or 7900HT Sequence Detection System (ABI, CA, USA). Commercial kits from Applied Biosystems were used when available, and if this was not possible, designed unlabeled PCR primers and fluorogenic TaqMan® MGB probes were used (Assay by Design). Universal PCR conditions, as previously described, were followed in either 25 µl (ABI Prism 7000) or 5 µl (7900HT) reaction volume depending on the sequence detection system used. The genotypes were selected manually (ABI Prism 7000) or called up automatically by the SDS software (7900HT) from the allelic discrimination tab.

Table 3a. Genotyped *INDO* SNP*s.*

SNP location	mRNA location	dbSNP rs-number	Alleles	Amino acid	ABI Assay ID
Promoter		888447	G / T		C___7481100_10
		3808606	C / T		C___2037693_10
		3824259	G / T		C__27491530_10
		10089084	C / G		C__30475151_10
Exon 1	124	35059410	A	Thr	C__25608081_10
			G	Ala	
Exon 3	344	35099072	A	His	C__25608082_10
			G	Arg	
Exon 4	428	12545877	A	Lys	
			G	Arg	Assay by Design
	434	4463407	G	Ser	
			T	Ile	
Intron 4		4613984	A / G		C__26317191_10
		7820268	C / T		C__31027194_10
Intron 5		10108662	A / C		C___2037684_10
Intron 7		7010461	C / T		C__31027191_10
Intron 8		2302843	A / G		C__25472128_10
Intron 9		3739319	A / G		C___2037680_1_

Table 3b. Genotyped *INDOL1* SNPs.

SNP location	mRNA location	dbSNP rs-number	Alleles	Amino acid	ABI Assay ID
Promoter		2729489	A / T		C__15925609_10
Exon 2	184	4736794	G	Val	C__25988509_20
			A	Ile	
Intron 5		2543080	G / T		C___2037587_10
Exon 6	508	10109853	T	Trp	C___2037583_10
			C	Arg	
	520	35212142	A	Thr	C___2037582_10
			T	Ser	
Exon 8	843	4503083	A	Ter	C__27902198_10
			T	Tyr	

2.3. Determination of atopy

Atopy was determined by means of skin prick testing performed by specially trained nurses with a panel of 22 common allergen extracts (ALK A/S, Copenhagen, Denmark). The allergens were selected to cover exposures in both urban and rural environments, and they included dog, cat, horse, cow, *Acarus siro*, *Tyrophagus putrescentiae*, *Dermatophagoides farinae*, *D. pteronyssimus*, *Lepidoglyphus destructor*, birch, alder, mugwort, meadow foxtail, timothy grass, barley, barley flour, oats, wheat flour, rye flour, *Aspergillus fumigatus*, *Alternaria alternata* and *Cladosporium herbarum*. Skin prick test responses were considered positive if at least one allergen caused a wheal with a diameter at least 3 mm larger than that produced by the negative control. A person was defined as atopic if one or more of the 22 allergens tested gave a positive response.

2.4. Helicobacter pylori measurements

H. pylori IgG antibodies were measured from serum samples using the enzyme-linked immunosorbent assay (ELISA, Pyloriset-EIA-G III, Orion Diagnostica, Espoo, Finland). Titres of 30 or higher were considered positive for *H. pylori* antibodies.

2.5. Determination of cardiovascular risk factors

The data on cardiovascular risk factors used in Study IV were recorded during the follow-up conducted in 2001. For the determination of serum lipid levels, venous blood samples were drawn after an overnight fast. All the measurements were performed in duplicate in the same laboratory. Standard enzymatic methods were used for measuring levels of triglycerides and HDL cholesterol. LDL cholesterol concentration was calculated by the Friedewald formula (Friedewald et al. 1972). The fasting plasma CRP concentrations were analysed by a high-sensitive latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany), where the detection limit was 0.06 mg/l. BMI was calculated as participants' weight in kilograms divided by the square of their height in meters. Systolic and diastolic blood pressure were measured with a random zero sphygmomanometer and the mean of three measurements was used in the analysis. Smoking habits were elicited by a questionnaire.

In Study V, serum total cholesterol and triglycerides were determined by commercial automated enzymatic methods (Olympus System Reagent, Hamburg, Germany). Direct enzymatic methods were used for HDL cholesterol determinations (Roche Diagnostics, Mannheim, Germany). The analyses were performed on an Olympus AU400 (Germany) clinical chemistry autoanalyser. LDL cholesterol was calculated with the Friedewald formula. CRP concentrations were determined by a chemiluminescent immunometric assay (Immulite, Diagnostic Products, Los Angeles, CA). Participants' height and weight were measured and BMI was calculated. Blood pressure was measured using the automatic Omron M4 manometer (Omron Matsusaka, Japan, and Omron Healthcare Europe, Hoofddorp, the Netherlands). The average of the three measurements was used in the analysis. Current smoking was elicited by a questionnaire. Those who were currently smoking were defined as smokers and the rest of the subjects as nonsmokers.

2.6. Carotid artery measurements

For determining the IMT in Study IV, the left common carotid artery (CCA) was scanned by ultrasound technicians following a standardized protocol (Raitakari et al. 2003). Ultrasound studies were performed using Sequoia 512 ultrasound mainframes (Acuson, CA, USA) with 13.0-MHz linear array transducers. A magnified image was recorded from an angle that showed the greatest distance between the lumen-intima interface and the media-adventitia interface. A moving scan of five seconds duration, which included the beginning of the carotid bifurcation and the CCA was also recorded. Digitally stored scans were manually analysed by a single reader blind to participants' details.

In Study V, high-resolution B-mode carotid ultrasound examination of the right carotid artery was performed according to a standardized protocol using a 7.5 MHz linear array transducer (Niiranen et al. 2007). Examinations were made by centrally trained and certified sonographers, and one reader was responsible

for reading all images. The IMT was measured from three digitized end-diastole images of the CCA (lateral angle) and the carotid bulb (three interrogation angles). Three summary measurements were calculated: the mean of the three average IMT of the CCA, the mean of the three average IMT of the carotid bulb and the mean of these two means (mean IMT). Mean IMT was used for the analysis in this study.

2.7. Statistical methods

In Studies I-III, where the variables were not normally distributed, statistical methods appropriate for non-parametric data were used. Differences between two groups were analysed using the Mann-Whitney *U*-test, and differences between three or more groups using the Kruskall-Wallis test. In Studies IV and V, Student's *t*-test and chi square test were used for normally distributed variables and log-transformed skewed variables. Correlations were calculated with the Pearson correlation coefficient test. Multivariate linear regression analysis was performed to analyse the independent effect of cardiovascular risk factors on IMT. Statistica software (ver. Win 6.1, StatSoft Inc., Tulsa, OK, USA) and SPSS for Windows (ver. 13.0 and 14.0, SPSS Inc., Chicago, IL, USA) were used for the statistical analyses. Findings were considered statistically significant at $P < 0.05$.

LD calculations were performed with Haploview software, version 4.1. (Barrett et al. 2005). Haplotype blocks were created using the default algorithm taken from Gabriel et al. (2002). In this method 95 % confidence limits on D' are generated and each comparison is called "strong LD", "inconclusive" or "strong recombination". A block is created if 95 % of informative (i.e. non-inconclusive) comparisons are "strong LD".

2.8. Ethics

The ethical committee of the Finnish Red Cross Blood Transfusion Centre approved the use of human blood in Study I. Studies II and III were approved by the ethical committee of Tampere University Hospital, and informed consent to participate was obtained from all participants. In Study IV, the Cardiovascular Risk in Young Finns Study plan was approved by the local ethics committees of the participating university hospitals, and all the subjects gave written informed consent. In Study V, the protocol of the Health 2000 Survey was approved by the Epidemiology Ethics Committee of the Helsinki and Uusimaa Hospital District, and the participants of the survey signed an informed consent form.

Results

1. Effect of *IFNG* polymorphism on IDO enzyme activity (Study I)

The role of *IFNG*+874 genotype in the regulation IDO activity was investigated by analysing genotypes from a group of healthy adults, and correlating these to their plasma trp and kyn levels. The measured levels and kyn/trp ratio are shown in Table 4a. Trp level was similar in males and females, but the level of kyn and kyn/trp ratio were significantly higher in males. As the levels were dependent on gender, the effect of the SNP was analysed separately in male and female subjects. Distribution of the *IFNG*+874 genotype followed the Hardy-Weinberg equilibrium.

Trp and kyn levels and kyn/trp ratio of the female subjects by their *IFNG*+874 genotype are shown in Table 4b. According to our results, women with the genotype TT had the highest ratios. This effect was also observed in both trp and kyn concentrations. In males the SNP did not have any effect.

Table 4a. Serum tryptophan and kynurenine concentrations and kyn/trp ratio (median, interquartile range) of 309 healthy blood donors.

Variable	**Females** (n = 139)	**Males** (n = 170)	*P**
Trp, µmol/l	80.51 (69.78–91.41)	78.81 (70.65–80.31)	0.179
Kyn, µmol/l	1.96 (1.65–2.27)	2.14 (1.79–2.44)	**0.005**
Kyn/trp, µmol/mmol	24.31 (20.96–28.91)	27.00 (23.63–32.05)	**< 0.001**

*Mann-Whitney *U*-test

Table 4b. Serum tryptophan and kynurenine concentrations and kyn/trp ratio (median, interquartile range) of 139 female blood donors by IFNG+874 T>A genotype.

Variable	**TT** n = 55	**TA** n = 61	**AA** n = 23	*P**
Trp, µmol/l	75.30 (67.44–89.16)	84.24 (72.62–93.05)	78.87 (66.99–90.88)	0.081
Kyn, µmol/l	2.01 (1.69–2.40)	1.98 (1.65–2.27)	1.88 (1.57–2.00)	0.092
Kyn/trp, µmol/mmol	26.22 (22.84–30.90)	24.41 (20.65–27.97)	22.40 (19.56–25.68)	**0.023**

*Kruskal-Wallis test

2. Effect of the atopic phenotype and risk-modifying environmental factors on IDO activity (Study II)

We hypothesized that the effect of atopic phenotype and that of the known risk-modifying environmental factors could be reflected as differences in IDO activity. Trp and kyn concentrations and *H. pylori* IgG antibodies were measured from a cohort of 392 non-asthmatic adults of whom 149 were atopics. In this cohort, 148 individuals gave anamnestic information about childhood on a farm. The results showed that atopics had a significantly lower kyn/trp ratio than non-atopics, which was observed in both males and females (Mann-Whitney $P = 0.005$). Kyn/trp ratio was elevated (Mann-Whitney $P = 0.002$) and atopy less frequent (33.2 vs. 44.3 %, chi-square test $P = 0.022$) in *H. pylori* seropositive subjects. Reduction of atopy risk by childhood on a farm was not significant in this study material (chi-square test $P = 0.445$), but its effect on the kyn/trp ratio was clear (Mann-Whitney $P = 0.031$). In our cohort *H. pylori* and farm effects were independent; childhood on a farm did not increase the *H. pylori* seropositivity rate.

3. Effect of *TGFB1* and *CTLA4* polymorphisms on IDO activity in *Helicobacter pylori* seropositive individuals (Study III)

In Study II we showed that IDO activity is lower in a Th2 –associated disease, atopy, and higher in the presence of *H. pylori* infection. In this study we wanted to investigate whether the increase in IDO activity caused by *H. pylori* infection is genetically regulated. As candidate genes we used *TGFB1* and *CTLA4*. From each gene we chose one SNP that has been associated with high protein production (TGFB1) or expression (CTLA4). Their effect on IDO activity was studied in *H. pylori* seropositive and seronegative individuals.

Trp and kyn levels and *H. pylori* IgG antibodies were measured and *TGFB1*-509 and *CTLA4*+49 genotypes analysed from 391 adults. In this cohort there were 200 *H. pylori* seropositive and 191 seronegative individuals. The genotype distributions of *TGFB1* and *CTLA4* SNPs were in Hardy-Weinberg equilibrium. Our results showed there was no significant difference in IDO activity between the carriers and non-carriers of the high producer/expression genotype of the genes *TGFB1* and *CTLA4*. However, IDO activity was increased in *H. pylori* seropositive individuals compared to the seronegative subjects (Mann-Whitney *U*-test $P = 0.003$), and this difference was observed only in carriers of the high producer/expression genotype of the genes studied (*TGFB1*: Mann-Whitney *U*-test $P < 0.001$, *CTLA4*: $P = 0.004$).

4. Association of IDO activity with the grade of atherosclerosis and the risk factors in coronary artery disease (Studies IV and V)

T lymphocytes in atherosclerotic plaques are mainly of the Th1 subtype, secreting e.g. IFN-γ (Hansson 2005), which is a strong inducer of IDO. Our aim in Study IV was to test the hypothesis that IDO is involved in the regulation of inflammatory responses associated with the development of early atherosclerosis. We investigated the activity of IDO, i.e. kyn/trp ratio, in 986 young adults, and measured their IMT as well as traditional risk factors for atherosclerosis. The population cohort used in Study IV comprised young or middle-aged individuals (24-39 years of age). In Study V we aimed to replicate the finding in a cohort of 921 older people (age range 46-76 years), i.e. in subjects with more advanced atherosclerotic changes.

In Study IV, the mean (± s.d.) kyn/trp was 27.0 ± 7.2 μmol/mmol in females and 27.9 ± 7.3 μmol/mmol in males. Kyn/trp ratio correlated with mean IMT in female subjects ($r = 0.131$, $P = 0.002$), while in males there was no correlation between kyn/trp and IMT ($r = 0.040$, $P = 0.402$). The risk variables for atherosclerosis that showed significant correlation with kyn/trp ratio were in females age, HDL and LDL cholesterol, triglyceride, BMI, waist circumference,

waist-to-hip ratio and CRP. In males the significant variables were HDL cholesterol, BMI, waist circumference, waist-to-hip ratio and CRP.

In addition to IDO activity, age, BMI and systolic blood pressure were significant predictors of IMT in females, when investigated with a univariate analysis (age: $r = 0.278$, $P < 0.001$, BMI: $r = 0.147$, $P = 0.001$, systolic blood pressure: $r = 0.101$, $P = 0.019$). To find out the possible independent effect of kyn/trp on IMT in females, a multivariate linear regression model was constructed with IMT as the dependent variable and the traditional cardiovascular risk factors (age, HDL and LDL, triglyceride concentration, BMI, systolic and diastolic blood pressure, smoking), concentration of CRP and kyn/trp ratio as independent variables. IDO activity was not an independent predictor for IMT in this multivariate model; the variables which remained significantly associated with IMT were age and BMI.

In Study V, the mean (± s.d.) kyn/trp was 32.4 ± 9.1 μmol/mmol in females and 31.9 ± 8.7 μmol/mmol in males. IDO activity correlated significantly with IMT both in females ($r = 0.208$, $P < 0.0001$) and in males ($r = 0.168$, $P = 0.001$). IDO activity also correlated with several cardiovascular risk factors in both sexes. In females those risk factors were age, BMI, HDL cholesterol, triglyceride and CRP concentration, and both systolic and diastolic blood pressure. In male subjects a significant correlation with IDO activity was seen in age, LDL and total cholesterol, CRP concentration and diastolic blood pressure.

As in Study IV, a multivariate linear regression model was constructed to assess the independent effect of the IDO activity on IMT. The analysis was done separately for female and male subjects. IMT was used as a dependent variable, and age, HDL, LDL, triglyceride concentration, systolic and diastolic blood pressure, CRP concentration, BMI and smoking status as cofactors. As in Study IV, in these multivariate models IDO activity did not remain as an independent predictor of IMT in either females or males: age and systolic blood pressure were independently associated with IMT in female subjects and age, triglyceride and smoking habits in males.

5. Effect of *INDO* and *INDOL1* polymorphisms on IDO activity (unpublished data)

We investigated the possible role of *INDO* and *INDOL1* polymorphisms in the regulation of IDO activity. Genotypes were determined from four different study populations; 309 Finnish blood donors, 392 control participants of an adult asthma study, 986 Cardiovascular Risk in Young Finns Study participants and 284 nonagenarians. From six of the studied 14 *INDO* SNPs only one genotype was found, which means they may be artefacts in the database or simply have a very low minor allele frequency. The rest of the polymorphisms studied were from the promoter area or intronic SNPs not causing an amino acid change in the sequence. We did not find any significant or repeatable associations with the trp

catabolism, and the results were also negative after constructing haplotypes from the genotyped SNPs.

From the six *INDOL1* SNPs selected for genotyping only one (rs 4503083) was significantly associated with the kyn/trp ratio in the same way as reported by Metz et al. (2007). The subjects who were homozygous for the allele causing the premature stop codon had the lowest kyn/trp ratio (P = 0.001). However, this effect was significant only in female nonagenarians (n=215). Also, the frequency of the minor allele causing the stop codon was low (0.18), and among the female nonagenarians there were only 10 carriers of this affecting genotype. As in the case of *INDO*, *INDOL1* haplotypes did not show any significant association with IDO activity.

LD was calculated between all the *INDO* and *INDOL1* SNPs studied from which the genotype data was received. Figure 2 shows the Haploview LD plot created from the genotypes of the Finnish blood donors. LD is presented in D' values and the (dark) red color indicates strong LD. Our genotype data comprised three haplotype blocks; two in the *INDO* gene area and one in the *INDOL1* gene.

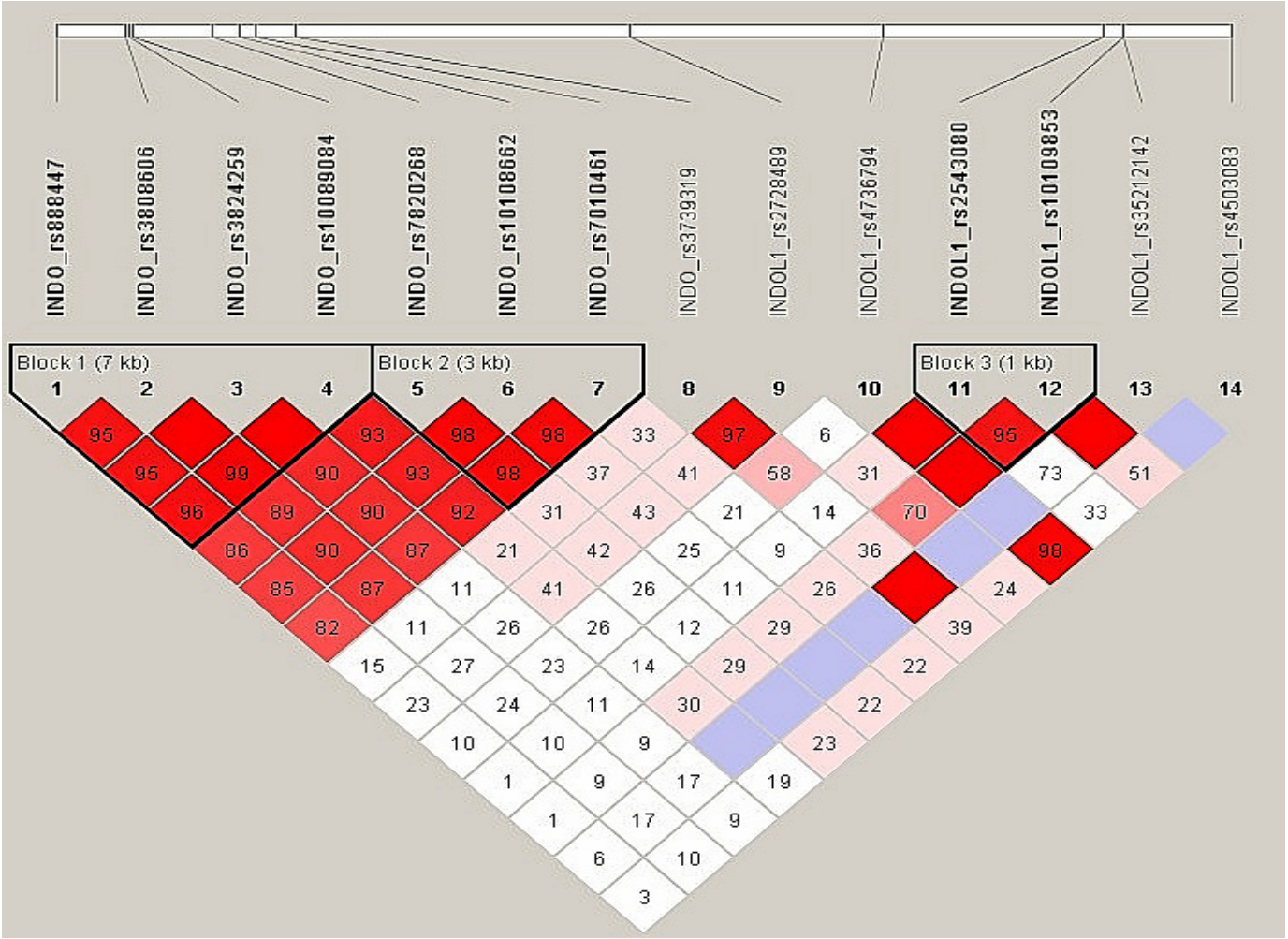

Figure 3. *LD values (D') and haplotype blocks of the INDO and INDOL1 polymorphisms studied on chromosome 8. The bar at the top represents the relative positions of the SNPs.*

Discussion and conclusions

1. Association of gene polymorphisms with IDO activity

1.1. IFNG+874

Among healthy Finnish blood donors the *IFNG* polymorphism +874 T>A was associated with the kyn/trp ratio, i.e. IDO enzyme activity. As the measured levels of kyn and the kyn/trp ratio were dependent on gender, the effect of the SNP was analysed separately in male and female subjects. The association was significant only in females. Women with the *IFNG*+874 genotype TT had the highest kyn/trp ratios, and the same effect was observed in both trp and kyn concentrations. The difference in results between male and female subjects could be explained by the hormonal influence and the generally greater immune reactivity of females (Verthelyi 2001). The expression of the *IFNG* gene may also be subject to direct hormonal control (Fox et al. 1991). However, the association between *IFNG*+874 SNP and IDO activity was not found in other study materials; control participants of an adult asthma study (Studies II and III) and young adults participating in the Cardiovascular Risk in Young Finns Study (Study IV) (unpublished data).

Since IFN-γ is the most potent inducer of the IDO enzyme, the results from Study I were quite as expected. The SNP +874 lies within a binding site for the transcription factor NF-κB, which is known to induce IFN-γ expression. Electrophoretic mobility shift assays have shown specific binding of NF-κB to the *IFNG* sequence containing the +874 allele T, thus T and A alleles correlate with high and low IFN-γ expression respectively (Pravica et al. 2000).

There are no previous publications about an association between *IFNG* polymorphisms and trp catabolism. However, +874 SNP has been associated with several clinical conditions, e.g. the development of tuberculosis (Lio et al. 2002, Lopez-Maderuelo et al. 2003, Rossouw et al. 2003, Sallakci et al. 2007), symptomatic parvovirus infection (Kerr et al. 2003), recurrent pregnancy loss (Daher et al. 2003) and spontaneous preterm delivery (Speer et al. 2006). Our study report is the first concerning the genetic regulation of IDO activity. The data show that *IFNG* gene controls trp catabolism, which may thus be operative in diseases associated with the polymorphisms of the *IFNG* gene.

1.2. TGFB1-509

We aimed to find out whether the increase in IDO activity caused by *H. pylori* infection (Study II) is genetically regulated. *TGFB1* and *CTLA4* were selected as candidate genes, because they are known to be involved in the activation of T-reg cells. *TGFB1*-509 promoter polymorphism -509 C>T alters a Yin Yang 1 (YY1) transcription factor consensus binding site; allele T enhances YY1 binding and *TGFB1* promoter activity, and is associated with higher circulating concentrations of TGF-β1 in plasma (Grainger et al. 1999, Silverman et al. 2004).

We did not find a significant difference in IDO activity between the carriers and non-carriers of allele T of the *TGFB1*-509 SNP when both *H. pylori* seronegative and positive subjects were studied together. However, the increased IDO activity caused by *H. pylori,* which was shown in 392 control participants of an adult asthma study (Study II), was found to be *TGFB1*-509 T allele dependent. This allele dependence was observed in the same study population and only in *H. pylori* seropositive subjects (Study III). I.e. seropositive subjects carrying the high TGF-β1 production allele (genotype TT or CT) had higher kyn/trp ratios than seronegative subjects.

According to our results, *H. pylori* induced IDO activity is regulated by the *TGFB1* gene, and IDO might be involved in the T cell suppressive effects of this gene. However, the approach used in our study was not informative in localizing the site of action of TGF-β1.

1.3. CTLA4+49

As was the case with the *TGFB1* SNP, the +49 A>G SNP in the first exon of the *CTLA4* gene, which leads to a threonine/alanine amino acid substitution, did not directly affect the IDO activity in control participants of an adult asthma study. However, in this case, too, *H. pylori* seropositive subjects carrying the genotype AA, which has been associated with higher cell-surface expression of CTLA4 (Ligers et al. 2001), had significantly higher kyn/trp than subjects with the genotype GG or AG (Study III).

CTLA4+49 SNP has been associated with several organ-specific autoimmune diseases, e.g. insulin-dependent diabetes, celiac disease, multiple sclerosis and thyroid diseases (Kristiansen et al. 2000), and also with multiple types of cancer (Sun et al. 2008). Increased IDO activity in individuals with the high expression genotype AA is an expected result, since CTLA4 is known to induce the *INDO* gene (Grohmann et al. 2002). In light of our findings, the increase in IDO activity caused by *H. pylori* infection is genetically regulated by the *CTLA4* gene, although the exact cellular mechanism is not yet clear.

1.4. INDO and INDOL1 polymorphisms

As mentioned in the review of the literature, there is no published information about the functionality of *INDO* polymorphisms or association studies of *INDO* polymorphisms and diseases, but some recent genetic studies have revealed variability in the *INDO* gene that may contribute to differences in the enzyme activity between individuals. Arefayene et al. (2006) resequenced the exons and intron-exon borders of the *INDO* gene in 96 DNA samples from the DNA diversity panels from the Coriell DNA Repository. They found 15 variants, of which eight were novel findings, including a non-synonymous SNP in exon 3. Further studies by the same group revealed a 9 bp deletion in exon 7, replacing amino acid chain alanine-leucine-leucine-glutamic acid with an aspartic acid, although this deletion was rare; it was detected only in one of the 48 Afro-American samples (Arefayene et al. 2007). Expression of the wild-type and variant cDNAs in COS-7 and HEK293 cells showed that the variants caused >90 % reduction in IDO activity compared to the wild-type, demonstrating the presence of nonfunctional alleles of the *INDO* gene in human population. These variants did not appear to affect the mRNA concentration following a transient transfection; however, even though the wild-type protein was easily detectable by western blot, the variant proteins were not detectable. These data are certainly of interest, since several clinical studies have demonstrated that interindividual IDO activity is highly variable. However, larger studies are needed to confirm the findings of Arefayene et al., and the possible variability between different ethnic groups should also be considered.

We investigated the possible role of 14 *INDO* polymorphisms in the regulation of IDO activity. However, we did not find any significant and replicable associations with the trp catabolism, and the results were also negative after constructing the haplotypes from the genotyped SNPs (unpublished data).

Currently there is only one published study focusing on the polymorphisms of *INDOL1* (IDO2). Metz et al. (2007) identified two SNPs that abolished the enzyme activity; R248W and Y359stop. The nonsynonymous substitution R248W in exon 9 reduced catabolic activity by about 90 % in T-REX cells. The other SNP Y359stop generates a premature stop codon, which abolished the enzyme activity. According to their data, these SNPs could have an extensive effect on IDO2 activity in humans, since both SNPs are common, especially in individuals of European and Asian descent.

We analysed the possible association of six *INDOL1* SNPS with trp degradation. Among these were the two SNPs investigated by Metz et al.; R248W (rs 10109853) and Y359stop (rs 4503083). From the SNPs detected only Y359stop was associated with the kyn/trp ratio, as previously reported by Metz et al. However, this effect was significant only in female subjects (n=215). Because the frequency of the minor allele causing the premature stop codon was low (0.18), there were only 10 carriers of the affecting homozygous genotype among the female nonagenarians. Associations of the *INDOL1* haplotypes with IDO activity were not significant (unpublished data).

Due to the lack of genetic association studies on *INDO* and *INDOL1*, it cannot be concluded that IDO activity is clearly regulated by the polymorphisms of these two genes. The findings from one population have not so far been generalizable to other study populations. Our study populations were quite small, and more significant associations could possibly be found in a larger number of subjects. It is also possible that the most effective SNP(s) still remain to be identified, or have not yet been investigated. Further studies are required in order to understand the genetic regulation of trp degradation and IDO activity, and also the possible impact of *INDO* and *INDOL1* on human disease.

2. IDO activity and atopy

We investigated the possible association of IDO activity and a Th2-associated disease atopy in a cohort of 392 non-asthmatic adults, of whom 149 were atopics. The activity was significantly lower in atopic individuals (Study II). This association was seen both in female and male subjects. *H. pylori* infection has previously been reported to associate with the decreased risk of atopy (Liu and Murphy 2003), and confirming this, we observed that atopy was less frequent in *H. pylori* seropositive subjects. Childhood on a farm, which is probably related to increased exposure to LPS, is also a factor known to be associated with reduced atopy risk (Liu and Murphy 2003). Although in our study we observed no such effect, childhood on a farm was significantly associated with increased IDO activity.

According to the so-called “hygiene hypothesis”, first evinced by Strachan (1989), frequent contacts with microbial components in childhood favor differentiation of T cells towards the Th1 direction thus reducing the risk of Th2-associated diseases in later life. IDO is known to be induced by the activators of the Th cell differentiation in the Th1 direction, e.g. LPS and IFN-γ, and Hayashi et al. (2004) showed that induction of IDO activity with bacterial components via TLRs inhibited Th2-cell driven murine experimental asthma. Further studies by these scholars demonstrated that the mechanism behind this is the trp metabolite 3-HAA, which suppresses TCR-triggered NF-κB activation by directly inhibiting pyruvate dehydrogenase kinase (PDK1) phosphorylation. This leads to dysfunction and cell death of activated Th2 cells, which in turn suppresses experimental asthma (Hayashi et al. 2007). The variation in IDO activity observed in our study is also in line with the Th1/Th2 paradigm.

Supporting our results, Kositz et al. (2008) found significantly higher serum trp levels in atopic patients than in healthy controls, which may result from lower IDO activity. However, contradictory reports have also been published. Von Bubnoff et al. (2003) suggested that the expression of IDO is differentially regulated in atopic versus non-atopic monocytes. They demonstrated an increase of about 4½ times in induction of the *INDO* gene after FcεRI crosslinking or IFN-γ exposure in atopic compared to non-atopic monocytes. Xu et al. (2008a) reported that IDO promotes Th2 responses and allergic inflammation in murine

lung DCs. Lung has at least two resident cell types, epithelial cells and myeloid DCs, which constitutively express IDO protein, and two others, the plasmacytoid DCs and eosinophils that can be recruited in larger numbers during infections or allergic inflammation. It appears that the function of IDO is stimulatory (Odemuyiwa et al. 2004) or inhibitory (Hayashi et al. 2004, Grohmann et al. 2007) depending on the target cell, the stimulus, and the specific model (Xu et al. 2008a).

Our data suggests that IDO activity is closely associated with the immune mechanisms involved in the pathogenesis of atopy, although more studies are needed to clarify the role of IDO in Th differentiation and Th1/Th2 related diseases.

3. IDO activity and atherosclerosis

Inflammation and immune activation play a role at all stages of the pathogenesis of atherosclerosis and cardiovascular disease. Accordingly, inflammatory markers are elevated in patients with clinical and subclinical atherosclerosis and predict cardiovascular events (Hansson 2005). Increased trp degradation and kyn/trp ratio has been reported in coronary heart disease patients compared with healthy controls (Wirleitner et al. 2003). We found an association between IDO activity and mean IMT, which can be used as a presymptomatic predictor of atherosclerosis, in female subjects participating in the Cardiovascular Risk in Young Finns Study (Study IV). However, IDO activity was not an independent predictor for IMT when analysed by a multivariate model, thus IDO does not have an independent role in the pathogenesis of atherosclerosis. Only age and BMI remained significantly associated with IMT in the multivariate model.

IDO activity and trp degradation are known to increase during normal ageing (Frick et al. 2004, Pertovaara et al. 2006), and this was also seen in our study populations in Study IV and V. The mean kyn/trp ratio of the participants of the Health 2000 Survey in Study V was higher than the mean ratio of the younger participants of the Cardiovascular Risk in Young Finns Study in Study V. An association between IDO activity and IMT was also found in the older study cohort, where more advanced atherosclerotic changes were expected. In this population the association was seen in both female and male subjects. As was the case in our earlier study, IDO activity did not remain as an independent predictor of IMT in a multivariate model. In female subjects age and systolic blood pressure were independently associated with IMT, and in male subjects the significant risk factors were age, triglyceride and smoking habits.

Our studies are the first to show an association between IDO enzyme activity, cardiovascular risk factors and IMT. When the association between IDO activity and the traditional risk variables for atherosclerosis was investigated in participants of the Cardiovascular Risk in Young Finns Study, a significant correlation was found in females with age, HDL and LDL cholesterol, triglyceride, BMI, waist circumference, waist-to-hip ratio and CRP. Except for

the LDL cholesterol and triglyceride, these were also significant in male subjects (Study IV). In the Health 2000 Survey participants' IDO activity also correlated with several cardiovascular risk factors in both sexes. In females those risk factors were age, BMI, HDL cholesterol, triglyceride and CRP concentration, and both systolic and diastolic blood pressure. In male subjects a significant correlation with IDO activity was seen for age, LDL and total cholesterol, CRP concentration and diastolic blood pressure (Study V).

It is not clear, whether increased IDO activity is deleterious or advantageous in the process of atherosclerosis development. Increased activity may be simply a consequence of inflammation, i.e. merely a non-specific reactive marker of inflammation. If IDO activity is an attempt to downregulate the immune response induced e.g. by oxidized LDL or some other antigen, IDO would have a protective role. T-regs have recently been suggested to counteract disease initiation and progression in atherosclerosis (Mallat et al. 2005). If increased IDO activity induced T-reg cell activity in early atherosclerosis, increased IDO activity would constitute a favorable response. However, as IFN-γ is a key player in atherogenesis and in the development and progression of cardiovascular disease, biochemical reactions induced by IFN-γ may have harmful consequences for host cells (Schröcksnadel et al. 2006a). As IFN-γ is one of the main inducers of IDO, and has both pro- and anti-atherogenic properties, it is also possible that IDO plays a pro-atherogenic role.

Concluding remarks

Indoleamine 2,3-dioxygenase is a cytosolic enzyme that catalyses the oxidative metabolism of tryptophan to kynurenine. By removing trp from the cell environment IDO enables the host to inhibit the growth of various infectious pathogens, and it also regulates the local function of the immune cells. In the study presented we investigated the regulation of IDO enzyme activity and its role in certain clinical conditions. Serum trp and kyn concentrations were measured from four different study populations, and calculated kyn/trp ratio was used as an indicator of IDO activity.

We observed that IDO activity was significantly lower in subjects with a Th2-associated disease, atopy, compared with healthy control subjects. Environmental factors know to reduce the atopy risk, *H. pylori* and childhood on a farm, were shown to increase the enzyme activity (Study II). These results suggest that IDO activity is associated with the immune mechanisms involved in the pathogenesis of atopy. More studies are required to clarify the role of IDO in Th differentiation and Th1/Th2 related diseases.

We found that IDO activity associated with the grade of atherosclerosis in two different study populations, when the carotid artery intima-media thickness was used as an indicator, although it was not an independent predictor for IMT when analysed by a multivariate model. IDO activity also correlated with several cardiovascular risk factors both in female and male subjects (Studies IV and V). Our studies are the first to show an association between IDO activity, cardiovascular risk factors and IMT. However, the exact role of IDO and increased trp catabolism in atherosclerosis development, whether it is deleterious or advantageous, remains to be clarified.

The genetic effect on the regulation of IDO activity was studied by analysing the association of the genotypes from certain immune related genes with the measured kyn/trp ratio. In our study IDO activity was shown to be regulated by *IFNG* polymorphism +874 T>A in healthy female blood donors (Study I). Studied *TGFB1* and *CTLA4* polymorphisms did not have a direct effect on IDO activity, but the previously found increase in IDO activity caused by *H. pylori* infection was shown to be regulated by these genes; *TGFB1* polymorphism -509 C>T and *CTLA4*+49 A>G (Study III). We investigated also the possible effect of several *INDO* and *INDOL1* polymorphisms, and found only one *INDOL1* polymorphism, Y359stop, affecting the enzyme activity (unpublished data). There is a lack of published genetic association studies concerning IDO, thus further studies with larger study populations are required in order to understand the genetic regulation of trp degradation and IDO activity.

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Original communications

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Association of Interferon-gamma +874(T/A) Single Nucleotide Polymorphism with the Rate of Tryptophan Catabolism in Healthy Individuals

A. Raitala *, M. Pertovaara†, J. Karjalainen‡, S. S. Oja *§ & M. Hurme *¶

*Department of Microbiology and Immunology, Medical School, University of Tampere; †Department of Internal Medicine, Section of Rheumatology; ‡Department of Respiratory Medicine; §Department of Clinical Physiology; and ¶The Laboratory Centre, Tampere University Hospital, Tampere, Finland

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Correspondence to: A. Raitala, MSc, Department of Microbiology and Immunology, Medical School, University of Tampere, 33014 Tampere, Finland. E-mail: annika.raitala@uta.fi

Abstract

Mechanisms induced by tryptophan (trp) catabolism are important in the regulation of both normal and pathogenetic immune responses. The key enzyme is indoleamine-pyrrole 2,3-dioxygenase (EC 1.13.11.42) (IDO) which converts trp to kynurenine (kyn), the main toxic metabolite. It is known that interferon-gamma (IFN-γ) is able to activate IDO. We wanted to analyse whether the strength of this mechanism would be under genetic control. To this end, we analysed the IFN-γ +874(T/A) genotypes, which are known to have an effect on IFN-γ production, of 309 healthy blood donors and correlated these to the levels of trp and kyn in their blood. The data obtained demonstrate that the presence of the high producer T allele was associated with increased IDO activity (i.e. elevated kyn and kyn/trp levels), but this effect was observed only in females. These data show that trp catabolism is genetically controlled by the IFN-γ gene and may thus be operative in those disease conditions associated with the polymorphisms of the IFN-γ gene.

Introduction

Tryptophan (trp) is an essential amino acid, which is catabolized by two distinct biochemical pathways. Trp 5-hydroxylase (EC 1.14.16.4) converts trp into the neurotransmitter serotonin and secondly, it is transformed to kynurenine (kyn), a reaction where two different enzymes perform: trp 2,3-dioxygenase (EC 1.13.11.11) (TDO) and indoleamine-pyrrole 2,3-dioxygenase (EC 1.13.11.42) (IDO) [1]. These enzymes catalyse oxidative degradation of trp, which is the first, rate-limiting step in trp catabolism by the kyn pathway [2]. IDO is expressed in antigen-presenting cells (macrophages and dendritic cells) [3], and there is now increasing evidence showing that activation of IDO during the inflammatory response leads to a decrease in local trp levels [1]. These decreased levels (and/or the increased levels of toxic metabolites) have an inhibitory effect on proliferation of T lymphocytes, directly or indirectly via activation of regulatory T cells [2]. It has recently also been demonstrated that IDO is expressed in some tumour cells, and its activation may be responsible for the inhibition of T-cell reactivity against these tumours [4].

IDO production is induced by several inflammatory mediators including interferons (IFN), the most potent of which is interferon-gamma (IFN-γ). Unlike IDO, TDO seems to be a homeostatic gene and is not induced or regulated by signals from the immune system. IFN-γ pathway is required for the normal upregulation of IDO expression during infection [2]. Production of IFN-γ is genetically controlled, and there are two well-known polymorphisms in the IFN-γ gene. It has been reported that the 12 CA repeat microsatellite allele in the noncoding region of the first intron is associated with a higher level of *in vitro* cytokine production [5]. The same group has also reported complete linkage disequilibrium between the 12 CA repeat allele and the presence of the T allele at the +874 position (+874T/A) from the translation start site. This polymorphism lies within a binding site for the transcription factor NF-κB, and electrophoretic mobility shift assays showed specific binding of NF-κB to the allelic sequence containing the +874T allele [6]. As this transcription factor induces IFN-γ expression, +874T and A alleles probably correlate with high and low IFN-γ expression, respectively [7].

The ratio between kyn, the first product of IDO, and trp, the substrate of the enzyme, allows an estimate of IDO activity *in vitro* and *in vivo*. This ratio seems to be a sensitive indicator for IFN-γ-induced trp degradation

and therefore an activated immune system. Thus, simultaneous measurements of kyn and trp, allowing calculation of the ratio, enable indirect examination of endogenous IFN-γ formation [8]. The aim of this study was to find out the possible role of IFN-γ +874 genotype in the regulation of trp catabolism in healthy individuals.

Materials and methods

Blood samples of 309 healthy adults (170 men and 139 women) were obtained from the Finnish Red Cross Blood Transfusion Center, Tampere. The mean age of the subjects was 45 years (range 21–64 years). Trp and kyn concentrations in peripheral blood were measured by reverse-phase high-performance liquid chromatography as described [9]. Trp was monitored by fluorescence detection at 266-nm excitation wavelength and 366-nm emission wavelength; kyn was measured by ultraviolet absorption at 360 nm wavelength. Kyn/trp ratio was calculated by relating concentrations of kyn (μmol/l) to trp (mmol/l) allowing an estimate of IDO activity [9].

The base-exchange polymorphism at +874 of the IFN-γ gene was analysed by the amplification refractory mutational system-polymerase chain reaction method (PCR) described by Pravica *et al.* [6]. DNA specimens from buffy coat blood samples were prepared using standard methods. Briefly, genomic DNA was amplified with the use of Thermoprime^{PLUS} DNA polymerase (Abgene, Surrey, UK) in two different PCR reactions; each reaction employed a generic antisense primer and one of the two allele-specific sense primers. To assess the success of PCR amplification, one internal control of 426 bp was amplified using a pair of primers designed from the nucleotide sequence of the human growth hormone. The amplified products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide. The PCR was performed using 10 cycles (95 °C for 15 s, 62 °C for 50 s and 72 °C for 40 s), followed by 20 cycles (95 °C for 20 s, 56° for 50 s and 72 °C for 50 s). Characteristics of the primer sequences are summarized in Table 1.

The statistical method selected is indicated in the *Results* section as used. The analyses were carried out with Statistica software (ver. Win 6.1; StatSoft, Tulsa, OK, USA). The findings were considered significant in case of a *P*-value of less than 0.05.

Results and discussion

Trp and kyn blood levels of 309 healthy blood donors (170 men and 139 women) are shown in Fig. 1. The levels of trp were similar in males and females, but the levels of kyn and kyn/trp ratio were higher in males (Mann–Whitney *U*–test: kyn $P = 0.005$; kyn/trp $P = 0.0004$). Age did not have any effect on the levels in female donors. In males, kyn/trp ratio correlated weakly with age (Spearman: $P = 0.003$, $r = 0.229$).

As the levels were dependent on gender, we analysed the effect of the IFN-γ +874(T/A) single nucleotide polymorphism (SNP) separately in male and female blood donors. The genotype distribution was in Hardy–Weinberg equilibrium both in male and in female donors. The data demonstrate that women with the genotype TT had the highest kyn/trp ratios (Fig. 2; Kruskal–Wallis: $P = 0.023$). This effect was observed in both trp and kyn concentrations. In males, the IFN-γ SNP did not have any effect.

A decrease in serum trp and a parallel increase of kyn due to IDO activation is found in various diseases associated with T-cell activation [2]. IDO-inducing IFN-γ is a multifunctional cytokine that is essential in the development of T-helper 1 cells, defence against viruses and intracellular pathogens and in the induction of immune-mediated inflammatory responses [10]. The CA repeat microsatellite polymorphism in the first intron of IFN-γ gene and the linked +874(T/A) SNP have been associated with several autoimmune and chronic inflammatory conditions [10], e.g. higher production of IFN-γ *in vitro* [6], allograft fibrosis in recipients of lung transplants [11], the incidence and severity of acute kidney rejection [12], aplastic anaemia [13], the development of tuberculosis [7, 14, 15], symptomatic parvovirus infection [16] and recurrent pregnancy loss [17]. It has also been suggested that the presence of IFN-γ +874A allele is advantageous for the longevity [18]. Whether these polymorphisms exert their action through trp catabolism-mediated mechanisms remains to be studied.

Table 1 Characteristics of the primers used for genotyping the polymorphism of IFN-γ by amplification refractory mutational system-polymerase chain reaction method

Primer	Product size (bp)	Sequence
IFN-γ generic primer (antisense)	261	5′-tcaacaaagctgatactcca-3′
IFN-γ primer T (sense)	261	5′-ttcttacaacacaaaatcaaatct-3′
IFN-γ primer A (sense)	261	5′-ttcttacaacacaaaatcaaatca-3′
Internal control primer 1	462	5′-gccttcccaaccattccctta-3′
Internal control primer 2	462	5′-tcacggatttctgttgtgtttc-3′

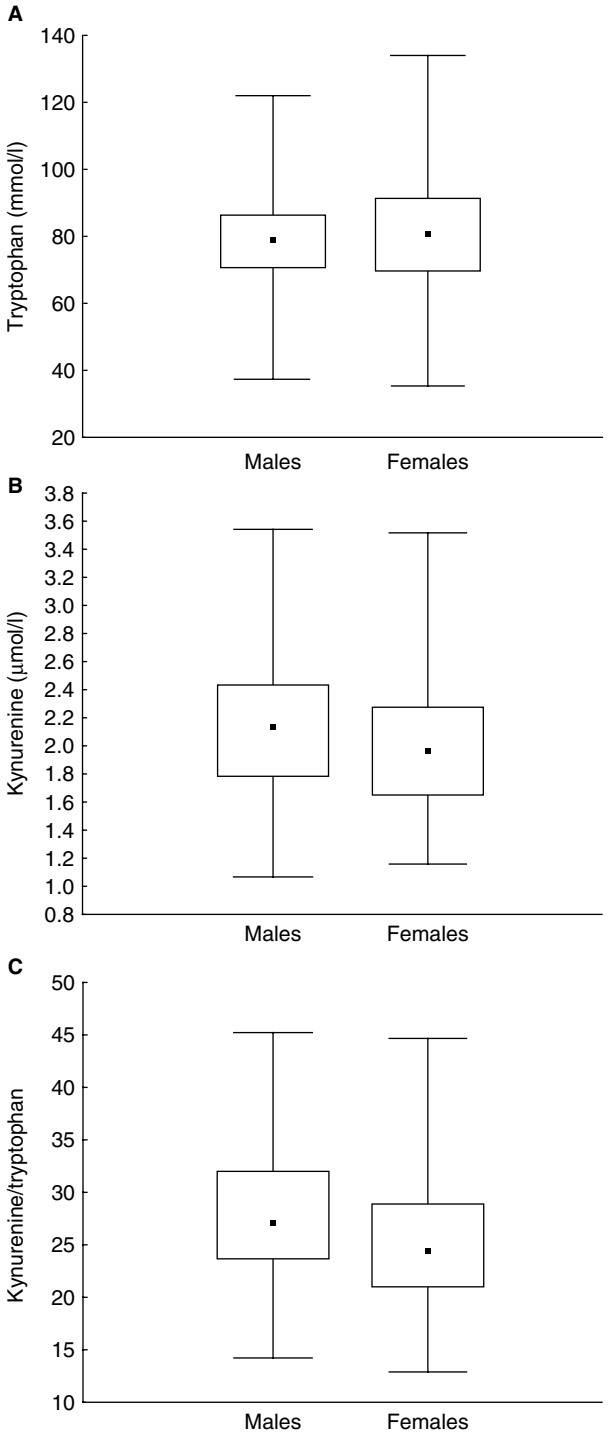

Figure 1 Plasma concentrations (median, 25–75% quartiles, min–max) of (A) tryptophan, (B) kynurenine and (C) the kynurenine (μmol)/tryptophan (mmol) ratio of 309 healthy blood donors.

In this study, we analysed the IFN-γ +874(T/A) genotype of 309 healthy blood donors and correlated these to the levels of trp and kyn in their plasma. Our results show that the presence of the high producer T allele of IFN-γ +874 is associated with increased IDO activity (i.e. elevated kyn and kyn/trp levels), though this effect was observed only in females. These data show that trp catabolism is genetically controlled by the IFN-γ gene and may thus be operative in those disease conditions associated with the polymorphisms of the IFN-γ gene.

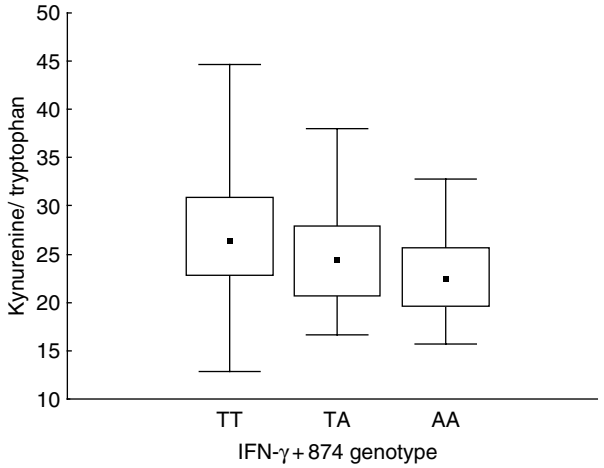

Figure 2 The association between the IFN-γ +874 genotype and the kynurenine (μmol)/tryptophan (mmol) ratio (median, 25–75% quartiles, min–max) in female blood donors.

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Indoleamine 2,3-dioxygenase enzyme activity correlates with risk factors for atherosclerosis: the Cardiovascular Risk in Young Finns Study

M. Pertovaara,*† A. Raitala,* M. Juonala,‡ T. Lehtimäki,§¶ H. Huhtala,** S. S. Oja,††¶ E. Jokinen,‡‡ J. S. A. Viikari,§§ O. T. Raitakari¶¶ and M. Hurme*¶

*Department of Microbiology and Immunology, University of Tampere, Medical School, Finland, †Department of Internal Medicine, Tampere University Hospital, Tampere, Finland, ‡The Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland, §Department of Clinical Chemistry, University of Tampere, Medical School, Tampere, Finland, ¶The Centre for Laboratory Medicine, Tampere University Hospital, Tampere, Finland, **Tampere School of Public Health, University of Tampere, Finland, ††Department of Physiology, University of Tampere, Medical School, Tampere, Finland, ‡‡Hospital of Children and Adolescents, University of Helsinki, Helsinki, Finland, §§Department of Medicine, University of Turku, Turku, Finland, and ¶¶Department of Clinical Physiology, University of Turku, Turku, Finland

Accepted for publication 14 December 2006
Correspondence: Marja Pertovaara MD, PhD, Department of Internal Medicine, Tampere University Hospital, PO Box 2000, FIN-33521 Tampere, Finland.
E-mail: marja.pertovaara@uta.fi

Summary

Indoleamine 2,3 dioxygenase (IDO), an enzyme involved in the catabolism of tryptophan, suppresses T cell activity and is up-regulated by various inflammatory stimuli. The ratio of kynurenine, the main metabolite of tryptophan, to tryptophan (kyn/trp) reflects IDO activity. We calculated IDO activity and measured carotid intima-media thickness (IMT), a presymptomatic predictor of atherosclerosis, in 986 young adults (544 female, 442 male) for whom data on levels of high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride, high sensitive C-reactive protein (CRP), body mass index (BMI), waist circumference, waist-to-hip ratio, systolic and diastolic blood pressure and smoking habits were available. IDO activity correlated significantly with IMT in female subjects, but not in males. In a multivariate linear regression model, IDO did not correlate independently with IMT in female subjects. However, IDO activity correlated significantly with several risk factors for atherosclerosis in females, i.e. with age, LDL-C, BMI, weakly with CRP and inversely with HDL-C and triglyceride. In males IDO activity correlated significantly with CRP and inversely with HDL-C. In conclusion, our results suggest that the IDO enzyme is involved in the immune regulation of early atherosclerosis, particularly in young female adults, and could constitute a novel marker of immune activation in early atherosclerosis in females.

Keywords: atherosclerosis, indoleamine 2,3-dioxygenase, inflammation

Introduction

The inflammatory nature of atherosclerosis is currently well recognized (for reviews, see [1–3]). The atherosclerotic plaque contains inflammatory cells such as macrophages and T lymphocytes, most of which are of the Th1 phenotype, i.e. proinflammatory cells. The plaque macrophages take up oxidized low-density lipoprotein (LDL) or are activated by microbial components or autoantigens (e.g. heat shock proteins), leading to production of proinflammatory cytokines and presentation of the antigenic material to the Th1 cells. Thereafter the macrophages differentiate gradually to lipid-loaded foam cells, i.e. activated macrophages. This chain of events resembles a normal immune response. The normal immune/inflammatory response induced by an antigen is followed by activation of several inhibitory mechanisms, which is also the case in the immune response associated with atherosclerosis. The immunoregulatory cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β1 have been associated with protection from atherosclerosis in mouse models [4,5] and in human disease [6].

Recently, indoleamine 2,3-dioxygenase (IDO) has drawn considerable attention as another mechanism of immune regulation [7–10]. IDO is an enzyme involved in the catabolism of the essential amino acid tryptophan, and the ratio of kynurenine, the main metabolite of tryptophan, to

tryptophan (kyn/trp) can be used to reflect IDO activity. IDO is expressed mainly in antigen-presenting cells (APCs), i.e. at the initiation of the immune response. Its activation leads to a decrease in the tryptophan concentration in local microenvironments, thus suppressing the activation of surrounding T lymphocytes ('suppression by starvation') [8].

T cells in atherosclerotic plaques are mainly of the Th1 subtype, secreting, e.g. the proinflammatory cytokine interferon (IFN)-γ [2]. IDO is up-regulated in response to various infectious and inflammatory stimuli and IFN-γ, a Th1 cytokine, is also a strong inducer of IDO. Our aim here was to test the hypothesis that IDO is involved in the regulation of inflammatory responses associated with the development of early atherosclerosis. To this end we investigated the activity of IDO, i.e. kyn/trp, and measured carotid intima-media thickness (IMT), a presymptomatic predictor of atherosclerosis, as well as traditional risk factors for atherosclerosis in 986 (544 female, 422 male) young adults.

Materials and methods

Subjects

The Cardiovascular Risk in Young Finns Study is a prospective multi-centre cohort study being conducted in five university hospital cities in Finland; it was initiated in 1980, with the latest control visits made and laboratory data gathered in 2001. The cohort in the current study comprised 986 participants (544 female, 442 male aged 24–39 years) from two of the centres, the cities of Helsinki and Turku and their rural surroundings.

The data on cardiovascular risk variables in the cohort in 2001 (levels of high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride, body mass index (BMI), waist circumference, waist-to-hip ratio, systolic and diastolic blood pressure, smoking habits) had been recorded by methods described previously [11]. Plasma high sensitive C-reactive protein (CRP) concentrations had been analysed by latex turbidometric immunoassay (Wako Chemicals GmbH). Carotid IMT measurements were performed in 2001, as described elsewhere [11].

Tryptophan and kynurenine determinations

Tryptophan (μmol/l) and kynurenine (μmol/l) concentrations in peripheral blood were measured by reverse-phase high-performance liquid chromatography (HPLC), as described previously [12]. Tryptophan was separated with a Shimadzu liquid chromatograph LC-10AD VP (Shimadzu Co, Kyoto, Japan) using a 50-mm BDS Hypersil C18 5 μm column (Thermo Electron Co, Bellefonte, PA, USA). It was monitored by fluorescence with a Shimadzu RF-10A XL detector at 266 nm excitation and 366 nm emission wavelengths. Kynurenine was separated with a Hewlett Packard 1100 liquid chromatograph (Palo Alto, CA, USA) using Merck LiChroCart 55–4150 mm cartridge containing a Purospher STAR RP-18 3 μm column (Merck Co, Darmstadt, Germany). It was determined by ultraviolet absorption at 360 nm wavelength with a Hewlett Packard G13144 detector. Kyn/trp (μmol/mmol) was calculated by relating concentrations of kyn (μmol/l) to trp (mmol/l), this allowing an estimate of IDO activity.

Statistical analysis

Values for CRP and triglycerides were transformed logarithmically prior to statistical analyses due to their skewed distributions. Smoking was regarded as a dichotomous variable (current and ex-smokers *versus* never-smokers). Comparisons of continuous and dichotomous variables were performed by Student's *t*-test and χ^2 test, respectively. Correlation was calculated with the Pearson correlation coefficient. Multivariate stepwise linear regression analysis was performed to analyse the independent effect of kyn/trp on IMT. Findings were considered statistically significant at $P < 0{\cdot}05$. Statistical analyses were performed with SPSS version 13·0 for Windows.

Ethical considerations

The study was approved by the local ethics committees and all subjects participating in the study gave written informed consent.

Results

The data on various risk factors for atherosclerosis in female and male subjects, as well as tryptophan and kynurenine concentrations and kyn/trp, are presented in Table 1.

The mean (± s.d.) kyn/trp was 27·4 ± 7·2 μmol/mmol in all subjects, 27·0 ± 7·2 μmol/mmol in females and 27·9 ± 7·3 μmol/mmol in males. Kyn/trp correlated weakly with mean IMT in all subjects ($r = 0{\cdot}091$, Pearson's correlation coefficient, $P = 0{\cdot}004$). In females the correlation was more pronounced ($r = 0{\cdot}131$, $P = 0{\cdot}002$), while in men kyn/trp did not correlate with IMT ($r = 0{\cdot}040$, $P = 0{\cdot}402$). The correlation coefficients between kyn/trp and the risk variables for atherosclerosis in female and male subjects are shown in Tables 2 and 3, respectively.

In addition to IDO activity, age, BMI and systolic blood pressure proved significant predictors of IMT in univariate analysis in females (Table 4). To assess the independent effect of kyn/trp on IMT in females, a multivariate linear regression model was constructed with IMT as the dependent variable and the traditional cardiovascular risk factors (age, HDL-C and LDL-C, logarithmically transformed concentration of triglyceride, BMI, systolic and diastolic blood pressure, smoking) and the logarithmically transformed

Table 1. Risk factors for atherosclerosis, tryptophan (trp) and kynurenine (kyn) concentrations and kyn/trp ratio in 986 young (24–39 years) adults.

Variable	Females, $n = 544$	Males, $n = 442$
Age, years	31·5 ± 4·9	31·9 ± 5·0
HDL, mmol/l	1·40 ± 0·30	1·13 ± 0·28*
LDL, mmol/l	3·11 ± 0·76	3·45 ± 0·91*
Triglyceride, mmol/l	1·15 ± 0·58	1·55 ± 1·09*
BMI, kg/m^2	24·0 ± 4·3, $n = 525$	25·7 ± 3·9*, $n = 433$
Waist circumference, cm	78·0 ± 11·1, $n = 531$	89·3 ± 10·8*, $n = 441$
Waist-to-hip ratio	0·78 ± 0·06, $n = 529$	0·89 ± 0·06*, $n = 441$
Systolic blood pressure, mmHg	115 ± 13, $n = 541$	129 ± 13*, $n = 438$
Diastolic blood pressure, mmHg	71 ± 9, $n = 541$	75 ± 9*, $n = 438$
CRP, mg/l	2·43 ± 5·11	1·61 ± 0·60**
IMT, mm	0·57 ± 0·08, $n = 541$	0·60 ± 0·11*, $n = 439$
Serum tryptophan, μmol/l	84·8 ± 13·7	93·3 ± 14·2*
Serum kynurenine, μmol/l	2·25 ± 0·56	2·56 ± 0·59*
Kyn/trp, μmol/mmol	27·0 ± 7·2	27·9 ± 7·3
Current or ex-smokers (%)	49·1	62·2*

trp: Tryptophan; kyn: kynurenine; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; BMI: body mass index; CRP: C-reactive protein; IMT: common carotid artery intima-media thickness. Values are mean ± s.d. unless indicated otherwise. $^*P < 0{\cdot}0001$, $^{**}P = 0{\cdot}004$, Student's *t*-test or χ^2 test.

concentration of CRP (logCRP) and kyn/trp as independent variables (Table 4). Kyn/trp was not an independent predictor for IMT in this multivariate model; the variables which remained associated significantly with IMT in female subjects were age and BMI.

Discussion

There is previous evidence from a small series ($n = 35$) of subjects with angiographically verified coronary heart disease that IDO activity is increased in patients with clinically evident coronary heart disease compared with healthy controls [13]. Increased carotid artery IMT measured by ultrasound is a surrogate marker for early atherosclerosis which predicts cardiovascular events in population groups [14]. We aimed here to test the hypothesis that IDO is involved in the immune regulation of early atherosclerosis in a large population-based sample of healthy young adults for whom both IMT measurements and detailed data on various cardiovascular risk factors were available.

Table 2. Correlations of risk factors for atherosclerosis with kyn/trp (μmol/mmol) in 544 young (24–39 years) female adults.

Variable	Correlation (*r*) with kyn/trp	*P*-value
Age	0·129	0·003
HDL	–0·090	0·037
LDL	0·111	0·010
logTriglyceride	–0·129	0·003
BMI, $n = 525$	0·219	< 0·0001
Waist circumference, $n = 531$	0·234	< 0·0001
Waist-to-hip ratio, $n = 529$	0·166	< 0·0001
Systolic blood pressure, $n = 541$	0·006	0·898
Diastolic blood pressure, $n = 541$	0·038	0·380
logCRP	0·085	0·048
IMT, $n = 541$	0·131	0·002

kyn: Kynurenine; trp: tryptophan; HD: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; logTriglyceride: logarithmically transformed concentration of serum triglyceride; BMI: body mass index; logCRP: logarithmically transformed concentration of C-reactive protein; IMT: common carotid artery intima-media thickness.

Table 3. Correlations of risk factors for atherosclerosis with kyn/trp (μmol/mmol) in 442 young (24–39 years) male adults.

Variable	Correlation (*r*) with kyn/trp	*P*-value
Age	0·066	0·163
HDL	–0·155	0·001
LDL	–0·013	0·791
logTriglyceride	0·017	0·718
BMI, $n = 433$	0·094	0·050
Waist circumference, $n = 441$	0·110	0·020
Waist-to-hip ratio, $n = 441$	0·103	0·030
Systolic blood pressure, $n = 438$	–0·086	0·072
Diastolic blood pressure, $n = 438$	–0·064	0·178
logCRP	0·234	< 0·0001
IMT, $n = 439$	0·040	0·402

kyn: Kynurenine; trp: tryptophan; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; logTriglyceride: logarithmically transformed concentration of serum triglyceride; BMI: body mass index; logCRP: logarithmically transformed concentration of C-reactive protein; IMT: common carotid artery intima-media thickness.

Table 4. Univariate correlations between risk factors for atherosclerosis and multivariate stepwise linear regression model of the relationships between the risk factors for atherosclerosis and IMT (mm) in 541 young (24–39 years) female adults.

	Univariate analysis		Multivariate analysis	
	Correlation (r) for IMT		B ± s.e.	
Risk variable	($n = 541$)	P-value	($n = 521$)	P-value
Age	0·278	< 0·001	0·004 ± 0·001	< 0·001
Kyn/trp	0·131	0·002		
HDL	–0·035	0·422		
LDL	0·080	0·063		
logTriglyceride	–0·062	0·148		
BMI, $n = 522$	0·147	0·001	0·002 ± 0·001	0·003
Systolic blood pressure	0·101	0·019		
Diastolic blood pressure	0·072	0·095		
logCRP	0·013	0·764		

IMT: common carotid artery intima-media thickness; kyn: kynurenine; trp: tryptophan; HD: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; logTriglycerid: logarithmically transformed concentration of triglyceride; BMI: body mass index; logCRP: logarithmically transformed concentration of C-reactive protein. Variables in the stepwise linear regression model: age, kyn/trp, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, logarithmically transformed concentration of triglyceride, body mass index, systolic blood pressure, diastolic blood pressure, logarithmically transformed concentration of C-reactive protein, smoking habits (current smoker or ex-smoker/never smoker).

Our main finding was that IDO activity correlated with IMT and several other risk factors for atherosclerosis in female subjects in univariate analysis, suggesting that IDO is involved in the regulation of the inflammatory response of early atherosclerosis. However, IDO activity did not have an independent effect on IMT in females when analysed in a multivariate model simultaneously with traditional risk factors for atherosclerosis and with CRP.

In addition to a significant correlation with IMT in females in univariate analysis, IDO activity correlated significantly with several risk factors for atherosclerosis. Similarly, in clinical epidemiological studies the marker of inflammation, CRP, has also been found to correlate with several conventional cardiovascular risk factors in women, including age, BMI, blood pressure and cigarette smoking [15] and to predict the occurrence of cardiovascular events, also among women [16]. However, there was only a weak correlation between IDO and CRP in females in the present study, implying that these markers of inflammation act differentially in the process of early atherosclerosis in females; neither IDO nor CRP correlated independently with IMT in a multivariate model in our cohort of young female adults. However, on the basis of the univariate analysis the IDO enzyme was a more potent predictor of carotid IMT in female subjects than CRP. In contrast, a clear correlation emerged between IDO and CRP in men, implying that these two separate markers of inflammation also act in a different way in females and males.

In addition to IDO, the degradation of tryptophan to kynurenine is also regulated by the hepatic enzyme tryptophan 2,3-dioxygenase (TDO) [9]. The possible role of TDO in the enhanced tryptophan degradation in the preclinical stages of atherosclerosis cannot be excluded, although IDO seems a more probable activator, as TDO regulates basal serum tryptophan concentrations and IDO is up-regulated in response to inflammatory conditions, which early atherosclerosis represents [9].

The positive correlation between IDO activity and age was to be expected, as IDO activity is known on the basis of previous studies to increase with age [17,18]. There was also a significant correlation between IDO and BMI, waist circumference and waist-to-hip ratio in both sexes. In parallel with this finding, in another recent study tryptophan depletion and thereby increased IDO activity was observed in morbidly obese patients, persisting even after weight reduction and leading to chronic immune activation [19].

In general, we do not know whether increased IDO activity is deleterious or advantageous in the process of development of early atherosclerosis. The first alternative is that increased IDO activity is purely a consequence of inflammation, i.e. merely a non-specific reactive marker of inflammation. However, if IDO activity is an attempt of the body to down-regulate the immune response induced by, e.g. ox-LDL or some other antigen, IDO would have a protective role against atherosclerosis. A specific type of T cells, i.e. regulatory T cells (T_{reg}), has been suggested recently to counteract disease initiation and progression in atherosclerosis [20]. IDO-expressing dendritic cells (DC) have in turn been postulated to generate T_{reg} [9]. If increased IDO activity induced T_{reg} cell activity in early atherosclerosis, increased IDO activity would constitute a favourable response.

However, as IFN-γ is one of the main inducers of IDO and as there is substantial evidence speaking for its importance in atherosclerosis (reviewed in [2]) it is also possible that IDO plays a pro-atherogenic role. At present, the nature of the role of IFN–γ/IDO interaction in atherosclerosis can only be speculated, as IDO expression can also be induced by an IFN-γ independent mechanism [21], and IFN-γ itself has both pro- and anti-atherogenic properties (reviewed in [22]).

In support of the alternative that IDO would protect from atherosclerosis, IFN-γ, a strong inducer of IDO, has been shown to inhibit oxidation of LDL-C in human mononuclear cells [23]. Here IDO activity correlated significantly with LDL-C in females, and it is possible that increased IDO activity is directed to down-regulate the immune response. Because the correlation between IDO activity and LDL-C was observed only in females, it would seem that this mechanism does not operate in the regulation of the inflammatory reaction of early atherosclerosis in males, but only in females. The inverse correlation between IDO activity and the anti-atherogenic HDL-C is in line with the finding of a direct correlation between LDL-C and IDO. However, the inverse correlation of IDO with triglyceride remains without explanation, particularly as there was a positive correlation between IDO and different indices of weight.

The fact that IDO was not a predictor of atherosclerosis in male subjects, as judged by the lack of correlation between IDO and IMT, suggests that hormonal factors might influence the effects of IDO. Oestrogen has indeed been found to up-regulate one type of APC, i.e. DC, to express IDO, which can limit T cell responses [24]. Moreover, IDO has been found to be elevated in autoimmune diseases with a female preponderance, for example systemic lupus erythematosus [25] and Sjögren's syndrome [26]. On the other hand, either early atherosclerosis or precociously increased IMT has been found in both these autoimmune diseases [27,28].

To conclude, our results suggest that the IDO enzyme is involved in the immune regulation of early atherosclerosis in young female adults. On the basis of our results we cannot definitely exclude the possibility that IDO is just another non-specific marker of inflammation, but we presume that IDO functions to down-regulate the inflammatory response, i.e. that its action is targeted to limit the inflammatory response. However, it is as such also a novel marker of immune activation in early atherosclerosis in females.

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