Preface:

One of the most exciting moments in my research years, which changed my career path, was on July 5th, 1996. That day I found, to my amazement, that the p40 subunit of the heterodimeric cytokine IL-12 can be mixed with the other subunit called p35, that was released from necrotic cells, extracellularly and generate all the biological activities that had been assigned to the intact heterodimeric IL-12. This was truly a "Eureka" moment for me, a rare and exhilarating experience. This finding was contrary to what was known about how this cytokine was supposed to work, because originally it had been suggested, that to generate biologically active IL-12p75 both subunits had to be expressed within the same cell (Trinchieri 1995).

This observation and a few others that came along during validation of this finding (from an *in vivo* model of sepsis in which I measured p40 and IL-12p75 levels in response to LPS) made me think that perhaps we were looking at the wrong protein (IL-12p75). I noticed that throughout the literature investigators measure the p40 subunit but draw conclusions as if they were actually detecting the IL-12p75 heterodimer. In addition, to induce the IL-12p75 heterodimer production from DCs or macrophages, it was a common practice by many investigators to pre-incubate DCs with IFN-γ, or with IFN-γ and simultaneously other stimuli. I found such experimentation to be misleading because, under normal conditions, cells that are exposed to a pathogen during the early stages of innate immunity are not simultaneously exposed to IFN-γ. Thus, deductions from various experiments had suggested that IL-12p75 is

the protein secreted early in direct response to pathogens proved to be incorrect. This mistake (measuring p40 and calling it IL-12) has been made since the discovery of IL-12 in the early 1990's, and over ~80% of references to IL-12 in PubMed refer only to the p40. The IL-12p75 is composed of two proteins, p35 and p40, linked by a disulfide bond and in order to get biologically active IL-12 both subunits normally need to be expressed within the same cell. However, one cannot measure only one subunit and draw accurate conclusions about the secretion of the whole molecule (heterodimer).

The above observations coupled with the *in situ* data by Bette et al., published in 1994- demonstrating that after an LPS challenge, p35 and p40 were expressed in different anatomical sites in the spleen led me to this question: If the two subunits were expressed in two different anatomical sites, how would they ever form the IL-12p75 heterodimer? Consequently, I started thinking differently about IL-12 and its role during the early host response to pathogens. My own observations in conjunction with data from the literature led to the deduction that each subunit is independently regulated, and that p40 can be secreted in direct response to pathogens independent of IL-12p75. This formed the bases for a hypothesis that I spent the next five years refining and validating until finally it was published in 2002 (Chapter-I). These observations led to a quest to understand the underlying biological significance of secreted p40 in various inflammatory conditions and its role during innate immune responses.

The aim of this thesis is to show specifically that it is p40 and not p75 that is rapidly secreted in response to bacteria/bacterial products from antigen-presenting cells. Furthermore, the p40 secretion is an independent event that does not involve the IL-12p75 heterodimer. In contrast, secretion of IL-12p75 from antigen presenting cells is tightly controlled and requires help from antigen-activated T cells. Thus, each chapter specifically addresses a question regarding p40 and p75 and how the secretions of these proteins are controlled.

Although Chapter-I was published in 2002, there has not yet (2010) been a major shift in the old thinking about the role of IL-12p75 during the early host response to pathogens. Chapter-I is the theoretical basis of this thesis with detailed analysis and discussion about the current paradigm. It challenges the original view that IL-12p75 is a bridge between the innate and adaptive immunity and that it is secreted by antigen presenting cells in direct response to LPS stimulation, a notion that is so well-established, it is in the immunology text-books (Cellular and molecular immunology, Abbas, Lichtman, Pober).

Chapter-II demonstrates the role of endogenous IL-12p75 in the generation of CTL and shows that recombinant p40 homodimer inhibits endogenous IL-12. The *in vivo* relevance of these observations regarding the p40 monomer and homodimer are not clear yet. Chapter-III is a thorough study that utilizes a novel ELISPOT assay to measure IL-12p75 at the single cell level. This study demonstrates that LPS alone cannot induce IL-12p75 secretion from dendritic cells (DCs) and further supports the notion that IFN-γ

is an important contributor to IL-12p75 production. Without IFN-γ, LPS alone is not sufficient to induce IL-12p75 secretion from DCs. Furthermore, only antigen-activated T cells and not naïve T cells are capable of inducing IL-12p75 secretion from DCs. This chapter makes the important conclusion that bacteria/bacterial products alone cannot induce IL-12p75. This work challenges the current paradigm, in which argues that IL-12p75 is the instigator of T_H1 responses. Chapter-III further supports the view that p40 might be an integral component of early inflammatory responses.

Having shown in Chapter-III that IFN-γ is a critical factor in LPS induced IL-12p75 production, Chapter-IV shows that secretion of IL-12p75 from DCs in the presence of antigen-activated $CD4^+$ T cells is actually IFN-γ-independent, thus resolving one of the outstanding issues that exists concerning modulation of the IL-12-IFN-γ axis during the formation of immunological synapses between T cells and antigen-bearing DCs. The question is, which comes first, IL-12 or IFN-γ? Data from this chapter suggests that in the presence of antigen-activated that are IFN-γ deficient but not naïve T cells, IL-12p75 can be secreted from LPS-activated DCs. This chapter demonstrates that during cognate interactions between antigen-activated T cells and antigen-bearing DCs, initial secretion of IL-12p75 is independent of IFN-γ from T cells.

Finally, Chapter-V challenges the current thinking that emphasizes that exposure to LPS renders DCs "exhausted" such that, if they are re-stimulated again with CD40L, they cannot produce IL-12p75 and instead skew T cell responses from a T_H1 to a T_H2 phenotype (Langenkam et. al. 2000; Reiner et al. 2007). This

chapter demonstrates that LPS-activated DCs are perfectly capable of secreting IL-12p75 if they are restimulated with antigen-activated T cells (or certain combinations of T-cell products) but not with LPS or LPS plus IFN-γ. The deduction from this chapter is that DCs have absolutely no problem in secreting IL-12p75 when they receive appropriate signals from antigen-activated (but not naïve) T cells.

Introduction:

Some 3.5 billion years ago, when life emerged on earth, so did death and injury. As life evolved and became more complex, leading to the emergence of the multicellular organisms, those new organisms had to deal with injuries and find a way to repair themselves. They also had to deal with rapidly growing bacteria. It seems that the battle between cells and bacteria led to the process of eating and drinking (phagocytosis and pinocytosis). Swallowing the obnoxious bacteria by cells was a simple way of getting rid of them. This fundamental process exists throughout the animal kingdom; however, in multicellular organisms, the process of cleanup and defense against bacteria was taken over by specialized cells commonly known as professional phagocytes. The combination of phagocytic cells, which are either fixed or extravasated from the vessels into the tissue and mixed with fluid phase factors that simmer away in tissue, is commonly known as an inflammatory response, a process typical of vascularized tissues, whereby fluid and white blood cells accumulate at the site of injury. Inflammation can be a result of chemical agents, mechanical trauma, or detrimental colonization of a host organism by a foreign species (bacteria, fungi etc.), which is commonly referred to as an infection. The inflammatory response is the essence of the host defense mechanism. The word inflammation was used in the Smith Papyrus, a scroll written in Egypt millennium ago. However, the Roman writer of the first century A.D., Cornelius Celsus who provided a clinical definition of inflammation, a definition that is still used today which is known as four cardinal signs: Rubor et Tumor cum Calore et

Dolore ["redness and swelling with heat and pain"] Nevertheless, the first person who actually described the phenomena of inflammation using the mesentery and the tongue of the frog was Julius Cohnheim (1839-1884); he explained that vasodilation of vessels account for rubor, the increased blood for calore, exudation of the fluid for tumor and pain for dolore. Moreover, when Cohnheim was looking at the vessels, he made a seminal observation; he noticed that white cells began to line the wall of venules and stuck there, while RBCs passed them by. He also noticed that some of the attached white blood cells crept across the wall of the vessel and ended up in the extravascular spaces; this process is now called diapedesis. At the time he could not explain why this occurred, and there was no explanation until the remarkable discovery by Iya Ilyich Mechnikov (1845-1916) who received the Nobel Prize in 1908 for his observations using star-fish-larvae to show that cells were capable of engulfing foreign matter, including bacteria. He concluded that the purpose of inflammation was to bring phagocytes to the site of injury in order to engulf invading bacteria or any other foreign matter. This discovery led to the establishment of the field currently known in academia as cellular immunology.

Despite the tremendous progress that has been made in the field of medicine and immunology in the past century, we still do not have a clear understanding of inflammatory processes. It is still debated that how the immune responses are initiated. One school of thought argues that the host defense is activated upon recognition of endogenous or exogenous "danger" signals (Matzinger, 1994) and another view argues that the immune system discriminates between

self and non-self via conserved pattern recognition receptors that are fixed into the host genome to detect pathogen associated molecular patterns (PAMPs) associated with pathogens via the host's pattern recognition receptor (PRR) (Janeway, 1989).

During the innate phases of the immune response, various cells, including neutrophils, mast cells, macrophages, and dendritic cells (DCs) play an important role in the orchestration of inflammatory responses within the tissue to contain the pathogens before the recruitment of more elaborate arms of the immune system.

It is thought that macrophages and DCs are the primary antigen-presenting cells at the front line of the host defense confronting almost any invading organism/antigen or stimulus within the tissue before recruitment of neutrophiles into the inflammatory site. These cells capture antigens and have an ability to process and present the antigenic peptide in the context of the major histocompatability molecules to the T cells. Upon activation, these cells have the ability to make various soluble factors that contributes to the inflammatory responses.

The functions of DCs and macrophages are unique: while macrophages have evolved to ingest bacteria through phagocytosis, DCs have evolved to perform a different mission. DCs are recognized as the professional antigen-presenting cells that play an essential role in the initiation of host primary T cells responses (Banchereau and Steinman, 1998). DCs are mobile immunological sentinels embedded in various tissues where they have the ability to capture antigens and migrate into the secondary lymphoid organs. Once

activated, DCs express costimulatory molecules, secreted chemical signals such as cytokines and chemokines that diffuse into the surrounding tissue; this will further activate and recruit various cells into the inflammatory site during the early host response to pathogens. In the secondary lymphoid organs, DCs present the processed antigenic peptide (signal 1) to naïve T cells in the context of major histocompatibility complex in combination with costimulatory molecules (signal 2). This will prime the naïve T cells.

The current thinking is that the earliest host immune response to microbial pathogens is mediated through recognition of pathogens or their products (such as Lipopolysaccharide (LPS), Lipoteichoic acid, peptidoglycan, or bacterial CpG) via Toll-Like receptors (TLRs) leading to the secretion of soluble mediators. Among various soluble mediators, IL-12p75 (also known as IL-12p70) is a heterodimeric cytokine composed of two unrelated proteins--namely p35 and p40-- that can be secreted from DCs. The 40 kDa subunit is formed exclusively in DCs, Macrophages, and monocytes. In contrast, the expression of p35 mRNA has shown to be constitutive and ubiquitous (Wolf et al., 1991). It is widely believed that, during the innate phases of the immune response, when DCs encounter pathogenic microbes or their products they recognize these pathogens via TLRs. This recognition will lead to the activation and secretion of the heterodimeric cytokine IL-12p75 (Medzhitov, 2001). The heterodimeric cytokine, IL-12p75, is thought to be one of the initial early signals secreted by DCs that represents a functional bridge between the innate and adaptive immune systems (Trinchieri, 1995). These interpretations led to the

original paradigm that early secretion of IL-12p75 acts on natural killer cells (NK) leading to the secretion of IFN-γ which in turn acts on naïve T cells to skew adaptive immunity toward a T_H1 phenotype (Trinchieri, 1995). The premise is that IL-12p75 and the nature of antigens likely operate as a crucial checkpoint to determine whether a T_H1 immune response should result. When, where and how the host made such decisions to recruit and mount a more elaborate defense is not clear.

I disagree with this paradigm. Therefore, the studies in this thesis argue a different view. This thesis propose that the initial signal(s) (intrinsic "danger" or extrinsic pathogen-associated molecular patterns) that activate the immune system during the innate phase of the immune response will not lead to the secretion of IL-12p75 from DCs, but rather results in the secretion of large quantities of the p40 subunit. Secretion of IL-12p75 is tightly controlled and its induction is highly specific and requires antigen-activated T cells. Consequently, in this model IL-12p75 would be involved in the maintenance of an ongoing T_H1 response, rather than being a part of the immunological decision-making process. This model further proposes that it is p40 and not IL-12p75 which is secreted early in response to "danger" and/or PAMPS during the inflammatory phase of the immune response (Chapter-I). First let us start with a description of the cytokine and its biology.

The IL-12p75 Molecule:

Biologically active IL-12 is a heterodimeric glycoprotein with a molecular weight of approximately 75-kDa and it is composed of a 35-kDa protein and a 40-kDa protein covalently linked together through a disulfide bond. IL-12p75 was originally identified as a factor produced by the human EBV-transformed B cell line RPMI 8866, ADP, NC37 and it was named by one group "natural killer stimulatory factor" (NKSF) (Kobayashi et al., 1989) and by another group "cytotoxic lymphocyte maturation factor" (CLMF) (Stern et al., 1990). It was revealed that both NKSF and CLMF were identical; therefore it was named IL-12 (Gubler et al., 1991).

The p40 gene encodes a 328-amino acid polypeptide with a 22-amino acid hydrophobic signal sequence. p40 contains ten cysteine residues and four possible N-linked glycosylation sites, and one consensus heparin-binding site (Gubler et al., 1991; Wolf et al., 1991). p40 is primarily regulated at the transcriptional level directly by bacteria or bacterial products via various Toll-like receptor ligands such as LPS, CpG etc (D'Andrea et al., 1992; Hsieh et al., 1993). The p40 subunit is closely homologous to the extracellular domain of the hemopoietic cytokine receptor family containing ciliary neurotropic factor receptor a chain (CNTF-Rα), IL-6 receptor and G-CSF receptor (Merberg et al., 1992)

p40 displays many structural features associated with the hemopoietin receptor family. It has one tryptophan and four cysteine residues in the conserved sequence WSEWAS, similar to the consensus WSXWS motif in this receptor family (Taga and Kishimoto, 1992). Although, p40 is analogous to hemopoietin

receptors, it is readily secreted from antigen-presenting cells when TLRs are engaged. This important feature of p40 (being analogous to hemopoietin receptor) and its rapid secretion during the early host response to microbial pathogens or during the inflammatory response is an essential component for the hypothesis behind this thesis. Due to p40's unique structural features that resembles a cytokine receptor, it is proposed here that p40 is the molecule secreted early during host-pathogen interactions within the early inflammatory phase of the immune response and not the IL-12p75, in which p40 is instrumental in the biochemical translation of "danger" into host protection and survival (Hypothesis, Chapter-I).

This early response will contain the pathogen before the recruitment of more elaborate arms of the immune system. In contrast, p35 subunit is closely related to IL-6 and G-CSF with an a-helix-rich structure that is similar to many other cytokines (Merberg et al., 1992). RT-PCR analysis has revealed that p35 mRNA is expressed constitutively at low abundance in a large array of cells from a variety of lineages (D'Andrea et al., 1992; Wolf et al., 1991)

It has been shown that when p35 is expressed in the CHO cell line, it is inefficiently secreted in the absence of co-expression with p40 (Cassatella et al., 1995; D'Andrea et al., 1992; Wolf et al., 1991). The p35 gene encodes a 219-amino acid polypeptide with seven cysteine residues and three possible *N*-glycosylation sites. The mouse p35 gene consists of seven coding and one non-coding exons. Multiple transcriptional sites have been identified in p35 mRNA from B cell lymphoma line and bone marrow-derived DCs (Babik et al., 1999). Human p35 mRNA encodes two in-frame methionines at

its 5' end resulting in a potential additional 34-amino acid fragment at the *N*-terminus of the p35 signal peptide (Tone et al., 1996; Wolf et al., 1991), which is only observed (by amino acids sequence homology comparison) in human and non-human primates but not in other species (Abdi, K unpublished observations). Moreover, these two in-frame methionines at the 5' end of p35 can be detected in EBV-transformed B cells but not in human monocytes, which lack the 34 amino acid fragment (Hayes et al., 1998). However, the significant of this alternative initiation site and what is the role of first 34 amino acids is not clear.

p35 expression is mostly regulated post translationally and requires extensive post-translational modification by *N*-linked adducts and sialic acid during biosynthesis for its secretion in association with p40 to form the heterodimeric IL-12p75 (Aste-Amezaga et al., 1998; Carra et al., 2000; Ma et al., 1996). p40 biosynthesis requires only minor post-translational modifications. Pulse-chase experiments indicated that IL-12p75 persists intracellularly for a long period as an immature heterodimeric protein. Glycosylation is the key regulatory step that determines IL-12p75 secretion, therefore, this represent the marker that distinguishes the secreted mature versus immature intracellulare heterodimer (Carra et al., 2000). Consequently, expression and secretion of p35 is highly regulated and unlike p40 subunit, it is controlled transcriptionally as well as translationally. This is an important issue to consider when such measurements as intracellular staining are employed using flow cytometry techniques that are commonly used to measure various cytokines. In this

situation, intracellular detection of immature protein does not necessarily translate into its secretion. Moreover, when p35 is expressed without p40, it is not secreted. In contrast, p40 can be easily secreted without p35 co-expression and post-translational modifications (glycosylation) do not play a major role for its secretion. This points to an important fact: that p40, p35, and p75 have unique kinetics and they can be independently regulated.

IL-12p75 Genes:

The gene for human p40 is located on chromosome 5q31-q33, a region encoding several cytokines and cytokines receptors and in mice on the syntenic region of chromosomes 11 (Tone et al., 1996; Yoshimoto et al., 1996). The gene encoding the p35 chain is on human chromosome 3p12-3q13.2 (Sieburth et al., 1992) and in mouse using fluorescent *in situ* hybridization it was mapped to chromosome 6C (Yoshimoto et al., 1996) and in another study using interspecific backcross analysis on chromosome 3 (Tone et al., 1996). There are no homologies between the p35 and p40 sequences. The genes encoding the two subunits of IL-12p75 are separate and unrelated (Gubler et al., 1991; Wolf et al., 1991). However, the gene encoding the p35 subunit has some homology with other single-chain (a-helix-rich) cytokines (Merberg et al., 1992). The gene encoding p40 subunit has no homology to any other cytokines, but is homologous to the extracellular domain of the hemopoietic cytokine receptor family (Gearing and Cosman, 1991; Schoenhaut et al., 1992). p40 gene regulation is mostly at the transcriptional level in which IFN-γ has an enhancing effect on the rate of p40 gene transcription (Ma et al., 1996).

The IL-12p75 has been known to be a major inducer of IFN-γ production from NK and T cells (Chan et al., 1991). However, IFN-γ has an enhancing effect on IL-12p75 secretion when macrophages or DCs are simultaneously stimulated with IFN-γ plus LPS, but it is more effective when these cells are pre-incubated with IFN-γ for several hours before stimulation with LPS (Cassatella et al., 1995; Ma et al., 1996). The expression of the p35 mRNA is the limiting step in IL-12p75 production and IFN-γ strongly up-regulates the LPS-induced p35 mRNA expression, whereas the p40 mRNA was not affected (Snijders et al., 1996).

Following stimulation with LPS, macrophages or macrophage lines will transcribe p40, a process that involves nucleosome remodeling. It has been shown in transfection assays, that the p40 promoter was sufficient for transcriptional induction (Ma et al., 1996; Murphy et al., 1995; Plevy et al., 1997). Upon macrophage activation, nucleosome 1 is rapidly and selectively remodeled in a protein-synthesis-dependent manner (Weinmann et al., 1999). This nucleosome 1 spanning DNA elements are important for transcriptional induction of p40 promoter region. Suggesting that a remodeling complex is selectively targeted to this nucleosome during gene induction (Weinmann et al., 2001). It has been demonstrated that enhancing effects of IFN-γ on p40 induction does not influence nucleosome 1 remodeling, but rather is an event down-stream or independent of remodeling (Weinmann et al., 1999), supporting the notion that secretion of p40 can take place in the absence of IFN-γ. In contrast, secretion of IL-12p75 from DCs

stimulated with LPS requires IFN-γ and its presence is absolutely necessary (Chapter-III).

Recently, it was suggested that TLR-dependent nucleosome 1 remodeling at the p40 promoter takes place in the absence of *c*-Rel and possibly independent of C/EBP and other proteins that are required for activity of a stably integrated promoter (Weinmann et al., 2001). This study points to the direct effects of pathogens on transcription of the p40 gene and further supports the notion that IFN-γ has augmenting rather than initiating effects on p40 transcription. In contrast, induction of p35 requires the presence of IFN-γ, thus, arguing against the common view that IL-12p75 can be induced and secreted in direct response to TLR agonists during the early host response to pathogens in the absence of IFN-γ. This will place p40 and not the IL-12p75 as an early molecule secreted in direct response to engagement of TLRs by their ligands.

The IL-12p75 Receptors:

The biological effects of IL-12p75 on its target cells are mediated through an IL-12R complex, which consists of at least two receptors: IL-12Rβ1 and IL-12Rβ2 (Chua et al., 1994; Chua et al., 1995; Desai et al., 1992; Presky et al., 1996; Wu et al., 1997). Affinity cross-linking of surface bound ^{125}I-labeled-huIL-12 to activated human lymphoblasts identified a major complex of approximately 210-280kDa (Chizzonite et al., 1992). It is proposed that a functional IL-12 receptor is a heterodimer composed of at least one IL-12Rβ1 (100kDa) and one IL-12Rβ2 (130kDa) (Chua et al., 1994; Chua et al., 1995; Desai et al., 1992; Presky et al., 1996). Both receptors belong to the gp130 cytokine receptor superfamily,

which consists of type-I transmembrane glycoproteins with a strong homology to gp130 (a common receptor subunit for IL-6, IL-11, Leukemia-inhibitory factor, Oncostatin M, and ciliary neurotrophic factor), with a WSXWS motif in their extracellular domain (Chua et al., 1994; Chua et al., 1995). IL-12p75 was found to induce the tyrosine phosphorylation of JAK2, TYK2, STAT3, and it is the only cytokine in mice known to activate STAT4 upon binding to its receptor (Bacon et al., 1995; Schindler, 1995; Sinigaglia et al., 1999). The IL-12Rβ2 is likely to act as docking site for the STAT4 SH2 domain; in contrast to IL-12Rβ1, the IL-12Rβ2 cytoplasmic domain contains three tyrosine residues (Presky et al., 1996). Activated T and NK cells that respond to IL-12p75 express IL-12Rβ2, which is the signaling component of IL-12 receptor (Chizzonite et al., 1992; Desai et al., 1992). Moreover, after being cultured for three days, PHA activated T cells express low, medium and high affinity IL-12R receptors. The recombinant IL-12p75 binds with three affinities: K_d= 5-20pM; 50-200pM; 2-6nM (Chizzonite et al., 1992). The expression of the recombinant form of these two IL-12 receptors in COS cells has demonstrated that dimerization/oligomerization is dependent on ligand binding (Presky et al., 1996). Individual expression of each IL-12R subunit in COS cells resulted in IL-12 binding with low affinity (2-6nM) and expression of both subunits in the COS cells generates the intermediate affinity. The highest affinity of binding (5-20pM) has not been reconstituted in COS transfection experiments using both of the IL-12 receptor subunits (Chua et al., 1994; Presky et al., 1996). This suggests that an additional component of the IL-12 receptor might be required for

the high affinity binding that has not been reconstituted in the COS system. In fact, an additional surface protein of 85kDa has been identified that can associate with IL-12Rβ1 in human PHA-activated T cells upon stimulation with IL-12 (Kawashima et al., 1998). It has been demonstrated that this 85kDa protein can be rapidly phosphorylated on tyrosine residues (Kawashima et al., 1998). These data are consistent with the original observation that detected the presence of an 85kDa subunit during immunoprecipitations of IL-12 receptors (Chizzonite et al., 1992). The nature of this protein is unknown.

This finding implies that IL-12Rβ1 might be able to associate with yet unknown surface protein(s) and possibly have a functional signaling ability independent of IL-12Rβ2. IL-12p75 appears to interact with IL-2Rβ1 via domains on the p40 subunit and with IL-12Rβ2 via a heterodimer-specific regions of IL-12p75 to which both the p40 and p35 subunits may contribute (Presky et al., 1998). *In vitro* studies using recombinant p40 homodimer indicate that p40 interacts primarily with IL-12Rβ1. This was demonstrated using Ba/F3 cells expressing mouse IL-12Rβ1 alone, since mouse p40 homodimer bound to these cells with both high (Kd about 5pM) and low (Kd about 15nM) affinities (Wang et al., 1999). Moreover, mouse p40 homodimer binds to mouse Con-A blasts and activated B cells with both low and high affinities via the IL-12Rβ1 subunit without major contributions from the mouse IL-12Rβ2 subunit (Wang et al., 1999). These data suggest that p40 can independently bind to IL-12Rβ1 in the absence of p35.

Early T_H2 cells selectively lose their IL-12Rβ2 expression and functionally extinguish the IL-12R signaling pathway within 3 days after primary activation. This leads to the failure of phosphorylation of Jak2, STAT3, and STAT4 (Szabo et al., 1995; Szabo et al., 1997). Furthermore, ectopic expression of IL-12Rβ2 in developing and committed T_H2 cells did not lead to significant levels of IFN-γ production, preventing these cells from phenotypic reversal (Heath et al., 2000).

Both T_H1 and T_H2 cells bind to ^{125}I-IL-12p75 and have been shown to express high levels of IL-12Rβ1 that create 1000-1500 IL-12-binding sites with a dissociation constant ranging from 30-40pM (Rogge et al., 1997; Szabo et al., 1997; Szabo et al., 1995). This raises the question of why T_H2 cells that do not make IFN-γ should express IL-12Rβ1 and bind to IL-12? It would be of interest to determine if T_H2 cells express the 85kDa protein, and if they are capable of signaling through the IL-12Rβ1/p85 complex. This receptor complex would be a good candidate for p40 binding and signaling independent of IL-12p75 and IL-12Rβ2. In addition, it has been shown that various cells express IL-12Rβ1; for example, melanoma cell culture and melanoma cell lines have been shown to express IL-12Rβ1 (Yue et al., 1999). T cells isolated from BAL in patients with sarcoidosis (T_H1) and asthma (T_H2) (Rogge et al., 1999; Szabo et al., 1997), and superior cervical ganglion cells in mice have also been shown to express IL-12Rβ1 and IL-12Rβ2 (Lin et al., 2000). One important question remains to be addressed: what is

the role of IL-12Rβ1 in those cells that do not respond to IL-12, specifically T_H2 cells?

Recently, by using RT-PCR and RNase protection assay, a distinct form of IL-12Rβ1 that is expressed on T and NK cells has been shown to be constitutively expressed on splenic dendritic cells and on a dendritic cell line (CB1) (Grohmann et al., 1998). This IL-12Rβ1 is structurally and functionally distinct from that expressed by NK and T cells. Recombinant IL-12p75 binds directly to DCs with an affinity of 325pM, and allows recruitment of NF-κB to a major response element in the control region of the p40 transcription half sites, leading to induction of p40 (Grohmann et al., 1998). This is an intriguing finding that poses an important question: why do DCs that are the source of IL-12p75 express a distinct IL-12Rβ1 that is different from the T and NK cells? Moreover, IL-12p75 signaling in T cells occurs via STAT4, which is an essential step toward T_H1 development, since mice deficient in STAT4 have been shown to have defects in IFN-γ, CTL, and NK activities (Kaplan et al., 1996; Thierfelder et al., 1996). This was not the case for the structurally unique IL-12Rβ1 that was shown to be expressed on freshly isolated DCs: binding of IL-12p75 and signaling through this receptor involves members of the NF-kb family but not the STAT4 family (Grohmann et al., 1998). Do mononuclear phagocytic cells (MPs) also express such distinct IL-12Rβ1? Are they capable of signaling through the NF-KB pathway? Most importantly, is the establishment of such signaling pathways in human DCs due to the species-specificity of STAT4 activation via IFNα and T_H1

differentiation, since these pathways are distinct in humans but not in mice (Rogge et al., 1998; Szabo et al., 1995).

IL-12p75 Family:

IL-12p75 was the first heterodimeric cytokine that was discovered over eighteen years ago. This cytokine has a unique structure since it is composed of two unrelated proteins that are linked together by a disulfide bond. Even though this has been known for some times, there is no plausible explanation of what evolutionary purpose is served to have two unrelated genes encode two unrelated proteins that are assembled through an exquisite process to finally form one cytokine. Why evolutionary propose does it serve to go through all this trouble to generate one cytokine?

Ten years later in 2000, a group from DNAX Research Institute, through mixing and matching experiments, they discovered IL-23 (Oppmann et al., 2000). They found that when the p40 subunit of IL-12 was co-expressed with a novel protein named p19, which was discovered several year earlier, these two proteins associated together and were secreted as a heterodimeric protein that was named IL-23 (Oppmann et al., 2000).

p19 has overall a 40% sequence homology to the p35 subunit, and, like p35, cannot be secreted in the absence of p40. This discovery changed the way in which data had been interpreted for IL-12p75 and its biology and answered one important question. Before the discovery of IL-23, it was not clear why p35 KO mice should still developed severe disease in Experimental Autoimmune Encephalomyelitis (EAE), as it had been thought that IL-12 was a crucial component of this autoimmune disease. It was then rapidly

established that IL-23, rather than IL-12p75, is the essential player in the pathogenesis of this disease. Consequently, new helper T cells emerged from these studies, which are currently known as T_H17 cells. This led to the proposal that both IL-12p75 and IL-23 are members of a new emerging heterodimeric cytokine family with unique structure and functions (Brombacher et al., 2003).

The discovery of IL-23 further substantiated the principle hypothesis behind this thesis (Chapter I), which emphasizes that p40 can associate with other molecules independent of p35 to form novel heterodimeric proteins with independent functions. But why did it take almost ten years after the discovery of IL-12 before IL-23 was identified? The serendipitous discovery of p40's association with p19 further strengthens the proposed hypothesis in this thesis that p40 by itself is an important molecule that can form various heterodimers during the early host response to pathogens (Chapter I).

During the early years of IL-12p75 discovery, most investigators did not discriminate between p40 and p75. The battery of reagents and antibodies that were used to measure IL-12p75 mostly recognized and interacted with the p40 subunit, and they were not specifically chosen to measure IL-12p75. Thus, data that have accumulated around the existing paradigm--that IL-12p75 is secreted in direct response to pathogens during the innate immunity--have been based on incorrect measurements. Therefore, many investigators did not realize that p40 could be secreted in the absence of IL-12p75. Although, many investigators are now aware of this issue, there has not been a shift in this paradigm, and even

though IL-12p75 is not easily measurable, many investigators believe that the presence of p40 is a good indicator of IL-12p75 secretion. Therefore, it has recently been proposed that multiple TLRs are required to induce IL-12p75 from antigen-presenting cells during the early host response to pathogens (Trinchieri and Sher, 2007).

For this reason, I have proposed an alternative to the current view of the early host response to pathogens and the lack of a role of IL-12p75 during these early responses. I have argued that IL-12p75 is not the protein that is secreted during the innate immune response to TLR agonists. I have suggested that the engagement of TLRs only induces secretion of p40 and this protein plays an important role during the innate immune response. Thus the aim of this thesis is to address a very basic question: Is it p40 or IL -12p75 that is critical in the early host immune response following encounter of DCs with microbial pathogens?

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