Universidade da Beira Interior

Anacor Pharmaceuticals, Inc.

Small-Molecule Boron compounds to use against Interleukin-23

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TABLE OF CONTENTS

RESUMO

A plataforma-base da tecnologia desenvolvida na Anacor Pharmaceuticals é baseada no uso de compostos químicos que têm o Boro como átomo inicial das estruturas químicas sintetizadas. Esses compostos têm uma geometria única que lhes permite terem duas formas distintas e a capacidade de interagir de uma maneira inovadora com alvos biológicos até agora não atingidos utilizando compostos tradicionais com base carbonada. A reactividade do Boro permite a estes compostos interagir com um alvo biológico específico de uma determinada condição ou patologia, promovendo uma alteracão nesse alvo. As propriedades anti-inflamatórias de alguns compostos desenvolvidos na Anacor, juntamente com pesquisa na etiologia da psoríase, podem melhorar de forma considerável esta condição patológica. Inicialmente a origem da psoríase foi atribuída aos queratinócitos, mas hoje em dia é um facto conhecido de que é a relação próxima entre essas células e várias outras células imunitárias que originam os sintomas e lesões típicas desta patologia – eritemas bem demarcados, placas escamosas com hiperplasia epidermal (acantose) acompanhada por paraqueratose. Análises dos infiltrados celulares, assim como do tipo de citocinas e quimiocinas presentes nas lesões psoriáticas, levou inicialmente os investigadores a acreditarem que esta era uma doenca do subtipo T *helper* 1 (T_H1) (devido aos níveis elevados de Interleucina -12 (IL-12) e Interferão-γ (IFN-γ) e elevada presença de células $CD4^+$) ou uma doença auto-imune (em que o sistema imunitário reagiria contra queratinócitos do próprio). Com a descoberta de uma nova citocina – Interleucina-23 (IL-23) – que partilha a subunidade p40 e o receptor IL-12Rβ1 com a IL-12, mas não o receptor IL-23R, possivelmente um novo caminho de investigação foi descoberto. Ao contrário da IL-12 que actua em células *naive*, a IL-23 actua em células T de memória. A IL-12, inicialmente encontrada em lesões psoriáticas é, na realidade, a IL-23, tendo sido também estabelecida uma correlação entre esta última e uma outra citocina, a Interleucina-17 (IL-17). Uma investigação subsequente levou à descoberta de um novo grupo de células $CD4^+$ que produz IL-17 (e outras citocinas implicadas na psoríase) e que foi então designada T *helper* 17 (T_H17). A IL-23 é responsável por manter o crescimento deste subtipo de células T *helper* (T_H) e tanto a IL-23, como as células T_H17, estão implicadas na psoríase. Devido à semelhança estrutural com a IL-12 pensou-se que os mesmos mecanismos que inibem esta interleucina poderiam também inibir a produção da IL-23. A Fosfodiesterase 4 (PDE4) é a principal enzima que metaboliza o AMP cíclico (cAMP) encontrada em células inflamatórias e do sistema imunitário. Desse modo, e porque há um largo número de citocinas cuja produção é inibida por compostos que inibem a PDE4, a Relação Actividade/Estrutura (SAR) foi construída tendo dados de inibição da IL-23 e dados da inibição da PDE4 para compostos da Anacor e para compostos comercialmente disponíveis que se sabe serem inibidores da PDE4 (por exemplo, Rolipram, Ibudilast e outros). O presente trabalho mostra que a inibição da PDE4 não está correlacionada com a inibição da IL-23, não apenas pelo facto de haver vários compostos que inibem a citocina, mas que não inibem a enzima e vice-versa, mas também porque os compostos comercialmente disponíveis que se sabe serem inibidores da PDE4 não inibem a produção da IL-23. A continuação da

investigação e a pesquisa de compostos com estruturas químicas diferentes que promovam a inibição da IL-23, assim como a compreensão dos mecanismos que levam à sua produção, irão contribuir no futuro para aumentar a informação disponível sobre a regulação da produção desta citocina.

ABSTRACT

Anacor Pharmaceutical's core technology platform is based on the use of boron chemistry to develop novel therapies. Boron-based compounds have a unique geometry that allows them to have two distinct shapes, giving boron-based drugs the ability to interact with biological targets in novel ways and to address targets not amenable to intervention by traditional, carbon-based compounds. Boron's reactivity allows boron-based compounds to interact with a biological target that is specific to a particular disease or condition and create a change in that target. The anti-inflammatory properties of some Anacor compounds merged with research in the etiology of psoriasis could improve this pathological condition. Initially the origin of psoriasis was attributed to keratinocytes, but nowadays is a known fact that it's the close relationship between those cells and several others immune cells that creates the symptoms and typical lesions of this pathology – well-demarcated erythematous, scaly plaques with epidermal hyperplasia (acanthosis) accompanied by parakeratosis.

Analysis of the cell infiltrates as well as the type of cytokines and chemokines present in the psoriatic lesions, first lead researchers to believe that this was either a T helper 1 (T_H1) disease (due to the high levels of Interleukin-12 (IL-12) and Interferon-γ (IFN-γ) and $CD4^+$ cells) or an autoimmune disease (where the immune system would auto-react against own keratinocytes). With the discovery of a novel cytokine – Interleukin-23 (IL-23) - that shares the p40 subunit with IL-12 and also the IL-12Rβ1 receptor, but not the IL-23R, a new possible path was initiated. Unlike IL-12, the new discovered cytokine – IL-23 does not act on naïve T cells, but it does act on memory T cells. The IL-12 initially found in psoriatic lesions is actually IL-23 and a relationship was build between this and another cytokine – Interleukin -17 (IL-17). Further research lead to the findings of a $CD4^+$ subset that produces IL-17 (and other important cytokines) which was then designated T helper 17 (T_H17). IL-23 is responsible for maintaining the growth of this subset of immune cells and both IL-23 and T_H17 were implicated in psoriasis. Due to the structural similarity with IL-12, it was thought that the same inhibitory mechanisms that inhibit IL-12 could also inhibit IL-23 production. Phosphodiesterase 4 (PDE4) is the major cyclic AMP (cAMP)-metabolizing enzyme found in inflammatory and immune cells. Therefore and since there's a wide range of cytokines whose production is inhibited by PDE4 inhibitors, Structure Activity Relationship (SAR) was built having IL-23 inhibition data along with PDE4 inhibition data for Anacor compounds and commercially available compounds known to inhibit PDE4 (*i.e.* Rolipram, Ibudilast and others). The present work shows that the PDE4 inhibition does not correlate with IL-23 inhibition, not just by the fact that there are several compounds that inhibit the cytokine but not the enzyme and the other way around, but also because the commercially available compounds known to inhibit the PDE4 do not inhibit IL-23 production. Investigating further on and continue to look for IL-23 inhibition by structurally different chemical compounds synthesized as well as trying to understand the pathways by which IL-23 is produced would hopefully in the future increase the amount of information available about this cytokine regulation.

ABBREVIATION LIST

SAR – Structure activity relationship
QSAR – Quantitative structure activity relationship
IC_{50} – half maximal (50%) inhibitory concentration
TNF-α – Tumor necrosis factor- α
LB – Lamellar bodies
IFN-γ – Interferon-γ
IL-1β – Interleukin-1β
IL-1α – Interleukin-1α
TGF- α – Transforming growth factor α
TGF- β - Transforming growth factor β
EGF – Epidermal growth factor
EGFR – Epidermal growth factor receptor
IL-23, IL-17, IL-4, etc. – Interleukin-23, Interleukin-17, Interleukin-4, etc.
T_H1 – T helper 1
T_H2 - T helper 2
T_H17 – T helper 17
DC – Dendritic Cell
APC – Antigen Presenting Cell
TLR – Toll Like Receptor
MHC – Major histocompatibility complex
TCR – T cell receptor
STAT – Signal transducer and activator of transcription
IL-12Rβ1 – Interleukin-12 receptor β1 chain
IL-12Rβ2 – Interleukin-12 receptor β2 chain
IL-23R – Interleukin-23 Receptor
JAK – Janus kinases
PGE_2 – Prostaglandin 2
PDE – Phosphodiesterase
cAMP – cyclic adenosine monophosphate
CRE – cyclic adenosine monophosphate response elements
CREB – cyclic adenosine monophosphate response rlements-binding proteins
NF-kB – Nuclear factor kB
LPS – Lipopolyssacharide
IRAKs – Interleukin-1 receptor associated protein kinases
TAK1 – Transforming growth factor-β-activated kinase
TRAF6 – Tumor necrosis factor receptor associated factor 6
MAPK – Mitogen activated protein kinase
AP-1 – Activator protein 1

1. INTRODUCTION

1.1 BORON CHEMISTRY PLATFORM OVERVIEW

Anacor's core technology platform is based on the use of boron chemistry to develop novel therapies. Boron is a naturally occurring element that is ingested frequently through consumption of fruits, vegetables, milk and coffee (*source: Anacor Pharmaceuticals, Inc. archives*). In fact, organic compounds containing Boron have been known for over a century, and many aspects of their chemical properties and reactivity have been known for quite some time. Since the discovery and development of hydroboration, the resulting organoboranes are among the most widely used reagents and intermediates in organic synthesis including asymmetric reactions. It is not surprising that organoboron compounds exhibit such a plethora of useful properties. The electronic structure of boron and its strategic position on the periodic table adjacent to carbon makes trivalent boron compounds behave as *electrophilic* molecules with trigonal planar structures that are neutral yet isoelectronic to carbocations. However, formation of an additional bond to boron generates anionic tetravalent boron compounds that have tetrahedral structures and behave as *nucleophilic* molecules. Boron-containing compounds have an empty P orbital that allows for inclusion of "controlled activity" into targeted boron compounds. (*Source: Anacor Pharmaceuticals, Inc. archives*) Most notably, both of these types of boron compounds can be stable while retaining significant reactivity that defines their unique, versatile, interconvertible, and tunable chemical behavior (Petasis, 2007)

Nearly every common type of boron bond (B–H, B–B, B–C, B–N, B–O, B–F, B–Cl, etc.) has distinctive reactivity features that can be exploited. Indeed, this broad spectrum of electronic, structural, and reactivity behavior of organoboron compounds has led to a growing number of recent discoveries of useful chemical reactions, from metal-catalyzed processes to acid catalysis, asymmetric transformations, and multicomponent reactions. An added advantage of many of these processes is that their major boron by-

product is boric acid, an environmentally friendly (green) substance. However, the contributions of organoboron compounds are not limited to their unique and versatile nature as synthetic intermediates. In fact, a growing number of boron-containing compounds have been shown to have important biological activities and are even suitable as pharmaceuticals agents for clinical use (Hall, 2006).

Another unique feature of certain organoboron compounds, such as boronic acids and trifluoroborates, is that they retain reactivity even in the presence of a variety of functional groups. Despite their chemical stability, however, it is also possible to activate these molecules in situ towards suitable reactive intermediates, enabling new types of transformations. Based on this concept, some time ago it was introduced the use of organoboron compounds as key components in multicomponent reactions of amines and carbonyl compounds. Several variations of this type of reaction were introduced, including the synthesis of amino acids, amino alcohols, and amino phenols. Moreover, Petasis (2007) and others have shown that this type of process can be used for the synthesis of a large variety of novel amine derivatives, amino sugars, and heterocycles. A notable feature of this chemistry is that it proceeds under mild conditions and can generate directly many types of novel drug-like molecules, making it especially useful for diversity-oriented synthesis and applications in medicinal chemistry.

The use of organoboron compounds offers several advantages on the basis of their availability in a large variety of substitution patterns, and their tolerance of most common functional groups. The mild electrophilic nature of the boronic acid moiety has led to its use at the ‘warhead’ site of enzyme inhibitors, particularly for inhibiting proteases.

Several types of bioactive boron-containing molecules have been reported or are under investigation as therapeutic agents. These include certain boron analogues of biomolecules, the antibacterial and antimalarial agent diazaborine, various antibacterial oxazaborolidines, the antibacterial diphenyl borinic esters that inhibit bacterial cell wall growth, the antifungal agent benzoxaborole AN2690, and an oestrogen receptor modulator containing a B–N bond. (Petasis, 2007)

1.2 Structure Activity Relationship (SAR)

The replacement, in an active molecule, of a hydrogen atom by a substituent (alkyl, halogen, hydroxyl, nitro, cyano, alkoxy, amino, carboxylate, etc.) can deeply modify the potency, the duration, perhaps even the nature of the pharmacological effect. Structure-Activity Relationship (SAR) studies implying substituent modifications therefore represent a common practice in medicinal chemistry, all the more since half of the existing drugs contain easy-to-substitute aromatic rings. The perturbations brought by the substituent can affect various parameters of a drug molecule, such as its *partition coefficient, electronic density, steric environment, bioavailability, pharmacokinetics*, and finally its capacity to establish *indirect interactions* between the substituent and the receptor of the target. In reality is impossible to modify only one alone of these five parameters. Thus for example, the replacement of a hydrogen atom by a methyl group is going to play simultaneously on the five parameters listed above. Nevertheless, through a careful selection of the adequate substituent, it is possible to vary one of the considered parameters in a dominant manner.

The rational design of new chemical entities intended for use as drugs can be based in several methods. Besides SAR, also ADME (Absorption, Distribution, Metabolism, Excretion) parameters can be developed within a particular compound series. Even more powerful though to guide the chemist in the design process is Quantitative Structure-Activity Relationships (QSAR), using various statistical and mathematical tools.

First attempts to express quantitatively relationships between chemical structural and bioactivity go back to the beginning of last century. But only when computers and relevant mathematical methods became available were these approaches widely applied. The credit goes to Corwin Hansch and Toshio Fujita for introducing these quantitative methods into medicinal chemistry in the 1960's. Initially in their pioneering work, Hansch and coworkers focused their attention on the role of octanol/ water partition coefficients (log *P*) in drug transport processes which were thought to contribute crucially to the measured activities. As we know now, log *P* is the most predominant descriptor in many structure-activity correlation studies although there's other ways of describing chemical structure in a quantitative way.

The targets of a drug action may be quite diverse, including for example, membrane-bound receptors, ion channels, enzymes and DNA. Biologists developed a

broad variety of biological and pharmacological test systems producing different kinds of data. Some are quite simple and accurate, e.g. IC_{50} values as a measure of ligand affinity, while others are more complex with large errors e.g. *in vivo* data. Activity may be expressed as a continuous measure e.g. IC_{50} or % of inhibition at a specified concentration or as categorical data such as active versus inactive, agonist versus antagonist, or as strong, medium and weak.

1.2.1 Medicinal Chemistry

Taken in the *prospective sense* the objective of medicinal chemistry is the design and the production of compounds than can be used in medicine for the prevention, treatment and cure of human or animal diseases. Thus medicinal chemistry is a part of pharmacology, this latter being taken in its etymological sense ('pharmakon' + 'logos': study of drugs). Taken in the *retrospective sense*, medicinal chemistry also includes the study of already existing drugs, of their pharmacological properties, and their structure-activity relationships. An official definition of medicinal chemistry was given by an IUPAC specialized commission:

Medicinal chemistry concerns the discovery, the development, the identification and the interpretation of the mode of action of biologically active compounds at the molecular level. Emphasis is put on drugs, but the interest of medicinal chemistry is also concerned with the study, identification and synthesis of the metabolic products of drugs and related compounds.

Medicinal chemistry covers three critical steps:

- A discovery step, consisting of the choice of the therapeutic target (receptor, enzyme, transport group, cellular or in vivo model) and the identification (or discovery) and production of new active substances interacting with the selected target. Such compounds are usually called lead compounds; they can originate from synthetic organic chemistry, from natural sources, or from biotechnological processes.
- An optimization step, that deals with the improvement of the lead structure. The optimization process takes primarily into account the increase in potency, selectivity and toxicity. Its characteristics are the establishment and analysis of structure-activity relationships, in an ideal context to enable the

understanding of the molecular mode of action. However, an assessment of the pharmacokinetic parameters such as absorption, distribution, metabolism, excretion and oral bioavailability is almost systematically practiced at an early stage of the development in order to eliminate unsatisfactory candidates.

- A development step, whose purpose is the continuation of the improvement of the pharmacokinetics properties and the fine tunning of the pharmaceutic properties (chemical formulation) of the active substances in order to render them suitable for clinical use. This chemical formulation process can consist in the preparation of better absorbed compounds, of sustained release formulations, of water-soluble derivatives or in the elimination of properties related to the patient's compliance (causticity, irritation, painful injections, and undesirable organoleptic properties).

Medicinal chemistry is an interdisciplinary science covering a particularly wide domain situated at the interface of organic chemistry with life sciences, such as biochemistry, pharmacology, molecular biology, immunology pharmacokinetics and toxicology on one side, and chemistry-based disciplines such as physical chemistry, crystallography, spectroscopy and computer based information technologies on the other.

The knowledge of the molecular targets (enzymes, receptors, nucleic acids), has benefited from the progress made in molecular biology, genetic engineering and structural biology.

A certain number of terms more or less synonymous with medicinal chemistry are used: pharmacochemistry, molecular pharmacochemistry, drug design and selective toxicity.

At Anacor, one of the targets being developed in medicinal chemistry made *in house* is to produce anti-inflammatory compounds that will help to improve some pathological conditions that affect may people worldwide. Psoriasis is one of those pathological states.

The prevalence of psoriasis varies widely depending on ethnicity. Psoriasis occurs most commonly in Caucasians, with an estimated occurrence of 60 cases per 100,000/ year in this population. (Wermuth, 2003)

1.3 Psoriasis

The skin is the main organ connecting the body to the potentially harmful environment. To achieve homeostasis and to defend the body against microbial invaders, it serves not only as a physical barrier, but is also equipped with numerous immunological functions collectively called the skin immune system (Hilliard *et al.*, 2002). It is a primary lymphoid organ with an effective immunological surveillance system equipped with antigen presenting cells, cytokine synthesizing keratinocytes, epidermotropic T cells, dermal capillary endothelial cells, draining nodes, mast cells, tissue macrophages, granulocytes, fibroblasts, and non-Langerhans cells. Together these cells communicate by means of cytokine secretion and respond accordingly to stimulation by bacteria, chemical, ultraviolet (UV) light, and other irritating factors. In Figure 1 we can see a detailed schematic representation of the different cell layers that constitute this organ.

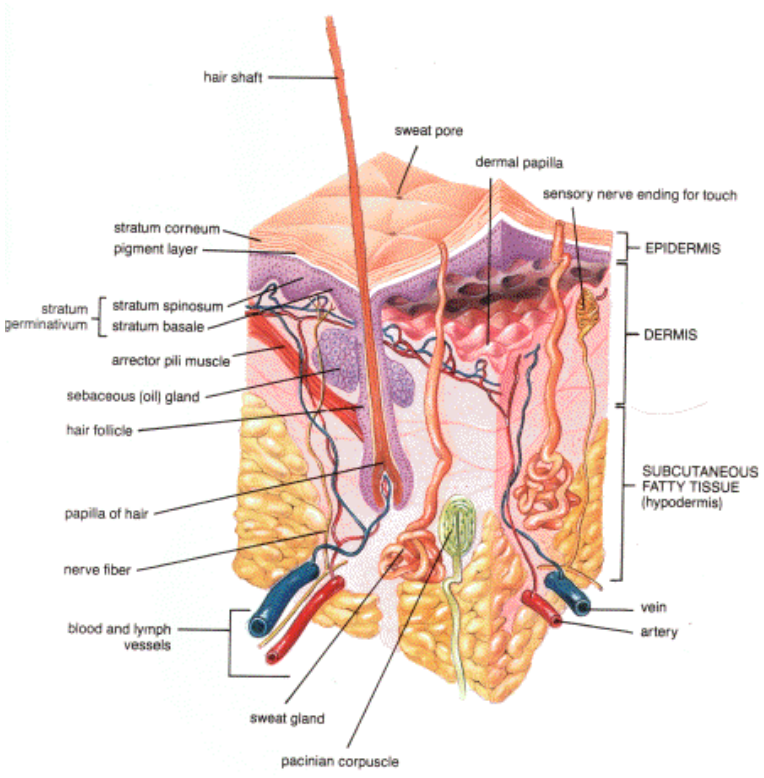

Figure 1 – Schematic representation of the skin showing the different layers and cell types existent in this organ. (*Adapted from www.wikipedia.org*)

The skin consists of two main tissue layers, the dermis and epidermis (Figure 1). Although it is much thinner than the dermal layer, the epidermis is of most importance for the skin's barrier function. The epidermis consists of several layers of keratinocytes and those layers have been identified as the *stratum basale*, *stratum spinosum*, *stratum ganulosum* and *stratum corneum* (Figure 1). The keratinocyte is the major cell type of the epidermis, making up about 90% of epidermal cells. Keratinocytes originate in the basal layer from the division of keratinocyte stem cells. They are pushed up through the layers of the epidermis, undergoing gradual differentiation until they reach the stratum corneum where they form a layer of dead, flattened, highly keratinized cells called squamous cells. The stratum corneum is then a highly cross-linked, lipid-rich anuclear structure that provides the first barrier to the environment. In a normal and healthy system, keratinocytes are shed and replaced continuously from the stratum corneum. The transit time (the approximate time that it takes for normal maturation of skin cells) from basal layer to shedding is 28 days (Traub and Marshall, 2007; Nickoloff, 1999).

The primary intrinsic anti-infectious protective shield of the skin is the stratum corneum, a non-viable surface layer of "mummified" anucleated cells derived from terminally differentiated keratinocytes. The stratum corneum consists of highly cross-linked proteinaceous cellular envelopes with extracellular lipid lamellae, consisting mainly of ceramides, free fatty acids, and cholesterol. A mild trauma can disturb this barrier altering transepidermal water loss, calcium ion gradients and renders the skin permeable to infectious agents and/ or their secreted products. One of the immediate protective responses of skin to barrier perturbation is the release of lamellar bodies (LB) containing pre-formed lipid and hydrolytic enzymes, as well as cytokines such as Tumor Necrosis Factor-α (TNF-α). Other early events include influx of neutrophils that release anti-microbial agents, production of anti-bacterial defensins by keratinocytes, and enhanced desquamation of the stratum corneum itself. In healthy individuals, these innate responses quickly restore cutaneous homeostasis, but in genetically predisposed patients with psoriasis, new lesions are created that can persist for decades. Because of the aberrant epidermal differentiation program in such lesions, psoriatic plaques lack a normal granular cell layer or intact stratum corneum (Nickoloff, 1999). In fact, regarding genetic predisposition for this condition, about 30% of individuals with psoriasis have a family history of the disease in a first- or second-degree relative and currently at least nine chromosomal susceptibility loci have been elucidated (PSORS1-9). HLA-Cw6 is a major determinant of phenotypic expression. An association with the PSORS has been found with functional polymorphisms in modifier genes that mediate inflammation (e.g., TNF-α) and vascular growth (e.g.,VEGF; Traub and Marshall, 2007).

Given their ability to link the innate and acquired immune systems, deregulated cytokine production (elevated levels of TNF, Interferon-γ (IFN-γ), Interleukin-1α (IL-1α), Interleukin-1β (IL-1β), Transforming growth factor-α (TGF-α), and other cytokines in

lesional skin) is postulated to establish chronic lesions by providing persistent pro-inflammatory signals to the skin (Chan *et al.*, 2006). As defended by some, the barrier defect in psoriasis might represent a consequence, rather than a cause, of infiltration by activated immunocytes that release cytokines that trigger an aberrant differentiation program in the epidermis. In corroboration to that, many drugs that selectively target activated T cells are very effective in producing remissions in psoriasis patients and that after an upper respiratory infection in psoriatic patients they develop numerous skin lesions that do not represent primary cutaneuous infections, and that are most likely a result of the bacterial infection that exit the pharynx and migrate to skin where cytokines are produced by resident immune cells present there. In fact, transgenic mice that constitutively produce IFN-γ in the epidermis, display profound barrier defects in the skin (Nickoloff, 1999).

Recent genetic and immunological advances have greatly increased understanding of the pathogenesis of psoriasis as a chronic, immune-mediated inflammatory disorder.

1.3.1 Histology and the role of keratinocytes in psoriasis

Psoriasis is a chronic inflammatory skin disease characterized by excessive proliferation and abnormal differentiation of epidermal keratinocytes, the growth and dilation of blood vessels, and the infiltration of leukocytes into the dermis and epidermis (Sa *et al.*, 2007).

The typical lesions in psoriasis are well-demarcated erythematous, scaly plaques. Histologically, there is marked epidermal hyperplasia (acanthosis) accompanied by parakeratosis (retention of keratinocyte nuclei in the stratum corneum) and a mixed dermal infiltrate, incuding $CD4^+$ T cells, dendritic cells (DC), macrophages and mast cells. Histological analysis of psoriatic skin reveals the absence of a normal stratum corneum. Psoriatic skin has little to no keratinocyte nuclei disintegration, which ultimately results in a modified stratum corneum that has retained keratinocyte nuclei (parakeratosis) – See Figure 2 (Chan *et al.*, 2006)

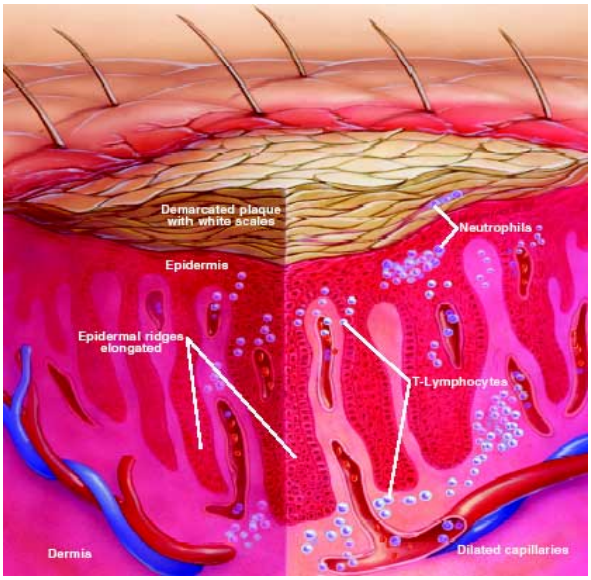

Figure 2 – Schematic representation of psoriatic skin. The T lymphocyte and its cytokines have been implicated in the changes seen in the skin of patients with psoriasis, including capillary dilation, epidermal ridge dilation, neutrophil proliferation, and plaque formation (*Adapted from www.uspharmacist.com.)*

The primary immune defect in psoriasis appears to be an increase in cell signaling from dendritic cells that recognize and capture antigens, migrate to local lymph nodes, and present them to T cells. The activation of T lymphocytes releases pro-inflammatory cytokines such as TNF-α that lead to keratinocyte proliferation. This hyperproliferative response decreases epidermal transit time to 2-4 days and produces the typical erythematous scaly plaques of psoriasis. This understanding of pathogenic mechanisms has led to the development and therapeutic use of TNF-α blocking agents (Traub and Marshall, 2007).

The mechanisms underlying abnormal keratinocytes hyper-proliferation and differentiation in psoriasis are not completely understood, although a number of epidermal growth factor (EGF) family growth factors have been proposed to play an important role. (Sa *et al.*, 2007) Human keratinocytes appear to require binding of EGF or other family member ligands to the EGF-receptor (EGFR) to sustain proliferation in culture (Piepkorn *et al.*, 1998). It was demonstrated (Austyn, 2000) that interruption of the activity of EGF-related autocrine growth factors in human keratinocytes by neutralizing antibody to the EGFR, or a specific inhibitor of the EGFR tyrosine kinase

(PD153035) not only causes cessation of keratinocyte proliferation, but also induces irreversible growth arrest and terminal differentiation (Austyn, 2000). Piepkorn *et al.,* 1998 studied the aberrant activity of the epidermal keratinocyte EGF receptor-ligand system and linked to the cutaneous inflammatory component of psoriasis.

Keratinocytes can, constitutively or after stimulation, produce many cytokines. Physical, chemical and pathogenic triggers induce keratinocytes to secrete pro-inflammatory cytokines, such as IL-1β and TNF-α, resulting in auto-activation of these cells to produce other inflammatory cytokines and chemokines. The pro-inflammatory cytokines may also activate other cells in the skin, such as DC, which, upon activation, start to mature and migrate to lymph nodes inducing adaptive immunity. (12)

Besides the cytokines mentined above, human keratinocytes can secrete low but significant levels of Interleukin-23 (IL-23) as stated by Piskin *et al.*, 2006 and functional analysis proved that these low levels of keratinocyte-derived IL-23 are biologically active and sufficient to amplify the IFN-γ production by memory T cells. These results not only support the view that keratinocytes contribute to cutaneous inflammation but also indicate that they can enhance immune responses through the expression of IL-23. In fact, keratinocytes in lesional psoriatic skin expressed markedly higher levels of IL-23 compared with keratinocytes in normal skin (Piskin *et al.*, 2006). It is likely that those higher levels are a result of the induction by cells in their neighborhood, since that cultured keratinocytes from normal skin and from nonlesional and lesional psoriatic skin display similar IL-23 staining by immunohistochemistry and secrete comparable levels of IL-23, which reinforces the concept that the increased expression of this cytokine is not an intrinsic aberration of the keratinocytes (Piskin *et al.*, 2006) .

Previously, psoriasis was considered a disorder of epidermal keratinocytes. In the current thinking, it is recognized primarily as an immune-mediated disorder and it is still yet unclear whether it represents a “T helper1 (T_H1) disease” a “T helper17 (T_H17) disease” or some combination of both. (Blauvelt, 2008)

Considering the facts that in mice a local excess of IL-23 in the skin causes cutaneous inflammation and that IL-23 is present at enhanced levels in the epidermis of psoriatic lesions, Piskin *et al*. 2006 speculate that keratinocyte derived IL-23 may participate in the perpetuation of psoriasis through the stimulation of T cells in their close proximity. In response, these T cells are stimulated to express pro-inflammatory cytokines like IFN-γ and IL-17, which in turn enhance the pro-inflammatory cytokine production by keratinocytes (Piskin *et al.*, 2006).

A complex relation exists between DCs, keratinocytes and T cells in the psoriatic lesion. Although DC and keratinocytes stimulate T cells to express IFN-γ by their secretion of the IL-23 heterodimer, IFN-γ production by $CD40L^+$ T cells enhances the expression of CD40, IL-1β, and IL-23 locally by keratinocytes and DC (Piskin *et al.*, 2006). In the

absence of sufficient suppressive potential, this mutual activation of the activated cells may develop into a self-amplifying loop that ends in a chronic inflammation as present in psoriatic lesions (Piskin *et al.*, 2006).

1.3.2 Types of Immune cells present in psoriatic lesions, infiltrates and related events

Persistent psoriatic lesions in humans contain immune infiltrates. (19) Analysis of infiltrates in psoriatic lesions have most consistently identified increases in myeloid-derived $CD11c^+$ dendritic cells and $CD4^+$ and $CD8^+$ T cells, with plasmacytoid dendritic cells ($CD11c^-$), monocytes/ macrophages, neutrophils, and mast cells also frequently found in increased numbers (Sa *et al.*, 2007). Adhesion molecules that promote leukocyte adherence are also highly expressed in psoriatic lesions (Traub and Marshall, 2007).

Initially, immature dendritic cells in the epidermis migrate to the lymph nodes and stimulate T-cells from lymph nodes to respond to an as yet unidentified antigen. It is known that in initial pre-pinpoint lesions, bacterial infections and physical trauma (Köebner's phenomenon) often precede lesion formation and neutrophil accumulation in the dermis, and an influx into the epidermis is observed (Chan *et al.*, 2006).

After T-cells receive primary stimulation and activation, a resulting synthesis of mRNA for interleukin-2 (IL-2) occurs, resulting in a subsequent increase in IL-2 receptors. The increased IL-2 from activated T cells and IL-12 from Langerhans cells ultimately regulate genes that code for the transcription of cytokines such as IFN-γ, TNF-α, and IL-2, responsible for differentiation, maturation, and proliferation of T cells into memory effector cells. Ultimately, T cells migrate to the skin, where they accumulate around dermal blood vessels. These are the first in a series of immunologic changes that result in the formation of acute psoriatic lesions.

Because the above-described immune response is a somewhat normal response to antigen stimulation, it remains unclear why the T-cell activation that occurs, followed by subsequent migration of leukocytes into the epidermis and dermis, creates accelerated cellular proliferation. A genetic predisposition for psoriasis is one of the explanations already stated in this work (Traub and Marshall, 2007).

1.3.2.1 Antigen Presenting Cells (APCs)

APCs are innate immune cells that are activated by microbial components via pattern-recognition receptors such as TLRs (Gutcher and Becher, 2007).

Dendritic cells, macrophages and B cells are all examples of APCs. Other cells like fibroblasts, glial cells and others can also have functions similar to those of APCs although they don't constitutively express the Major Histocompatibility Complex (MHC) proteins, and they can be stimulated to do so by IFN-γ (Austyn, 2000).

APCs provide three signals that are required for the activation of antigen-specific T cells, during an immune reaction (Figure 3)

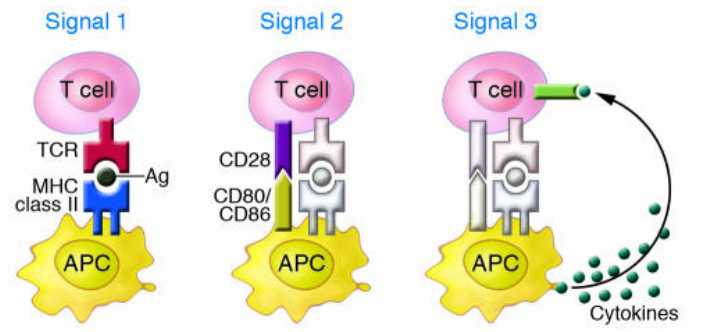

Figure 3 - Within the immune synapse formed between APCs and T cells, three signals are required for antigen-specific T cell activation. Signal 1 comprises the presentation of antigen peptide, in the context of MHC class II molecules, which is recognized by the antigen-specific TCR. Signal 2 involves the stabilization of the synapse through adhesion molecules and the generation of signals via costimulatory molecules present on the surface of APCs and T cells. CD80/CD86 on APCs interact with their receptor, CD28, on T cells to generate activatory signals, while interaction with cytotoxic T lymphocyte–associated protein 4 (CTLA4) generates inhibitory signals (not shown). The secretion of cytokines by APCs, which signal via cytokine receptors on T cells in order to polarize them toward an effector phenotype, produces signal 3. **Ag** - antigen. *Adapted from Gutcher and Becher, 2007.*

The first signal involves the presentation of antigen on the surface of an MHC class II molecule, which facilitates T cell recognition of the cognate antigen through the T Cell Receptor (TCR). In order from antigen-specific T cells to become activated and this population to expand in number, a second signal must be generated through the interaction of adhesion and co-stimulatory molecules present on the APC, such as CD80 and CD86, with CD28 in the surface of T cells. The third signal is the secretion of cytokines by APCs, which directs the differentiation of activated antigen-specific lymphocytes into an effector T cell subtype (IL-12 to T_H1-cell differentiation and IL-4 to

a T_H2-cell differentiation, Figure 4). The creation of a particular cytokine environment by APCs during immunity is critical for the determination of the appropriate type of immune response, which can be either cell-mediated or humoral (Gutcher and Becher, 2007).

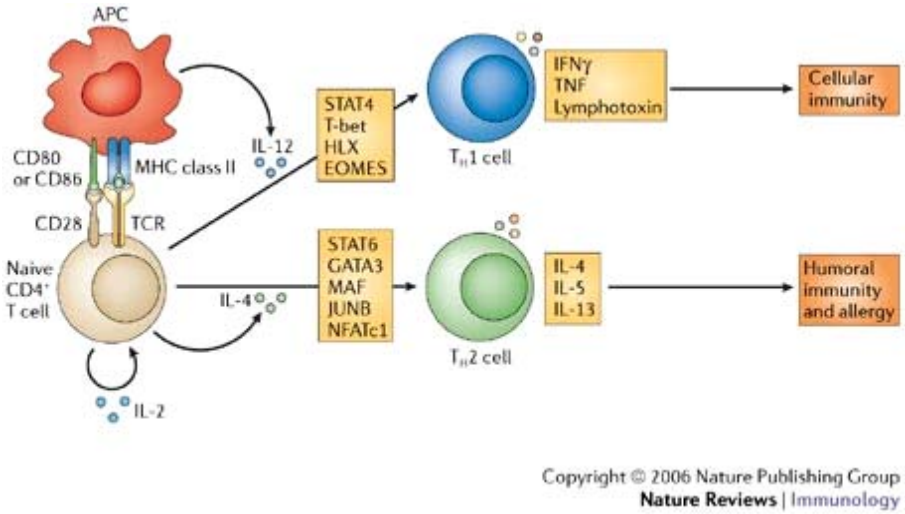

Figure 4 - T helper 1 (T_H1)- and T_H2-cell differentiation from naive CD4+ T cells and the factors known to mediate this process are indicated. T_H1 cells produce interferon-γ (IFNγ), and regulate antigen presentation and cellular immunity. Interleukin-12 (IL-12), which is produced by activated antigen-presenting cells (APCs), drives the process of T_H1-cell differentiation in which the transcription factors signal transducer and activator of transcription 4 (STAT4), T-bet, H2.0-like homeobox 1 (HLX1) and eomesodermin (EOMES) have important regulatory functions. T_H2 cells produce IL-4, IL-5 and IL-13, which are important regulators in humoral immunity and allergic responses. IL-4 is required for T_H2-cell differentiation. The transcription factors STAT6, GATA-binding protein 3 (GATA3), nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), MAF and JUNB regulate T_H2-cell differentiation and cytokine expression. **TCR** - T-cell receptor; **TNF** - tumor-necrosis factor. *Adapted from Dong, 2006.*

1.3.2.2 T cells present in psoriatic lesions

Several different subsets of T cells have been described, each with a distinct function. The subsets $CD4^+$ and $CD8^+$ are often found in psoriatic lesions. CD4 and CD8 are glycoproteins expressed in the membrane of T cells. $CD4^+$ cells can also be called T helper cells and $CD8^+$ cells can be called cytotoxic T cells. While the first ones are the "middlemen" of the adaptive immune system, secreting cytokines that "help" or regulate the immune response; the second ones destroy virally infected cells and tumor cells. (McCluskey, 1991; Bone and Bonilla, 1996). Through interaction with helper T cells, $CD8^+$ cells can be transformed into regulatory T cells, which prevent autoimmune

diseases such as experimental autoimmune encephalomyelitis. The $CD8^+$ role in psoriasis is still yet to be determined since they persist after treatment, which could be a signal of regulatory functions, but the existence of $CD8^+$ T cells, and their IFN-γ production capacity makes them possible effector cells in this disease. (Lowes *et al.*, 2008)

In 1986 Mosmann *et al.* initially proposed a model whereby $CD4^+$ T cells are subdivided into two independent subsets with distinct effector functions. They suggested that T_H cells could be segregated into T_H1 and T_H2 subsets on the basis of cytokine expression and bioactivities as well as helper function. Those and further experiments demonstrated that T_H1 cells develop in response to IL-12 and secrete predominantly IL-2, IL-3, TNF-α, and most notably IFN-γ and control cell-mediated functions such as the activation of macrophages, while the secretion of IL-4, IL-5, and IL-13 by T_H2 cells (which are developed in response to IL-4) leads to the stimulation of humoral immunity by aiding B cell activation and class switching. Interestingly, the cytokines of a particular T_H subtype are able to further promote the expansion of that subtype population while simultaneously inhibiting the development of the other subset. This allows each T_H subset to produce characteristic cytokines that in turn provoke the development of distinctive effector function specific for that immunogen. Thus, while T_H1 cells induce pro-inflammatory responses, such as delayed-type hypersensitivity, and eliminate intracellular infections, T_H2 cells mediate allergic reactions and are important for the elimination of parasitic infection (Lowes *et al.*, 2008; Wilson *et al.,*2007).

The inflammatory T_H1 phenotype which was initially determined to $CD4^+$ T cells in psoriasis due to elevated levels of IFN-γ, TNF-α, and IL-12. Consistent with this is the fact that an Ab anti-blocking the p40 subunit of IL-12, a cytokine essential for T_H1 development, has demonstrated early clinical efficacy in the treatment of psoriasis.

However, p40 is a common subunit for two cytokines, IL-12 and IL-23. Recently, IL-23 has been found to be the dominant p40-containing cytokine identified in psoriatic lesions. (Sa *et al.*, 2007) In addition to that, a new type of T cell, T_H17, has been described.

The T_H17 subset of T helper cells was identified on the basis of its ability to produce IL-17A, IL-17F and IL-22. T_H17 cells provide protection in certain infections but, more importantly, have also been linked to the development of autoimmune disease, a function previously assigned to T_H1 cells and IFN-γ. These cells were first recognized during assessment of the involvement of IL-23 in autoimmune disease. (Wilson *et al.*, 2006) After the discovery of a new T_H cell subset by Langrish *et al.*, a large number of articles in short succession were dedicated to the regulation of IL-17 expression by such T_H17 cells. IL-17 (also known as IL-17A) is a pro-inflammatory cytokine that induces the expression of IL-1, IL-6, granulocyte colony-stimulating factor (G-CSF), as well as

chemokines, by cells such as fibroblasts, stromal cells, and endothelial cells. $CD4^+$ T_H17 cells, which characteristically also produce IL-17F (another IL-17 family member, with the closest sequence identity to IL-17A), IL-6, TNF-α, and IL-22. Indeed, this T_H17 subset is now considered to constitute the critical population responsible for mediating pathogenesis during autoimmunity. (Gutcher and Becher, 2007) The schematic representation of the T_H subsets of cells is displayed in Figure 5.

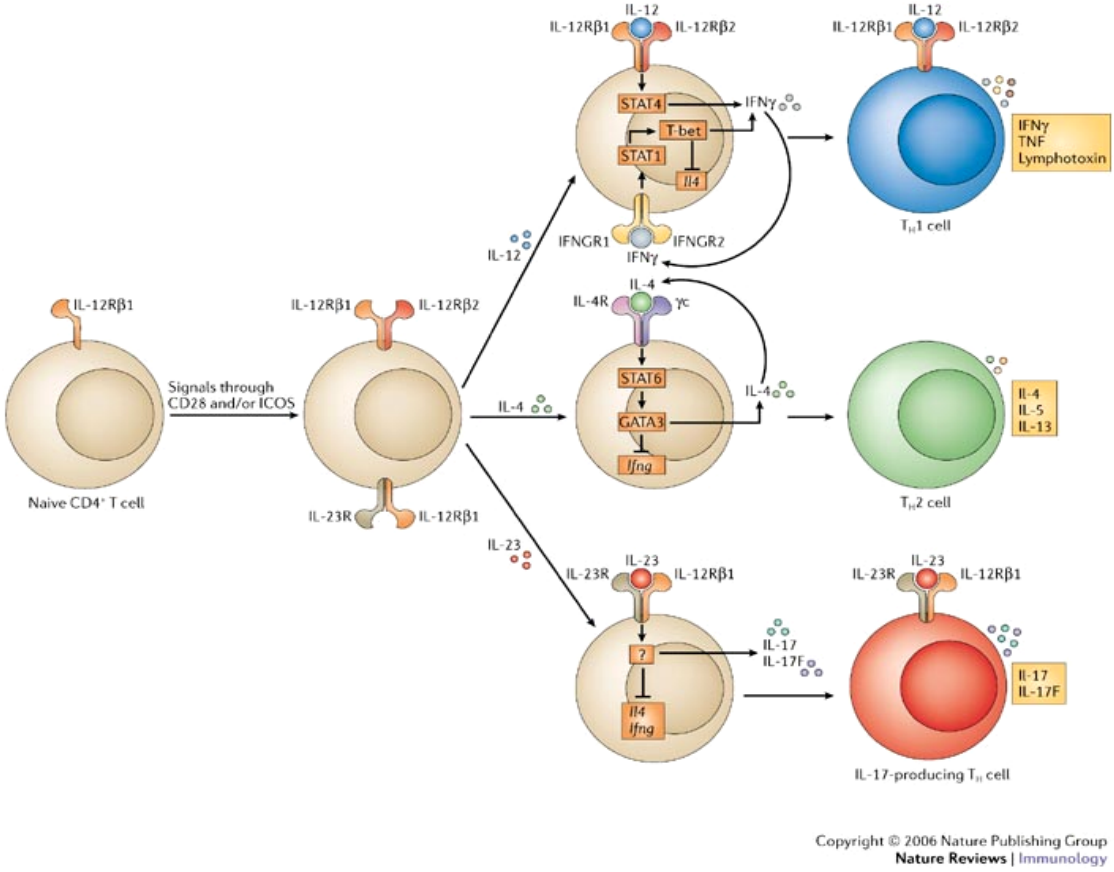

Figure 5 - T helper (TH)-cell differentiation from naive CD4+ T cells is remodeled to include the recent knowledge of the role of interleukin-23 (IL-23) on CD4+ T-cell differentiation. Naive CD4+ T cells that are activated by antigen-presenting cells (APCs) in the presence of co-stimulation through CD28 and/or inducible T-cell co-stimulator (ICOS) enter a state in which cytokines and cytokine receptors are co-expressed at low levels. The cytokine environment determines the terminal lineage commitment and cytokine expression profiles. IL-12, through the action of signal transducer and activator of transcription 4 (STAT4), potentiates the production of interferon-γ (IFNγ), which, through STAT1, increases the expression of the transcription factor T-bet. T-bet, in turn, increases IFNγ and IL-12R2 expression, inhibits IL-4 expression, and maintains TH1-cell-lineage commitment. By contrast, IL-4, through STAT6, increases expression of GATA-binding protein 3 (GATA3). GATA3 potentiates IL-4 expression, inhibits IFNγ expression and determines TH2-cell-lineage differentiation. IL-23 functions through still poorly understood transcriptional mechanisms, increases the differentiation of CD4+ T-cells to effector cells that produce IL-17 and IL-17F, and express high levels of IL-23R. TNF, tumor-necrosis factor; **IFNGR1** - IFNγ receptor subunit 1; **IFNGR2 -** IFNγ receptor subunit 2; **c** - common cytokine-receptor -chain. *Adapted from Dong, 2006*

1.3.2.1.1 T_H17 cells

Naive $CD4^+$ T helper (T_H) cells undergo initial T_H17-cell differentiation in the presence of transforming growth factor-β (TGF β) and interleukin-6 (IL-6), which leads to the expression of Interleukin-21 (IL-21). IL-21 further sustains T_H17-cell differentiation in an autocrine manner and establishes the transcriptional programme of T_H17 cells, including the expression of IL-23 receptor (Il-23R) and IL-1R1. IL-23 and IL-1, both of which are products of activated myeloid cells, possibly finalize the differentiation programme of T_H17 cells and help to maintain the differentiated T_H17 cells (Figure 6). This T cell lineage also expresses preferentially IL-22, an IL-10 family member, as demonstrated by Liang *et al* (Liang *et al.*, 2006).

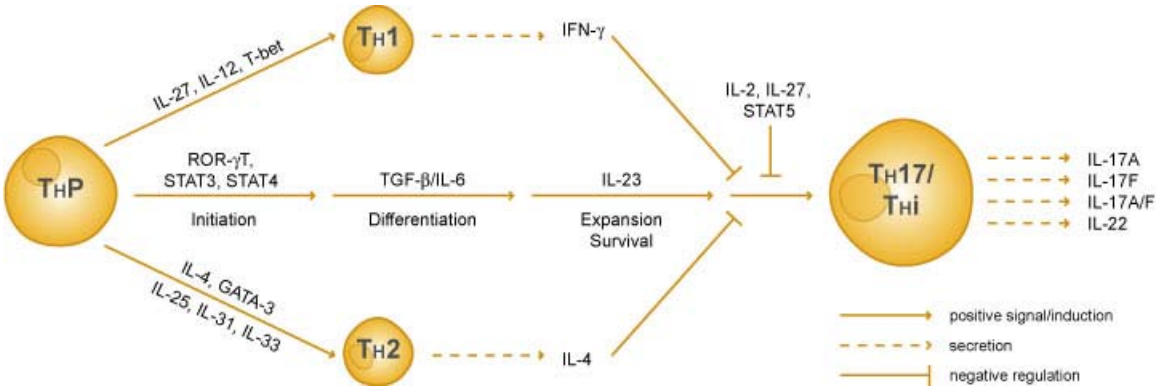

Figure 6 - Transforming growth factor-β (TGF-β), in the presence of IL-6, elicits the differentiation of naïve CD4+ T cells into T_H17 cells. The nuclear receptor RORγt acts as a key transcription factor in this lineage-commitment process. In addition, IL-1 and tumor necrosis factor enhance T_H17 development, whereas IL-4, IFN-γ, IL-27 and IL-2 suppress T_H17 differentiation. *Adapted from www.eBioscience.com*

T_H17 cells probably have a specific role in normal immune function through the coordinated action of their effector cytokines and chemokines, similar to the well established functions of T_H1 and T_H2 cells in regulating cellular immunity and antibody production. The 'signature' cytokine and chemokine profile of T_H17 cells suggests that these cells regulate the immune function of epithelial cells rather than cells of the classical immune system. The IL-23-regulated expression of IL-17A, IL-17F, IL-22 and IL-26 by a subset of $CD4^+$ T cells is particularly notable, as receptors for IL-17A, IL-17F, IL-22 and IL-26 are expressed on the epithelial and stromal cells of tissues that include skin, lung, colon and brain. (Wilson *et al.*, 2007)

A delicate balance of cytokines promotes the differentiation of naïve $CD4^+$ T cells into either polarized T_H17 cells or autoimmunity-suppressing Tregs. (Figure 7).

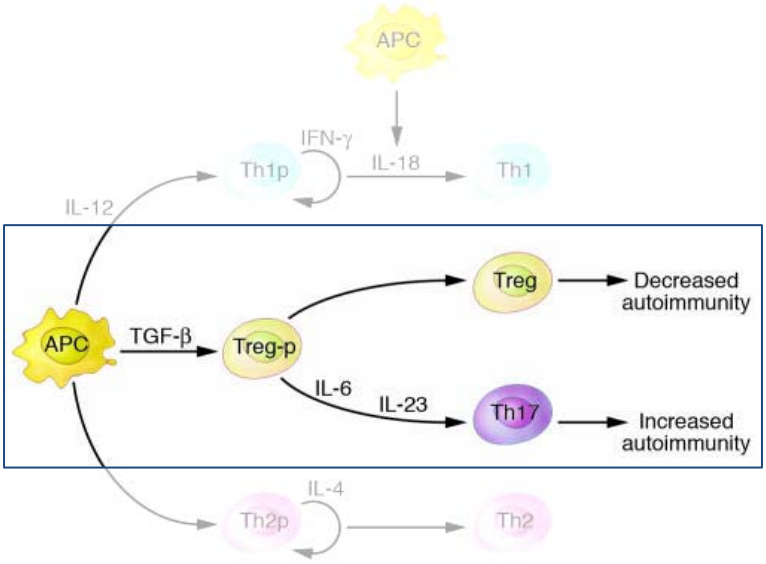

Figure 7 - APC-derived cytokines guide the differentiation of naive T cells into an effector T cell subtype. TGF-β secretion can polarize naive cells toward a regulatory phenotype or an auto-aggressive phenotype, depending on the cytokine environment: secretion of TGF-β alone by APCs supports T_{REG} formation (which counteracts autoimmune inflammation) from T_{REG} precursor cells (T_{REG}-p cells). However, the additional presence of IL-6 results in the production of T_H17 cells, which are now considered to be the pathogenic T cell population during autoimmunity. The pathogenic, APC-derived cytokine IL-23 is critical for the maintenance and survival of these auto-reactive T_H17 cells. *Adapted from Gutcher and Becher, 2007*

While TGF-β stimulates polarization of $CD4^+$ T cells into T_{REG}s in the absence of IL-6 *in vitro*, the additional presence of IL-6 evidently shifts the balance toward the pro-inflammatory T_H17 phenotype, a process that is amplified by IL-1β and is negatively regulated by T_H1 and T_H2 cytokines. Both *in vitro* and *in vivo* differentiation of T_H17 cells require induction of the transcription factor retinoic acid-related orphan receptor-γt (RORγt), which is characteristic of this new T cell subset. (Gutcher and Becher, 2007)

Interleukin-17

Interleukin-17A (IL-17A) and IL-17F are cytokines that induce multiple pro-inflammatory mediators, including chemokines, cytokines, and metalloproteinases, from epithelial and fibroblast cells. Both IL-17A and IL-17F are produced by T_H17 cells.

IL-17 has many pro-inflammatory effects in a wide variety of cells including keratinocytes, macrophages, and endothelial cells. Downstream effects of IL-17 include production of IL-1, IL-6, IL-8, TNF-α, granulocyte colony-stimulator factor, and GM-CSF, as well as antimicrobial peptides. (Lowes *et al.*, 2008)

1.4 Interleukin-12 and Interleukin-23

1.4.1 Interleukin-12

IL-12 is a heterodimeric cytokine that plays a key role in determining the nature of immune response to exogenous or endogenous antigens, since IL-12 not only augments gamma interferon (IFN-g) production by T_H1 lymphocytes and natural killer (NK) cells but also has been shown to diminish T_H2 lymphocyte development (Haskó and Szabó, 1999; Kincy-Cain *et al.*, 1996) IL-12 is comprised of two disulphide-linked protein subunits designated p35 and p40, which are covalently bonded and are encoded by two different genes. Both subunits have to be produced within the same cell to obtain the biologically active dimmer designated p70 (Haskó and Szabó, 1999) (Figure 8).

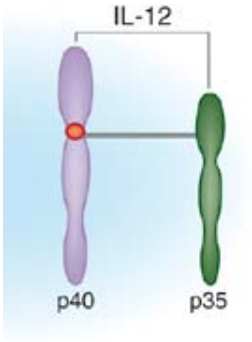

Figure 8 – Schematic representation from IL-12 showing the two subunits p40 and p35. *Adapted from Fitch et al., 2007*

Early in the immune response, IL-12 plays a critical role in directing the development of T_H1 versus T_H2 cell differentiation, characterized by an increased production of IFN-γ and IL-2 (T_H1 cytokines) and suppression of IL-4, IL-5 and IL-10 (T_H2 cytokines) formation. (Haskó and Szabó, 1999) The unique ability of IL-12 to direct T_H1 development and cellular immunity explains its key role in the development of certain inflammatory autoimmune disorders. Indeed, inhibiting the action of IL-12 has been shown to prevent development and block progression of disease in experimental models of autoimmunity. (D'Ambrosio *et al.*, 1998)

The production of IL-12 both by macrophages and dendritic cells can be induced by two different mechanisms – T-cell independent and T-cell dependent mechanisms. The T-cell independent pathway involves the interaction of the producer cells with various micro-organisms or microbial products. The T-cell dependent pathway involves stimulation of CD40 on antigen-presenting cells with membrane soluble CD40 ligand during T lymphocyte-antigen-presenting cell interactions.

1.4.1.1 IL-12 target

The main targets of IL-12 are T lymphocytes and natural killer (NK) cells, but IL-12 also affects the function of B lymphocytes and hematopoietic progenitor cells. (Haskó and Szabó, 1999) It exerts its effects by binding to specific cell surface IL-12 receptors on its target cells. The high affinity IL-12 receptor is formed by the co-expression of two subunits, the IL-12Rβ1 and IL-12R β2. While both subunits are responsible for providing

the binding energy, the IL-12Rβ2 is essential for signal transduction (Figure 9). IL-12 receptor stimulation in target cells activates the receptor-associated tyrosine kinases JAK2 and TYK2 and the transcription factors STAT3 and STAT4. (Haskó and Szabó, 1999)

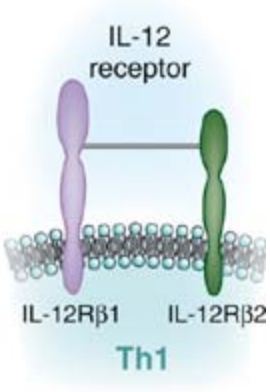

Figure 9 – Schematic representation from IL-12 receptor showing the IL-12Rβ1 and IL-12 Rβ2 subunits. *Adapted from Fitch et al., 2007*

1.4.1.2 Pharmacological modulation of IL-12 production

The induction of IL-12 production is a highly regulated process involving multiple pathways, which provides several targets for modulation by small molecular weight compounds. The most important targets are the cyclic AMP-protein kinase system, the glucocorticoid receptor and the transcription factor nuclear factor κB (NF-κB) .(Haskó and Szabó, 1999) These will be discussed below.

Cyclic AMP modulating strategies

Alterations in this intracellular cyclic nucleotide concentration have a profound effect on cytokine production by immune/ inflammatory cells. Inhibition of monocytes by prostaglandin E_2 (PGE_2), an agent known to elevate intracellular cyclic AMP levels, potently suppresses IL-12 production. Also, selective inhibition of Phosphodiesterases 4

(PDE4) by Rolipram for instance, also suppressed IL-12 production in rodent and primate models. (Haskó and Szabó, 1999, Schnurr *et al.*, 2005)

Elevation on intracellular cAMP has been generally associated with inhibition of lymphocyte activation. cAMP binds to and activates protein kinase A (PKA) that in turn phosphorylates several transcription factors that bind to cAMP response elements (CREs) in the DNA, named CRE-binding proteins (CREBs) (Figure 10). (Jimenez *et al.*, 2001)

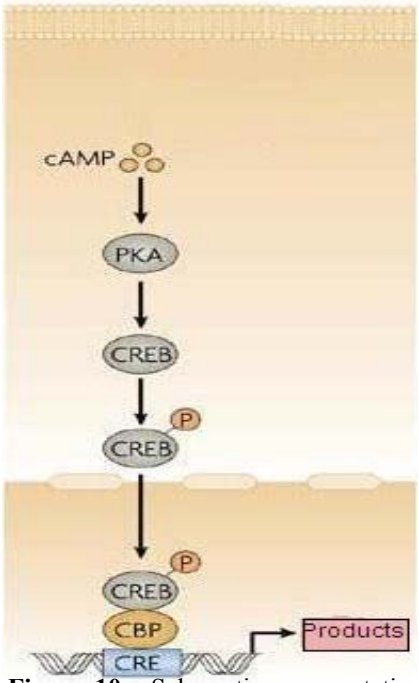

Figure 10 - Schematic representation of the cAMP pathway. When the cAMP concentration rises, it binds to the two PKA binding sites and the catalytic subunits are released. Those subunits catalyse the transfer of ATP and the CREB is activated. PKA – protein kinase A; CREB – CRE-binding proteins; CRE – cAMP response elements; P - phosphate group

PHOSPHODIESTERASE 4

The phosphodiesterase 4 (PDE4) enzymes, also termed cAMP-specific phosphodiesterases or high-affinity cAMP PDEs, specifically hydrolase cAMP with high affinity and are the targets for a class of drugs with anti-inflammatory and immunomodulatory functions.

PDE4 is encoded by four genes termed PDE4A, PDE4B, PDE4C and PDE4D. The catalytic region of PDE4 is highly conserved among the PDE4 subfamilies. Since PDE4 inhibitors bind to the catalytic site in a competitive manner, the high level of homology among PDE4 subfamilies may account for the difficulty in developing inhibitors that exhibit marked selectivity for these individual PDE4 subfamilies. The prototypic inhibitor of PDE4 is Rolipram. This compound exhibits at least a 100-fold selectivity for PDE4 relative to the other 10 PDE families. Binding of Rolipram to PDE4 appears to be to the catalytic site.

Jimenez *et al.*, 2001 tested the effect of Rolipram on cytokine production. They observed that cytokines like IL-5, IL-10 (type 2 cytokines), TNF-α and IL-2 were inhibited in the presence of that compound, but INF-γ and T cell proliferation in response to activation by CD3 are poorly affected. They also stated that the inhibition of TNF-α and IL-2 happened at the molecular level because a decrease in the transcriptional activity was noticed. Their results also demonstrate that Rolipram supresses NF-κB and NFAT activation. Therefore they defend that the effect of the PDE inhibitors - such as Rolipram – on cytokine transcription may be attributed to their ability to inhibit NF-κB and NFAT activation.

It is likely that the effect of PDE4 inhibitors in the *in vitro* transcription of a particular cytokine may depend on the relative contribution of AP-1 and CREB (enhanced by Rolipram) *versus* NF-κB and NFAT (inhibited by Rolipram) to the transcriptional activation of their respective promoters. Some activities resulting from PDE4 inhibition (such as TNF-α and NF-κB suppression) are likely due to stimulation of the cAMP-PKA pathway, whereas others are due to its ability to block other cellular functions such as NFAT activation, which does not seem to involve this pathway, since the attempt to mimic PDE4 by increasing the levels of cAMP with dBcAMP (Dibutyryl cAMP) did not inhibit NFAT activation. (Jimenez *et al.*, 2001)

Glucocorticoid Receptor

Glucocorticoids are used therapeutically as potent anti-inflammatory and immunosuppressive agents for a wide range of diseases, including auto-immune diseases, allergic states and other inflammatory illnesses. (Haskó and Szabó, 1999) It has been demonstrated that glucocorticoid hormones are important regulators of IL-12 production. It has been showed previously that Dexamethasone inhibits bioactive IL-12 release from monocytes, macrophages, dendritic cells, leading in all this cases to a decrease in the capacity of these cells to drive a T_H1 response but enhances their ability to direct T_H2 cytokine production. (Haskó and Szabó, 1999)

Targeting NF-κB

Several studies have identified putative NF-κB sites in the promoter regions of both IL-12 p35 and p40 genes. It has also been showed that inhibition of this transcription factor system can decrease IL-12 production. Also, the 1,25-dihydroxyvitamin D_3 inhibits IL-12 production not by binding to the vitamin D_3 receptor, but via inhibition of NF-κB binding to its consensus sequence on the IL-12p40 gene. (Haskó and Szabó, 1999)

1.4.2 Interleukin-23

The IL-23 was found during a genome scan for the IL-6/IL-12 cytokine family. IL-23 is a heterodimer, sharing a p40 subunit with IL-12 but having a distinct p19 subunit. IL-23 binds to IL-12Rβ1 but not to IL-12Rβ2. The receptor for this cytokine is heterodimeric and uses a novel second subunit, IL-23R, which is a member of the hematopoietin receptor family (Figure 11). The IL-23 production in monocytes occurs after stimulation via a Toll—Like Receptor by LPS. Although IL-12 strongly activates naïve T cells, IL-23 was reported to have preferential actions on memory T cells to increase IFN-γ production and proliferation. (Lee *et al.,* 2004)

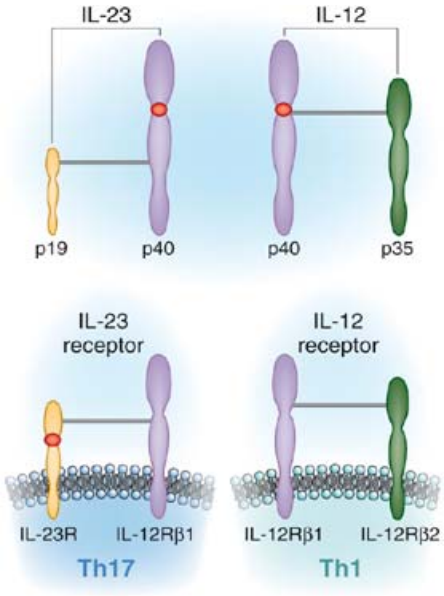

Figure 11–Schematic representation of the IL-12 and IL-23 sharing a common subunit p40 and a common subunit in their receptors – IL-12Rβ1. *Adapted from Fitch et al., 2007*

1.4.2.1 IL-23 and T_H17 in psoriasis

Wilson *et. al.*, 2007 identified a population of CD4+CD45RO+ memory T helper cells present in human peripheral blood that expressed IL-23R and produced more IL-17A than their IL-23R- counterparts. These IL-23R$^+$ memory T helper cells had the same distinct 'signature' cytokine and chemokine profile as *in vitro*-polarized T_H17 cells, suggesting that human helper T cells derived by IL-23 *in vitro* constitute an accurate representation of a subset of helper T cells normally present in the peripheral blood of healthy humans. IL-23 which makes T_H17 cells survive and proliferate has emerged as a master cytokine in the new paradigm of psoriasis pathogenesis. (Blauvelt, 2008) It is thought that the pathologies associated with T_H17 cells probably occur after deregulation of the appropriate 'checks' that control T_H17 cell proliferation or cytokine production. (Wilson *et al.*, 2007)

Chan *et. al.*, 2006 demonstrated that intradermal delivery of IL-23 protein in mice results in acute skin histopathology that shares many features with psoriasis, such as acanthosis (epithelial hyperplasia) and parakeratosis (keratinocyte nuclei in the stratum corneum).

An IL-12p40 antagonist that blocks IL-23 and IL-12p70 simultaneously was successful in a phase I psoriasis clinical trial, and IL-23p19, but not IL-12p35 gene expression was associated with human psoriasis (Chan *et al.*, 2006).

1.4.2.2 IL-23 and IL-17A in psoriasis

The human psoriatic data demonstrate that IL-17A gene expression is associated with this disease. (Chan *et al.*, 2006)

Chan *et al*., 2006 used a blocking monoclonal antibody to IL-17A to determine whether IL-17A is required for IL-23-dependent epidermal thickening. Anti-IL-17A did not inhibit IL-23 stimulated acanthosis, nor did it affect IL-23 dependent erythema, induration and parakeratosis.

These data and the observation that direct IL-17A delivery has minimal effect on epidermal hyperplasia suggest that IL-17A, although downstream of IL-23 and associated with human psoriasis, is not required for disease pathogenesis. (Chan *et al.*, 2006)

1.4.2.3 IL-23 and Tumor Necrosis Factor-α

In contrast to anti-IL17A, anti-TNF treatment partially inhibited IL-23-dependent acanthosis, and the degree of erythema and induration was decreased. (Chan *et al.*, 2006)

IL-23 stimulates macrophage TNF production and IL-23p19 transgenic mice have elevated serum TNF levels. Chang *et. al.* found that neutralizing TNF partially inhibited epidermal hyperplasia induced by IL-23. This observation is consistent with the efficacy of anti-TNF-direct therapies in psoriasis. TNF inhibition breaks the self-sustaining nature of psoriatic lesions by rapidly down-regulating several pro-inflammatory genes, including IL-23p19. (Chan *et al.*, 2006)

1.4.2.4 IL-23 genetic data

A genome-wide screen has identified the gene encoding IL-23R as a 'psoriasis-susceptibility gene'. Wilson *et. al.*, 2007 found that lesional skin samples from patients with psoriasis, but not skin samples from healthy donors, contained DCs that expressed the IL-23p19. These data confirm published results showing that IL-23 mRNA is upregulated in psoriasis and suggests that IL-23 may participate in the pathology of psoriasis. (Wilson *et al.*, 2007)

As a summary of the described above, deregulated cutaneous IL-23 production seems to set in motion several independent pathways. IL-23 contributes to the antimicrobial nature of psoriatic lesions by stimulating IL-17A and neutrophil recruitment. In parallel, IL-23 stimulates IL-19 and IL-24, which may directly act on keratinocytes in a TNF-regulated manner resulting in epidermal hyperplasia and/or altered keratinocyte differentiation. These functionally different arms suggest that IL-23 may have evolved as a "response to danger" cytokine invoking the body to protect itself by rapidly mobilizing anti-microbial components and instructing the epidermis to proliferate to provide additional protection from the environment. (Chan *et al.,* 2006)

Because of that and of the fact that there's an increased expression of IL-23 in the psoriatic lesional skin which contributes to the maintenance of the chronic inflammatory process, IL-23 is an interesting and valid target for pharmacological intervention. (Piskin *et al.*, 2006)

1.5 LIPOPOLYSACCHARIDE AND INTERFERON-γ PATHWAYS

The Lipopolysaccharide (LPS) is a large molecule consisting of a lipid and a polysaccharide (carbohydrate) joined by a covalent bond. It is the major component of the outer membrane of Gram-negative bacteria (and *Listeria monocytogenes*), contributing greatly to the structural integrity of the bacteria, and protecting the membrane from certain kinds of chemical attack. It is an endotoxin and induces a strong response from normal animal immune systems.

LPS acts as the prototypical endotoxin because binds the CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines in many cell types.

1.5.1 The Toll-Like Receptor 4 signaling

Toll-Like Receptors (TLRs) are members of the superfamily of Interleukin-1 receptors (IL-1Rs). They share significant homology in their cytoplasmatic regions.

Toll-like receptor 4, also known as **TLR4**, is a toll-like receptor. It detects lipopolysaccharide on Gram-negative bacteria and is thus important in the activation of the innate immune system.

The protein encoded by this gene is a member of the Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity. TLRs are highly conserved from Drosophila to humans and share structural and functional similarities. They recognize pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. The various TLRs exhibit different patterns of expression. This receptor is most abundantly expressed in placenta, and in myelomonocytic subpopulation of the leukocytes. It has been implicated in signal transduction events induced by lipopolysaccharide (LPS) found in most gram-negative bacteria. Mutations in this gene have been associated with differences in LPS responsiveness. Also, several transcript variants of this gene have been found, but the protein coding potential of most of them is uncertain.

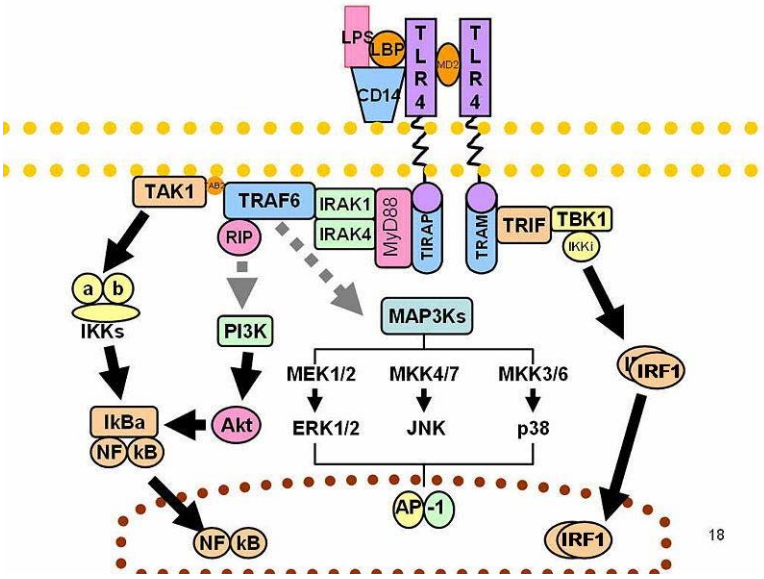

Figure 12 - The membrane proximal events in LPS signaling involve docking of MyD88 and IRAKs to th TLR, followed by recruitment of TRAF6 which then associates with downstream kinases of the MAP kinasekinase kinase family, resulting in activation of MAP kinase pathways and the IKK complex, ultimately leading to activation of the NF-kB. Other transcription factors activated by LPS include AP-1. **MyD88** – adaptor molecule, **IRAKs** – IL-1RI associated protein kinases, **TLR** – Toll Like Receptor, **TAK1** – TGF-β-activated kinase, **TRAF6** – tumor necrosis factor receptor-associated factor 6., **MAP kinase** – Mitogen-Activated protein kinase, **IKK** – IkB kinase, **AP-1** – Activator Protein 1. *Adapted from www.wikipedia.org*

MyD88 is recruited to TLR as a dimmer (Figure 12). MyD88 was originally isolated as a myeloid differentiation (MyD) primary response gene that is rapidly induced upon IL-6-stimulated differentiation of M1 myeloleukemic cells into macrophages. It consists in 3 domains: an N-terminal death domain (DD) separated from its C-terminal TIR domain by a short linker sequence. MyD88 promotes association with the IRAK1 and IRAK4. That association is mediated through a DD-DD interaction. If the C-terminal TIR domain of MyD88 is expressed alone, it acts as a dominant-negative inhibitor of TLR4 signaling, preventing the IRAK association with the receptors. IRAK4 phosphorylate IRAK1. TRAF6 is recruited to the IRAK1. The complex IRAK4/IRAK1/TRAF6 disengages from the receptor and reach the cell membrane. The complex than meets and interacts with another complex containing the TAK1. The TAK1 is phosphorylated and translocate with TRAF6 to the cytosol which leads to its activation and posterior activation of IKKs. Inactive IKKs sequesters NF-kappa B in the cytoplasm. IKKs activation leads to phosphorylation and degradation of IkappaB, and consequent

release of NF-kappaB. Activation of TAK1 also results in the activation of MAP kinases and JNK. (Paludan, 2000)

The activation of both NF-kB and AP-1 by TRAF6 involves a MAP3K which is activated along with TAK1, MEKK3 and ASK1, leading to downstream JNK, p38 and IKK activation. However, ASK1 is not activated by TRAF6 until it dissociates from thioredoxin (TrX), a process dependent on ROS presumably produced by LPS-activated membrane-bound NADPH-oxidases. Free ASK1 will then interact with TRAF6, resulting in p38-MAPK activation. ERK activation is mediated by the MAP3K Tpl2.

MyD88 also couples to interferon-regulatory factor 5 (IRF5) and IRF1. In the latter case, MyD88 traffics to the nucleus with IRF1. A bridging adaptor, MAL (MyD88-adaptor-like protein), is required for MyD88 recruitment. This is subject to regulation by BTK (Bruton's tyrosine kinase) and SOCS1 (suppressor of cytokine signalling 1), which promotes MAL degradation. IRF1 encodes interferon regulatory factor 1, a member of the interferon regulatory transcription factor (IRF) family. IRF1 serves as an activator of interferons alpha and beta transcription, and in mouse it has been shown to be required for double-stranded RNA induction of these genes. IRF1 also functions as a transcription activator of genes induced by interferons alpha, beta, and gamma. (Paludan, 2000)

1.5.2 The Interferon-γ signaling pathway

Interferon-γ (IFN-γ) is secreted by Th1 cells, Tc cells, dendritic cells and NK cells. Activation by IFN-γ is achieved by its interaction with a heterodimeric receptor consisting of IFNGR1 & IFNGR2 (interferon gamma receptors). IFN-γ binding to the receptor activates the JAK-STAT pathway. (Paludan, 2000)

The **JAK-STAT signaling pathway** takes part in the regulation of cellular responses to cytokines and growth factors. Employing Janus kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs), the pathway transduces the signal carried by these extracellular polypeptides to the cell nucleus, where activated STAT proteins modify gene expression. Although STATs were originally discovered as targets of Janus kinases, it has now become apparent that certain stimuli can activate them independently of JAKs. (Figure 13)

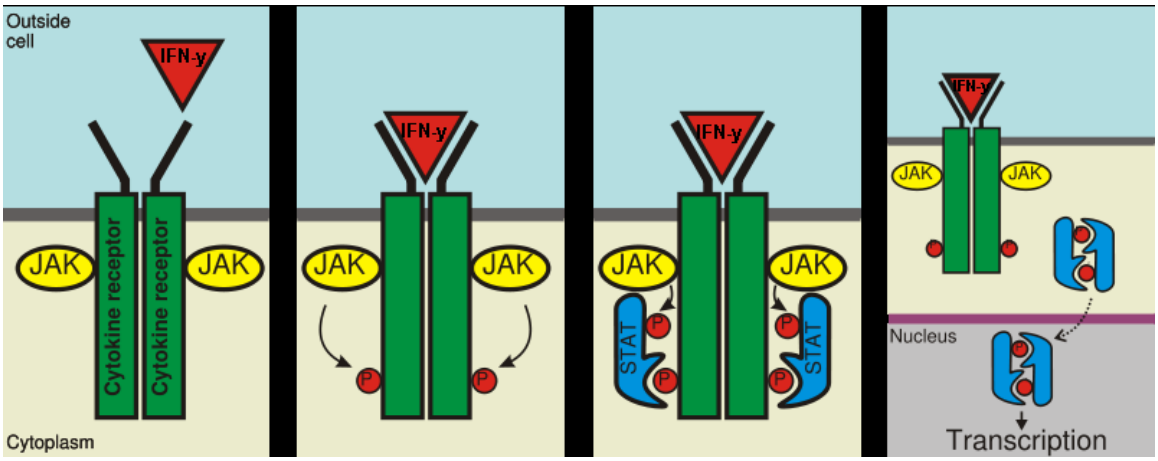

Figure 13 - Signaling from the IFN-γ receptor complex involves the tyrosine kinases Jak1 and Jak2, which become activated upon ligand binding. The principal substrae for IFN-γ-activated Jak1 and Jak2 is STAT1. Tyrsine-phosphorylated STAT1 dissociates from the IFN-γ receptor complex and forms homodimers that translocate to the nucleus and induce transcription via binding to GAS in IFN-γ-inducible promoters. One of the STAT1-induced genes is IRF-1. IRF-1 is itself a transcription factor that recognizes the sequence termed ISRE and plays, like STAT1, a central role in IFN-γ-induced gene expression. STAT1 – Signal Transducer and Activator of transcription 1, GAS – Gamma Activating Site, ISRE – Interferon Stimulation Response Element. Adapted from *www.wikipedia.org*

JAKs, which have tyrosine kinase activity, bind to some cell surface cytokine receptors. The binding of the ligand to the receptor triggers activation of JAKs. With increased kinase activity, they phosphorylate tyrosine residues on the receptor and create sites for interaction with proteins that contain phosphotyrosine-binding SH2 domain. STATs possessing SH2 domains capable of binding these phosphotyrosine residues are recruited to the receptors, and are themselves tyrosine-phosphorylated by JAKs. These phosphotyrosines then act as docking sites for SH2 domains of other STATs, mediating their dimerisation. Different STATs form hetero- as well as homodimers. Activated STAT dimers accumulate in the cell nucleus and activate transcription of their target genes. STATs may also be tyrosine-phosphorylated directly by receptor tyrosine kinases, such as the epidermal growth factor receptor as well as by non-receptor tyrosine kinases, such as c-src.

The pathway is negatively regulated on multiple levels. Protein tyrosine phosphatases remove phosphates from cytokine receptors as well as activated STATs. More recently identified Suppressors of Cytokine Signaling (SOCS) inhibit STAT phosphorylation by binding and inhibiting JAKs or competing with STATs for phosphotyrosine binding sites on cytokine receptors. STATs are also negatively regulated by Protein Inhibitors of Activated STATs (PIAS), which act in the nucleus through

several mechanisms. For example, PIAS1 and PIAS3 inhibit transcriptional activation by STAT1 and STAT3 respectively by binding and blocking access to the DNA sequences they recognize.

1.5.3 The synergy between pathways

Many inflammatory events triggered by LPS are enhanced synergistically by IFN-γ, thus enforcing the antibacterial and potentially host-damaging reactions at the sites of bacterial infection. (Paludan, 2000)

As described above, IFN- γ induces activation of the transcription factor STAT1, which in turn triggers production of IRF-1. These two proteins independently activate transcription and are believed to be largely responsible for the transcription of IFN- γ -induced gene. In addition to their independent action, recent studies have shown that a certain degree of cooperation between IRF-1 and STAT1 is observed in promoters containing binding sites for both factors [31–33]. Because IRF-1 is also induced by LPS this cooperation between IRF-1 and STAT1 could possibly contribute to synergistic gene induction.

The major contributor to generation of synergistic promoter activation, however, appears to be synergistic action of STAT1/IRF-1 with NF-κB. The vast majority of promoters induced synergistically by IFN- and LPS containing binding sites for STAT1 or IRF-1 and NF-κB. By a range of different experimental approaches it has been shown that NF-κB strongly enhances transcription from promoters activated by IRF-1 or STAT1. A number of studies have addressed in greater detail how the transcription factors induced the observed synergistic promoter activation. *A priori*, at least two scenarios can be envisaged. First, the transcription factors could bind independently to their respective sites on the promoter and generate a surface with enhanced interaction with the basal transcription machinery. Second, the transcription factors could interact physically on the promoter, thus forming a complex binding with higher avidity to the recognition sites than the transcription factors individually.

For the synergistic action of STAT1 and NF-κB, data seem to indicate that the two transcription factors bind DNA independently, thus supporting the first possibility described above as the mechanism. By contrast, the mechanism underlying the synergistic action or IRF-1 and NF-κB has been extensively studied and there is evidence by different experimental approaches that IRF-1 and NF-κB interact physically. It is

interesting that κB and the Interferon Stimulation Response Element (ISRE) sites are often juxtaposed in promoters induced synergistically by IRF-1 and NF-κB, so physical interaction is likely to play a functional role. A theoretical model based on the structures of IRF-1 and the Nf-κB p50 homodimer complexed to their respective binding sites in the IFN-beta promoter shows that the DNA bending imposed by IRF-1 actually brings IRF-1 into closer contact with NF-κB. The creation of bends and loops within promoters brings together DNA binding factors that interact with distantly located recognition sites. These transcription-factor-dense promoter regions then in turn interact with the basal transcriptional machinery and thereby contribute to synergistic effects on gene transcription. Such multi-factor transcription initiation complexes, generally termed enhanceosomes, have long been thought to be important for maximal and sustained transcription.

A study by Kovarik *et al.* showed that LPS augments IFN-g induced STAT1 activity independent of NF-κB activation. It has previously been described that phosphorilation of serine 727 of STAT1 enhances the trans-activating function of the transcription factor, and the authors found that this was the mechanism by which LPS brought about its effect on STAT1.

Collectively, the data available on the molecular mechanisms underlying the synergistic activity of IFN-g and LPS suggest cooperation between IFN-γ-activated STAT1/IRF-1 and LPS-activated NF-κB as the major players. (Paludan, 2000)

The IL-23 is a major player in the pathogenesis of psoriasis. Although previously mistaken by IL-12 due to the common subunit to both cytokines, nowadays the mechanisms triggered by IL-23 are known to be more related to psoriasis and other immune diseases than those of IL-12. Several mechanisms to inhibit IL-12 were thought to be of interest against IL-23. The Phosphodiesterase 4 inhibition is one of them. IL-23 is responsible for maintaining a recently discovered T helper subset - T_H17 and the mechanisms underlying both – cytokine and T cell subset are of great interest nowadays due to the possible relationship between them and pathological conditions previously not understood or mistaken as auto-immune or T_H1 derived conditions. IL-23 is produced after LPS stimulation and IFN-γ priming *in vitro* and its deregulation *in vivo* is yet to be understood. The comprehension of the underlying mechanisms of IL-23 production and the attempt to pursuit it as a target are the main interest of this work.

In psoriasis lesions, IL-23 is overproduced by dendritic cells and macrophages in response to bacteria and in keratinocytes in response to a yet indeterminate factor. IL-23 gene expression is increased in psoriatic lesions compared with uninvolved skin, but downstream consequences of IL-23 deregulation still unclear (13). This cytokine stimulates T_H17 cells within dermis to make "T_H17 cytokines", which include IL-17A, IL-17F, TNF-α, IL-21, and IL-22 (14). IL-23 seems to be more important for the survival and population expansion of T_H17 cells than for T_H17 lineage commitment. (Wilson *et al.,*2007)

Little is known about this cytokine. The aim of the present work is to understand better the mechanisms of regulation and production of IL-23. Due to its similarity with IL-12 and the fact that PDE4 inhibitors cause a decrease in IL-12 and other cytokines production, the mechanisms of IL-23 inhibition combined with already known data for PDE4 inhibitors are further investigated as a baseline of this work.

2. OBJECTIVES

The purpose of this work was twofold:

(1) a comparison between IL-23 and PDE4 inhibition to determine whether there are structural features that are necessary for inhibition of one activity and not the other, and

(2) Structure Activity Relationship (SAR), by comparing between different compounds using only the IL-23 inhibition values, to determine what structural features are critical for IL-23 inhibition.

3. Materials and Methods

3.1 Cell Culture and Activation

All compounds tested were obtained from Anacor Pharmaceuticals, Inc. (Palo Alto, CA, USA). They were prepared as a stock solution in 100% dimethyl sulfoxide (DMSO) at 10 mM or 40 mM. The final concentration of DMSO in the cultures was <1%.

Human THP1 monocytes were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured at 2.4 x 10^6 cells per flask in 75 cm^2 Falcon flasks in media containing RPMI (without L-Glutamine, Cellgro, VA, USA) supplemented with 10% (v/v) Fetal Bovine Serum (Cellgro), 2mM of L-Glutamine (Gibco,NY,USA), 10 mM HEPES (Gibco) 1mM of sodium pyruvate (Gibco), 0.05 mM of 2-Mercaptoethanol (Gibco) and 4.5 g/L of D-(+)-Glucose (Sigma, MO, USA). Cells were grown to a density of approximately 5 x 10^5 to 8 x 10^5/mL. THP-1 cells were harvested from two to four T75 Falcon flasks and resuspended at 10^6 cells/mL in culture media. Cells were seeded at 5 x 10^4 cells per well in 96-well plates and incubated overnight in a humidified atmosphere of 37 °C and 5% CO_2.

Overnight cultures of THP-1 cells were stimulated for 48 h with 100 ng/mL IFN-γ (R & D Systems, Minneapolis, MN, USA) and 1 µg/mL LPS (from *Escherichia coli* 0127:B8, purified by phenol extraction, Sigma-Aldrich) and with or without Anacor compounds. Stimulator and inhibitor compounds were added simultaneously to cells in culture Anacor compounds were added at a range of concentrations from 0.001 to 200 µM for EC_{50} determination or at just one concentration (10µM) for screening purposes. Stimulatory / inhibitory compounds were added so that each compound concentration, untreated and LPS+IFNγ has triplicate samples

Supernatants were collected after 48 hr and cells were pelleted by microcentrifugation. The cell-free supernatants were transferred to chilled Eppendorf tubes and frozen at -20 °C.

3.2 Enzyme-Linked Immunosorbent (ELISA) Assays

Cytokine levels in supernatants were measured using ELISA kits from R&D Systems (MN, USA). The range of detection for the ELISA kit was between 39 pg/mL and 2.5 ng/mL.

The quantitative sandwich enzyme immunoassay technique consists of having a polyclonal antibody specific for the IL-23p19 subunit coated in a 96-well plate. The samples and standards containing IL-23 are added to the plate and the protein (IL-23) binds the antibody attached to the plate.

The standards are built using a kit IL-23 powder that, after reconstituted, will give a standard solution of 10,000 pg/mL. That solution is further diluted to 2500 pg/mL and a set of 7 serial dilutions is made. A zero (containing only the dilution buffer) is added and used as a blank for all samples.

Wells are washed with washing buffer in order to discard all the unbound substances. A polyclonal antibody conjugated to horseradish peroxidase, specific for the IL-23p40 subunit, is added to the wells. The second wash again removes the unbound components and a substrate solution (conjugation of hydrogen peroxide and tetramethylbenzidine) is added to the wells. This solution is the substrate for the peroxidase and color develops in proportion to the amount of enzyme present, which is directly proportional with the amount of IL-23 captured in the plate. The color development is stopped with the addition of an acid solution (2N Sulfuric Acid) and the intensity of the color is measured at the wavelength of 450 nm with correction at 570 nm for optical imperfections in the plate.

3.3 Assay Optimization

Two aspects of the assay were optimized in order to give more reproducible results.

First two different kits, one from R&D and one from eBiosciences were compared in two different experiments. Cells were seeded and compounds and controls added to the different wells. The samples were measured in both kits. The eBiosciences kit repeatedly gave low values for the control (sample with IFNγ 100ng/mL and LPS 1µg/mL) although the range of detection stated by the kit was wider (15 pg/mL to 2.0 ng/mL) than the one of R&D (39 pg/mL to 2.5 ng/mL). The percent of inhibition for an Anacor compound at 10 µM was calculated as well as its $IC_{50,}$ and the eBiosciences kit failed to give reproducible results. The R&D proved to give reproducible results and was able to measure cytokine levels with good accuracy even at low concentrations, for example, the sample without compounds but with LPS and IFN-γ should give high values of IL-23, but

that failed to happen in eBiosciences but not in the R&D kit, giving values of -32.541 pg/mL for the first and 163.759 pg/mL in the second kit.

Since the amount of cells needed for each assay was enormous and in order to overcome the limiting step of cell growth, the assay was performed in a 96-well plate and compared to the 24-well plate model that was being used as standard Anacor procedure. Samples from a 24 and 96-well plates were tested in the R&D kit and the IL-23 levels compared. The percent of inhibition of Anacor compounds at 10 µM and its IC_{50} value were similar regardless of whether the assay was performed in 24- or 96-well plates. Therefore the 96-well plates were used from then on because they allow more compounds to be tested with the same amount of cells.

3.4 Data Analysis

The Optical Densities (ODs) were measured at the end of the ELISA using a plate reader set to wavelengths of 450 nm and 570 nm. A correction for optical imperfections in the is made subtracting the readings at 570 from the ones at 450 nm, the read outs of the samples and standards were processed in SoftMaxPro (Molecular Devices, Sunnyvale, CA, USA). The blank O.D. is subtracted from all values and a standard curve is made plotting the O.D.s read versus the standards solution concentrations. The curve is a 4-parameter fit with a general equation of y = (A-D)/(1+(x/C)^B)+D. The IL-23 concentration (pg/mL) of the samples is calculated by SoftMaxPro according to the curve equation and the O.D.s obtained from the plate reader. A standard deviation is calculated based on the triplicate values for the same sample.

The half maximal inhibitory concentration (IC_{50}) calculations were performed using GraphPad from Prism Software (v. 4.0, San Diego, CA, USA). A table of Logarithm of compound concentration versus the concentration of IL-23 in the sample is made and a graph is plotted. The IC_{50} is determined by Prism by calculating the concentration of Anacor compound needed to inhibit 50% of the IL-23 production in that assay. The maximum amount of IL-23 produced was the sample stimulated with IFNγ and LPS, and the minimum value should be the sample with the 200 µM of Anacor compound. Unless the compound is not effective inhibiting the IL-23, the curve should have a well defined sigmoid shape. All IC_{50} values should have a narrow 95% confidence interval.

The percent of inhibition for a single compound concentration was calculated using the formula: $100 - \left[\frac{IL-23 concentration_{sample with Anacor compound}(pg/mL)}{IL-23 concentration_{sample(LPS+IFN\gamma)}(pg/mL)} \times 100 \right]$

4. RESULTS

Screening different Anacor compounds in a model that measures cellular production of IL-23 enabled us to test a broad range of compounds at different concentrations, providing us with important data that allowed analysis of the relationship of compound structure to activity (ability to inhibit IL-23 production).

Since IL-23 is a recent target, little is known about its regulation and mechanism of action. In part because of that, putting together an assay that allowed us to optimize cytokine production and also finding a method that allowed us to quantify the IL-23 in the samples with a high throughput and with reproducible results, was not always a straight forward task. The IC_{50} (concentration at which 50% of the activity in the assay is inhibited) is a measure of the effectiveness of a compound to inhibit biological or biochemical function. So far, not all the compounds displayed have the correspondent IC_{50}, while others have already repeats. For these last ones, the IC_{50} value is displayed as an average of all results, the 95% confidence interval is displayed as the lower limit and the higher limit of all repeats and the R^2 is displayed as an interval of R^2 greater than the lowest value achieved for all repeats. Therefore prudence is needed once these results are analyzed since that the confidence interval and even the R^2 interval might seem too wide when in fact it just represents several intervals (as many as the number of experiments).

The purpose of this work was twofold (1) a comparison between IL-23 and PDE4 inhibition to determine whether there are structural features that are necessary for inhibition of one activity and not the other, and (2) Structure Activity Relationship (SAR), by comparing between different compounds using only the IL-23 inhibition values, to determine what structural features are critical for IL-23 inhibition.

For the compounds in Table 1 (Annex I) it is possible to establish a relationship between structure and activity, based only on IL-23, since just a few PDE4 data is available.

As one can see, the best compound listed on the Table 1 (Annex I) is AN2728, which allows one to conclude that the 5-position oxygen linker compounds are the most suitable to be basis of an SAR. In fact, the 5-position linker is effective only when a cyano is in the “para” position (compare AN2728 with AN2887).

The 6-position sulfur linkers do not have activity against IL-23. Several 6- position sulfur-linker compounds with different substituents in various positions were tested and

the percent of inhibition at 10μM was never higher than 43%. One should be aware that when sulfur, sulfoxide or sulfone linkers are used in macrophages, some of these compounds may be toxic for this cell type, therefore and until further investigation is made in the toxicity process, the chemical development of this group of compounds was temporarily suspended.

Taking AN2728 structure as a start, it was of interest to determine whether the cyano-phenoxy ring could be moved around the aromatic ring in the main structure. In Table 2 (Annex I), a set of 4 compounds is shown whose structures differ only in the position of the cyanophenoxy ring.

The only compound showing a reasonable half inhibitory value (IC_{50}) for both IL-23 and PDE4 was AN2728. These data enable us to see that the "para" cyano phenoxy ring is only active in the 5-position, not just for IL-23 but also for PDE4.

In order to determine the importance of the cyano group in the "para" position of the phenoxy ring, compounds containing non-cyano substituents were tested. Data are displayed in Table 3 (Annex I).

From data in Table 3, one may conclude that the cyano at the "para" position is critical for activity against IL-23, since no compound listed gave a percent of IL-23 inhibition at 10 μM greater than 49. So, from SAR data, one can conclude that the "para" cyano is critical for IL-23 inhibitory activity.

On the other hand for PDE4, other compounds like AN3675 show similar IC_{50} value to AN2728 (0.45 and 0.49 μM, respectively). Compounds like AN2903 and AN3165 show even better activity against PDE4 than AN2728 (0.1 and 0.29 μM versus 0.49 μM, respectively), implying that the "para" cyano is not critical for inhibition of PDE4 activity.

From these results, we can see clearly for the first time a lack of correlation between the ability to inhibit IL-23 and PDE4.

It was of further interest to determine whether the cyano could have activity in another position on the phenoxy ring besides the "para" position. Therefore, comparing the structures in Table 4 (Annex I), one can see that the "para" position cyano is the one that has activity against IL-23, since that compounds with IC_{50} values greater than 10μM are not considered to be potent cytokine inhibitors. In this particular case, the PDE4 data is also affected by the move of the cyano around the ring, increasing from 0.49 μM for

AN2728 ("para") to 3.72 and 4.40 μM for AN2876 ("ortho") and AN2906 ("meta"), respectively.

For IL-23 in particular, the best IC_{50} value so far obtained is still too high in the point of view of drug development. To further optimize the activity of compounds as IL-23 inhibitors, additional modifications were made to the AN2728 basic structure; some groups were added in the "meta" position, as shown in Table 5 (Annex I).

In some cases, there is an improvement in IL-23 inhibition. Compounds like AN2898 and AN3187 prove that. For the first 3 compounds of Table 5 (Annex I), the values for IL-23 and PDE4 vary in the same relationship, i.e., the compound with lower IC_{50} value for IL-23 is also the one that shows the lower IC_{50} value for PDE4 (compound AN2898 with 1.01μM IC_{50} value for IL-23 and 0.06μM IC_{50} value for PDE4), the one with the second lowest IC_{50} value for IL-23 is also the one with the second lowest value for PDE4 (compound AN3142 with 2.58μM IC_{50} value for IL-23 and 0.14μM IC_{50} value for PDE4), and finally AN3141 with 2.71μM IC_{50} value for IL-23 and 0.15μM IC_{50} value for PDE4.

For the remaining compounds that correlation does not always exist. Comparing AN2898, AN3187, AN3348, AN3398 and AN3399, we see that the IC_{50} values for PDE4 are in a similar range, i.e., we can say that they are equally potent inhibiting the enzyme. On the other hand for IL-23, all share similar percent of inhibition values, except for AN3348, which has almost no activity inhibiting the cytokine. Once again the fact that two compounds show similar potency inhibiting PDE4, doesn't necessarily allow us to predict how their behavior will be on IL-23 inhibition.

In a general manner, an addition of a second group in the "meta" position improved the compound activity; AN3163, AN3169, AN3348 and AN3437 are exceptions.

Regarding compounds with groups in the "ortho" position, they were grouped according to the type of substituent: a carbon-linked or single atom in the "ortho" position (Table 6, Annex I). In Table 7 (Annex 1) compounds are listed that have an oxygen-linked "ortho" addition.

Compounds AN3221, AN3274 and AN3275 in Table 7 of Annex I, have similar IC_{50} values for PDE4, however the IL-23 inhibitions at 10 μM vary significantly between these compounds.

The best compounds inhibiting IL-23 in Table 7, Annex I are AN3219, AN3275 and AN3296 having a percent of inhibition at 10 μM of 76, 88 and 82%, in that order. If one would look just to the values of percent of inhibition, one would think that AN3275 and

AN3296 were expected to be the best ones nevertheless, after IC_{50} determination, AN3219 seems to be not just the best of the three, but also the best of all compounds showed so far.

After a lot of additions were made in the basic structure of AN2728, it was thought that more profound alterations could be made in the ring itself to determine how these changes would affect IL-23 results. Therefore, a pyridine structure replaced the phenyl ring and these compounds were tested. In Table 8 of Annex I the pyridine analog of AN2728 are shown and compared to compounds containing the phenyl ring.

While for IL-23 the values are in the same range, for PDE4 the compound containing the pyridine seems to improve activity although the significance of the difference observed between both values might not be significant.

As shown in Table 9, the addition of a pyridine ring only improved IL-23 activity for the pairs AN3187/AN3317 and AN339/AN3827. In these compound substituting the pyridine ring resulted in no major change in PDE4 inhibition. On the other hand, comparing the pair AN3165/AN3171 in terms of PDE4 inhibition, one can say that the pyridine analog has better activity compared to the non-pyridine structure.

For the remaining compounds, comparisons couldn't be made in terms of IL-23 inhibition since that the percent of inhibition at 10μM is less than 40%, which makes it impossible to look for improvements between non-active compounds.

Other pairs of analog compounds must be built and conclusions have to be made regarding the advantage of including nitrogen on the ring.

One of the more interesting compounds in the pyridine-containing family of compounds was selected for further SAR analysis: AN3317.

Analogs of AN3317 were synthesized and the results are shown in Table 10 of Annex I.

Of the compounds displayed, AN3317, AN3715, AN3716, AN3783 and AN3826 all have IC_{50} values of less than 1μM. Also, while for PDE4 the IC_{50} values are all quite similar (from 0.01 to 0.04 μM), for IL-23 the range is wider going from 0.27 to 0.80 μM. When the IC_{50} value for PDE4 inhibition rises up to 0.19μM (AN3828) and then to 0.55μM (AN3874), the same happens to IL-23 IC_{50} values that are 1.48μM for AN3828 and 7.95μM for AN3874. Again and as seen in the first tables, the PDE4 and IL-23 do not correlate at 100%.

Regarding IL-23 the results seem promising in that so far this set of compounds shows best activity against this cytokine. The phenyl analogs of these compounds are either not part of the Anacor compound library or were never tested for IL-23.

Since the best IL-23 inhibitors seem to be those showed in Table 10, other compounds with the pyridine on their structure were tested and the results are present in the Table 11 (Annex I). All the compounds displayed in Table 11 (Annex I) are structures with a pyridine ring and with groups in the "para" position that are not the cyano molecule. Looking at those results, one can see that changes in PDE4 inhibition do not predict changes in IL-23 inhibition and that all compounds have medium to good PDE4 inhibition values, but none of them is a good IL-23 inhibitor. This shows the importance of the cyano in the "para" position for IL-23 inhibition.

Known PDE4 inhibitors which are commercially available were tested against IL-23 to check for inhibition results. As we can see in Table 12 (Annex I), no dose responses for IL-23 inhibition can be observed for any of the four known PDE4 inhibitors tested..

The results in Table 12 (Annex I) reinforce the statement that IL-23 and PDE4 inhibition aren't necessarily associated.

In agreement with these observations, one Anacor compound gave reasonable inhibition against IL-23, but not against PDE4: AN3056. The results are presented in Table 13 (Annex I).

Despite the similarity between AN3054 and AN3056, AN3054 has no activity against IL-23. Analogs for this pair were synthesized and the values for IL-23 and PDE4 (if available) inhibitions are displayed in Table 14 (Annex I).

The compounds AN3484 and AN3485 are the most potent ones inhibiting IL-23. It's interesting to notice that the addition of a Fluorine (F) and more important, a Chlorine (Cl) in the "ortho" position of AN3054 completely changes the potency of the compounds. This might be a beginning of an interesting SAR around this main structure.

As one can imagine, several different compounds were tested to search for activity against IL-23 in order not just to look for a good percent of inhibition or IC_{50}, but also to look for a better group of compounds even if they were structurally different from the basic molecule of AN2728. Listed in Table 15 (Annex I) are those compounds and their respective values for IL-23 and PDE4.

There are several aspects that need to be considered when comparing between IL-23 inhibition and PDE4 inhibition. One of them is that IL-23 assay is cell based, which differs from PDE4 assay. Other aspect is that the only information for IL-23 available so

far comes from a few scientific papers that point out several pathways for IL-23 synthesis. This means that the actual pathway for IL-23 biosynthesis is not known, and specific target molecules are not known. For PDE4 the crystal structure is available and Anacor compounds have been crystallized in the active site of the molecule.

Figure 14 illustrates a schematic representation of a PDE4 crystal structure and how a compound like AN2898 perfectly fits into the pocket of the active site of the enzyme and how AN3056 (no PDE4 inhibition) doesn't:

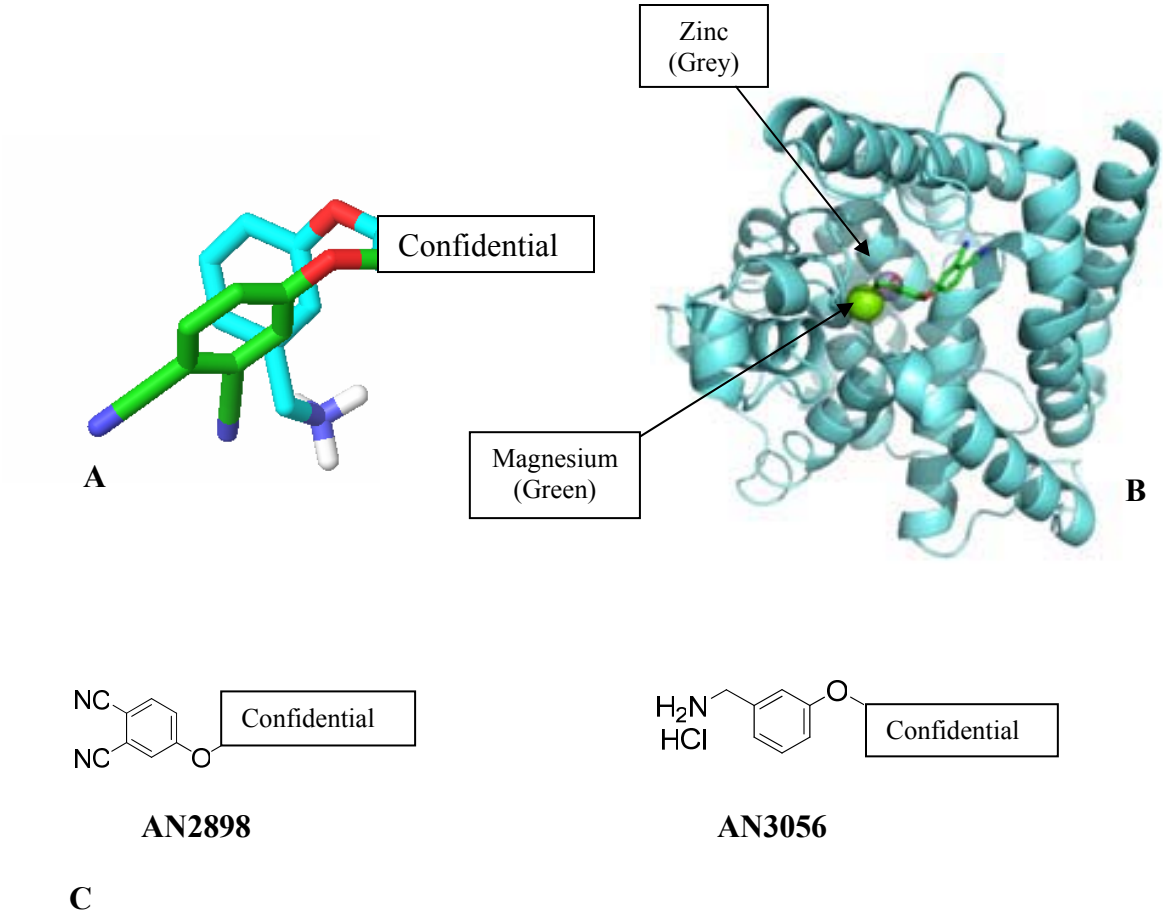

Figure 14 - Schematic representation of (A) AN2898 which is represented in green and AN3056 which is represented in light blue. We can see that the geometries of the two structures are completely different. (B) Schematic representation of PDE4 crystal structure. There are two metal groups in the enzyme marked in grey for Zinc and green for Magnesium (adopted from reference 11). (C) AN2898 and AN3056 molecules. *Adapted from Sanders et al.,2008*

The geometry of the two compounds is totally different which doesn't allow AN3056 to fit the active site pocket of PDE4. The two cyano in the "para" and "meta" positions form links with the enzyme – hydrophobic packing - fitting perfectly in the active site

pocket. Having AN3056 hydrophilic substituent's (-NH_2) there is no possibility of forming the hydrophobic packing mentioned above.

5. DISCUSSION

In order to improve activity of already existing compounds, some attachments were made to the chemical structures of different molecules. The Anacor library of compounds is built according to the effect that those small changes cause in the biological models of investigation.

In order to try to understand the relationship between IL-23 inhibition and PDE4 inhibition, a Structure Activity Relationship (SAR) study was performed. Anacor compound structures were compared with respect to effects on both IL-23 inhibition and PDE4 inhibition to attempt to determine whether specific molecular structures were necessary for one activity or the other.

Some compounds available at Anacor library, contain a sulfide, sulfoxide or sulfone linker between the "confidential" part of the molecular and the structures that have been discussed. These compounds failed to inhibit IL-23 production (Table1, Annex I, rows 4 to 11). Also the only compound containing a Nitrogen linker didn't show good activity against IL-23 (Table 1, Annex I, row 20). In a general way, the 6-position-oxygen linker compounds (Table 1, Annex I, Rows 12 to 19) are not IL-23 inhibitors. A compound containing a 5-position oxygen link gave better activity against IL-23; therefore the SAR was built by making successive slight changes in that compound (AN2728). Having AN2887 (see Table 1, Annex I) as basic structure (which has no additions to the phenyl ring) and adding different groups in the different positions of the phenyl ring, one can see the difference in inhibition values for IL-23. By comparing AN2887 and AN2728, observations can be made immediately. The addition of a Cyano group in the "para" position of AN2887 makes the compound shift from a non-active to an active one against IL-23. (0% on AN2887 versus 53% on AN2728, Table1, Annex I).

Several compounds were tested for IL-23 and PDE4 inhibition in order to understand if the "para" cyano-phenoxy could be moved around the aromatic ring without a loss in IL-23 and/ or PDE4 activity. As one can see in Table 2 (Annex I), only AN2728 has good inhibition against IL-23, meaning that the "para" cyano – phenoxy structure have to be in that specific location. Also for PDE4 the best structure is AN2728. The crystal structure of PDE4 has been elucidated (11) (Figure 13) and Anacor compounds have been modeled into the active site. AN2728 and 2898 have both been crystallized into the active site of the PDE4B. Those studies proved that the interactions between different parts of the molecules with the active site of the enzyme are critical for compound activity. Once the target is known, the compound synthesis can be adjusted to that knowledge and an improvement in inhibition can be achieved easier. We do know that for PDE4, the cyano in "para" and "meta" positions forms hydrophobic packing with PDE4 structure, being that hydrophobic packing necessary for PDE4 inhibition for this type of compounds. It is therefore important that the group in the "para" position allows hydrophobic interactions between the compound and the enzyme (for example, hydrophilic groups in that position wouldn't be part of an active compound against PDE4).

Regarding IL-23 and its regulation, one can only speculate about the target. To some extent a target profile can be drawn based on SAR information. After that, the existent candidate targets (for example, members of the NF-kB family and, in theory, all members of the IFN-γ and LPS signaling pathway – Figures 11 and 12) can be checked if they fit or not the profile, but in order to do so, numerous compounds have to be screened against these targets in a manner similar to what has been done for this study.

Having AN2887 as base-structure and a positive effect of adding a Cyano in the "para" position, other compounds were added in the same "para" position and IL-23 inhibition was tested for those. The compounds structure and results can be viewed in Table 3 (Annex I). All compounds showed percent of inhibition of IL-23 at 10 μM which was lower than 49%. It is legitimate to say that 49% is quite close to 53% obtained for AN2728 and therefore AN2893 and/ or AN3653 could also be compounds with interesting activities against IL-23. No IC_{50} data is available for the effect of AN2893 on IL-23 inhibition, but for AN3653 the IC_{50} value confirms that for this specific compound, the low percent inhibition means low activity inhibiting IL-23. One must not forget that the results for AN2728 were an average of 11 different experiments, and the result of AN3653 and AN2893 are an average of only 2 experiments.

Despite of all that, AN2893 with 49% IL-23 inhibition at 10 μM could still be a comparable compound to AN2728, but one can predict that it should have an IC_{50} value much lower than 3.6 μM of AN2728, overall bringing no contribution in improving capacity for IL-23 inhibition. All things considered and in a perfect situation where the values displayed for all compounds were the average of at least 3 experiments, one could

see if compounds like AN2893 with low to medium inhibition at 10 μM show an IC_{50} value suitable to pursuit SAR on those structures.

In Table 3 (Annex I), comparing all of the groups tested in the "para" position, one could be considered somewhat similar to cyano and this is the methoxy group of compound AN3654. The methoxy is a small group and is equally a hydrogen bonding acceptor like cyano but, as one can see, the percent of inhibition for IL-23 is only 5%, much lower than the inhibition for AN2728 (53%). That fact could be explained by the geometry of the methoxy group.

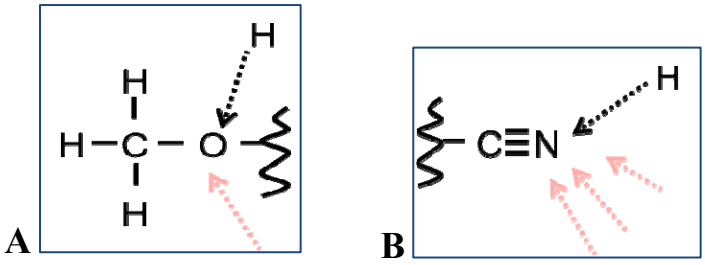

Figure 15 - Schematic representation of the differences between the geometry of a methoxy group (A) *versus* a cyano (B) group. The arrows represent multiple angles of hydrogen bonding to the oxygen (A) or to the nitrogen (B).

The fact that the cyano is a linear group allows an easier interaction with the water hydrogen since the nitrogen atom has no bulky attachments that could prevent or decrease the contact between the nitrogen group and the hydrogen from water. On the other hand, the oxygen in the methoxy is surrounded by the methyl group, which forms a bulky cap around it, decreasing the capacity of hydrogen bounding, in comparison to the cyano molecule on AN2728 (Figure 15).

Examining PDE4 inhibition of compounds in Table 3 (Annex I), one can see that some of them have better PDE4 inhibition values than to AN2728. AN2903 and AN3165 have lower IC_{50}'s against PDE4 but as we mentioned before, not against IL-23. From these compounds, we see for the first time a clear difference between effects on PDE4 inhibition and IL-23 inhibition. The cyano group in the "para" position seems required in order to have IL-23 inhibition, but for PDE4, an ester, ketone or trifluoro methyl (AN3165, AN2903 and AN3675, respectively) seem to keep or improve the compound potency to inhibit PDE4.

It was of interest to determine whether the Cyano group could be moved around the phenyl ring to improve compound activity against IL-23. The compounds described in Table 4 (Annex I) were synthesized. In this particular case, to inhibit both IL-23 and PDE4, the cyano must be in the "para" position. For PDE4, the geometry of AN2728, gives a perfect fit in the active site pocket of the enzyme and it's easy to understand why the cyano move around the phenyl ring makes the compound loose its activity. Apparently for IL-23, there is something unique about the cyano in the "para" position that allows AN2728 to have activity against IL-23 that no other compound described here has.

The next logical step was to investigate the combination of the cyano in the "para" position with other groups in "meta" and "ortho" positions.

Several compounds were added in the "meta" position and the results for PDE4 and IL-23 are shown in Table 5 (Annex I). The addition of another cyano in the "meta" position improved the activity against both IL-23 and PDE4. For PDE4 the second cyano in this position increased the hydrophobic packing of the molecule (comparing to AN2728), which provided a more potent inhibition of the enzyme. It's interesting to see that if there is a methoxy group in the "meta" position of a molecule that already has a cyano in the "para" position (AN3187), the compound has similar activity to the one that has two cyanos : one "para" and one "meta" (AN2898). If we have two methoxy groups in the "para" and "meta" positions (AN3674 in Table 15, Annex I, row 14), activity against IL-23 is lost. As discussed above, the methoxy is a group with similar strength in accepting an hydrogen bond, to cyano, but the activity of the compound with methoxy is only comparable to the one containing a cyano (AN2898) if the compound already has a cyano in the "para" position and the methoxy is a the "meta" position.

Interestingly, if we compare AN3187 (Table 5, Annex I, row 7) with AN3220 (Table 7, Annex I, row 2), the methoxy group must be in the "meta" position, because in the "ortho" position activity is lost against IL-23.

AN3163 (Table 5, Annex I, row 5) was expected to have activity against IL-23 as it did for other cytokines (a broad panel of cytokines is tested as part of Anacor routine procedures). In fact, pairs of esters and their correspondent carboxylic acids were synthesized in order to check for the assumed activity of the esters and assumed non-activity of their carboxylic acid analogs. This could potentially be of use in drug development since that the compound would be hydrolyzed by esterases in the blood stream, loosing its anti-inflammatory potency. This would be of use if there was a need to restrict distribution of a drug to specific, defined areas of the patient's body. This theory applied when other cytokines, for example TNF-α, IFN-γ, IL-2 etc were tested for

inhibition, but not for IL-23. The possibility of THP1 cells having rich content in esterases might explain the fact that the ester groups are hydrolyzed before they hit the target for IL-23.

Comparing AN3348 with AN3398, both compounds have esters in the "para" position but the fact that for AN3398 the ester is bulky and not linear as in AN3348, may results in protection against hydrolyses, which could explain the increased activity in AN3398 compared to AN3348.

AN3155 is more hydrophobic than AN2898, due to the trifluoro methyl group. This might explain the difference in IC_{50} values compared to AN2898. Increased hydrophobicity could explain the fact that AN3675 (Table 3, Annex I, row 13) showed no activity to inhibit IL-23compared to AN2728 (Table 1, Annex I).

Compounds with a second group in the "ortho" position (when the "para" position is occupied by a cyano group), were grouped according to the type of group in that position, whether it had a carbon or oxygen linking the group to the phenyl ring, or if there was single atom group in the "ortho" position.

From Table 6 (Annex I), the most potent compounds are AN3276 (row 9), AN3405 (row 12) and AN3153 (row 1). For AN3153, the R^2 and 95% Confidence Interval (C.I.) values are not as one could desire and since this compound has a single atom group in the "ortho" position, no further SAR analysis could be performed until other compounds with chlorine in other positions were synthesized.

Comparisons can be made between AN3276, AN3405 and others with similar structure. AN3276 and AN3278 (Table 6, Annex I, rows 9 and 10) are, respectively, an aldehyde and an alcohol. The fact that the aldehyde is more compact while the "-OH" from the alcohol can swing around the carbon, might explain the fact that the first has better activity against IL-23 than the second.

AN3405 and AN3406 (Table 6, Annex I, rows 12 and 13) can be compared since in the "ortho" position the AN3405 has a morpholine group while the AN3406 has a methyl-piperazine in the "ortho" position, differing from the first one only by a methyl and a nitrogen. Though, their activity against IL-23 shows a difference from 80% inhibition at 10μM for AN3405 to only 37% inhibition at 10μM for AN3406. That difference could be explained by the fact that the "ortho" group in AN3406 is more polar than the one on the same position for AN3405. The compound polarity can be other factor that influences the activity against IL-23. If we look to the pair AN3296 and AN3297 (Table 7, Annex I, rows 8 and 9), one can also see that there are some similarities in the "ortho" group structure and the most polar compound of these two (AN3297) is also the less potent.

The continuous compound screening will eventually enlighten more details about the type of groups that can or cannot be in the "ortho" and even the "meta" position in compounds with this general structure.

As we look at Table 7 (Annex I), the most potent compounds against IL-23 are AN3219 (row 1) and AN3275 (row 7).

It's curious to see that for compound AN3296 that has 82% inhibition at 10 μM, the IC_{50} value doesn't go below 3 μM, the same range as AN2728 that has 53% of inhibition at 10 μM. It is therefore important to calculate IC_{50}'s in several compounds in order to have a more accurate feeling for these possible results. Although the fact that the IC_{50} value for AN3296 is 3.81 μM doesn't rule out this compound as a good IL-23 inhibitor, it simply doesn't make him as potent as expected by looking just to the percent of inhibition at 10 μM.

Looking now for three particular compounds present in Tables 6 and 7 of Annex I, AN3220 (Table 7, Annex I, row 2), AN3202 (Table 6, Annex I, row 3) and AN3219 (able 7, Annex I, row 1), the "ortho" groups in these three compounds are somewhat similar. A methoxy (AN3220) group in the "ortho" position doesn't give activity against IL-23, while a methyl group improves the activity against the cytokine and a hydroxy group increases even more that activity. One possible explanation is that the size of the group in the "ortho" position is determinant to IL-23 inhibition. The fact that methoxy is bigger than methyl, which is bigger than hydroxy, could explain the different activities observed against IL-23. Again, for PDE4, the activities for these three compounds are quite similar.

So far, the polarity and/ or the size of group in the "ortho" position is important for activity against IL-23. As mentioned before, more data has to be collected in order to make safe conclusions about the type of substituent in the "ortho" position.

Making changes in the phenyl ring is another possibility to search for compound activity in IL-23 and PDE4. As we can see (Tables 8 and 9, Annex I), for IL-23 in compounds with low and medium potency against the cytokine, there is not an improvement on IL-23 inhibition (compounds with low potency: Table 8, Annex I, rows 1 and 2, Table 9, Annex I, rows 5 and 6). As for compounds with good IC_{50} against IL-23, Table 9, Annex I rows 3 and 4 and particularly rows 7 and 8, an improvement is observed. For PDE4 there is an improvement in all compounds except for AN3187 and AN3399 (Table 9, rows 3/ 4 and 7/8, respectively) which activity was maintained and AN2861/ AN3148 (Table 9, Annex I, rows 1/ 2) were active. From Table 9, Annex I we one can also see that also for the compounds containing the pyridine ring in their structure, the "para" cyano is important since only AN3317 has good IC_{50} (and its phenyl analog).

Another set of compounds containing a pyridine ring and cyano in the "para" position of that ring, were tested and the results are shown in Table 10 (Annex I). The main

difference between Tables 8, 9 and 10 (Annex I) is that in the last one, no phenyl analog was either tested or is available at Anacor library. All the compounds have the "para" cyano, while in the previous ones, a comparison between phenyl ring and pyridine ring was ment to be made. So, all compounds in Table 10 (Annex I) have good activity and so would probably have their phenyl analogs if they were available. As mentioned before, for this set of compounds, there seems to be a correlation between IL-23 and PDE4 inhibition. If one would just look to this table, one would easily assume that inhibiting PDE4 would lead to an IL-23 inhibition since for the compound AN3874 (row 9, Table 10) when compared to the remaining compounds in that table, has a lower potency inhibiting the PDE4 and that matches a decrease in potency against IL-23 for that specific compound and in comparison with all others in that table. This corroborates the importance of screening several compounds with several differences in their chemical structure which will prevent that conclusions like that are not made based only in the observation of a limited number of compounds behavior. The data from Table 10 (Annex I) together with all data showed here imply that much more complex mechanisms are present in IL-23 signaling pathway and that although the numerous similarities with IL-12, the modulation of IL-23 production happens by different pathways.

AN3199 (Table 11, Annex I, row 6) could be an interesting compound depending on their IC50 value since 43% inhibition could be an indicator of a reasonable activity against IL-23 and this compound not just doesn't have the cyano in the "para" position, as it has an ester there, which could be enough to build in a complete SAR for this particular structure. But once again, 43% is a result of a simple experiment therefore further analysis has to be made to determine with more accuracy the potency of such compound. The usefulness of screening tests is the ability to have a high throughput of data in a relatively short period of time. It is valuable to make comparisons between compounds, since all of them are tested in the same conditions and without having a positive control (meaning, a compound that is known to be potent against IL-23) the only possible comparison to be made is between Anacor compounds. Nonetheless, slight differences in potency can be overlooked in a search for a compound with better potency. The main objective of screening compounds is different from the one of this work. In screening, and having a compound with medium potency, the aim is to have a better one in every experiment made. In this work, one should always question a mid-potency compound not the importance of a medium value of percent of inhibition, but for it's contribution to the whole picture, meaning to the reveal of which type of chemical groups could improve the compound potency, if not that specific one, then potentially other with a quite different basic structure.

Once again the cyano in the "para" position is not required for PDE4 inhibition, but that seems not to be true for IL-23 inhibition.

Some of the known and commercially available PDE4 inhibitors like Rolipram, Ibudilast, Cilomilast and Roflumilast were tested for IL-23 inhibition and as expected, none gave a dose response (Table 12, Annex I). Actually from the 3 distinct times that Rolipram was tested, it appears that an increase of IL-23 production occurs at the highest concentration. That particularity was not seen in the other PDE4 inhibitors tested and showed in Table 13 neither for Anacor compounds that inhibit PDE4.

Table 13 (Annex I) shows a recent discovery and a whole new world of possibilities in terms of SAR. Two similar compounds AN3054 and AN3056 have different activities against IL-23 by itself and comparing to PDE4. AN3056 has a totally different chemical structure when compared to AN2728. The oxygen linker is in the 6-position and not on the 5-position, the substituent group is in the "meta" position and not in the "para" (which is AN3054 and has no activity) and that substituent group is hydrophilic unlike the cyano group, which is exactly the main characteristic for the loss of activity against PDE4. All the compounds tested for IL-23 and described here could only fit into two groups: compounds that inhibit PDE4 and also inhibit IL-23 or compounds that inhibit PDE4 but don't inhibit IL-23. With AN3056, a new group was started: compounds that don't inhibit PDE4 but inhibit IL-23. Although is impossible to say what characteristic of this compound allows it to inhibit IL-23, regarding PDE4 one can compare it structure and spatial conformation (Figure 14) with AN2898 and find out that not just the angles are different, but also the fact that AN3056 has an amine group that is hydrophilic and therefore doesn't allow the hydrophobic packing that makes AN2898 potent against PDE4. This particular fact, makes the screening of those type of compounds interesting from the point of view of IL-23 inhibition, since like was mentioned before, building an SAR with compounds that do not inhibit PDE4 and comparing that to compounds that inhibit IL-23 and PDE4 might be an interesting way of creating a profile of a compound of interest. It's interesting to see that the amine group moved to the "para" position doesn't make a potent compound against IL-23.

Interestingly and looking to Table 14 (Annex I), all compounds are derivatives of AN3054 and some of them like AN3484 and AN3485 showed activity against IL-23. Apparently, the addition of fluorine or even better, chlorine in the "ortho" position, improved the compound activity. There is an interesting path to follow here in terms of SAR for IL-23 inhibition where a single atom group could be placed in different positions of the AN3054 and AN3056 molecules and the IL-23 inhibition would be measured. As discussed previously, such compounds shouldn't fall in the category of "PDE4 inhibitors" due to its incapacity to form the hydrophobic packing necessary for PDE4 inhibition.

Further analysis of the signaling pathway by means of blocking separately each component of the proposed pathway and parallel compound screening will hopefully enlighten the method of inhibiting IL-23. Since it has been demonstrated by the work that has been presented here that some Anacor compounds have good activity to inhibit IL-23 and this may be an important tool in the treatment of pathological conditions like psoriasis, determining the mechanism of action of these compounds would be an important goal.

It was largely proved by this work that, unlike IL-12, which could be inhibited by a PDE4 inhibitor, and increases in cyclic AMP (cAMP) levels, the same is not true for IL-23. This may predict a difference in the signaling pathway for IL-23 compared to IL-12.

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