PARADOXICAL ONSET OF PSORIASIS AFTER IL-6 RECEPTOR BLOCKADE

by

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Psoriasis is a chronic inflammatory skin disease that affects approximately 2% of the world’s population. Accumulated knowledge regarding the role of IL-6 in psoriasis makes it a good candidate for therapy. However, several reports have now demonstrated the onset and/or worsening of psoriasis after treatment with the humanized anti-IL-6R antibody, tocilizumab. Given this paradoxical onset of psoriasis after tocilizumab treatment we hypothesized that under inflammatory conditions, the blockade of IL-6R causes IL-6 to be liberated into the periphery where it can bind alternative receptors causing off-target effects that, in the presence of TGF-β and IL-23, may cause the differentiation of naïve CD4 cells towards a Th17 phenotype and away from the suppressive regulatory T cell phenotype. Th17 cells can then produce other pro-inflammatory cytokines such as IL-17 and IL-22, which have been shown to be required for initiating and sustaining epidermal hyperplasia associated with psoriasis.
I. Introduction

Psoriasis is a chronic inflammatory disease of the skin that affects approximately 0.5-1% of children and 2-3% of the world’s population, which includes 7.5 million adults in the United States [2]. Psoriasis is a bimodally- distributed disease with a major age of onset at 20-30 years of age and a later smaller peak of onset at 50-60 years [7]. Psoriasis etiology remains unknown, it is thought to be multifactorial with key components including genetic susceptibility and environmental triggers in combination with skin barrier disruption and immune dysfunction [8]. There are five subtypes of psoriasis: vulgaris (plaque), guttate, pustular, inverse and erythrodermic. The most common variation of psoriasis is plaque psoriasis, which affects approximately 85-90% of psoriatic patients [8], [10].

Psoriasis has a detrimental impact on patient quality of life caused by significant physical and psychological distress and impairment [11],[12]. Based on a recent publication, the majority of psoriasis patients expressed feeling stigmatized by the disease as well as feelings of shame and guilt [13]. Psoriasis also affects interpersonal relationships of patients and impacts sexual well-being and capacity for intimacy primarily via poor self-esteem and sexual self-image [14, 15]. In addition, a negative social impact in the workplace associated with discrimination and difficulty in finding employment among psoriasis patients, have been observed. Nearly 60% of employed psoriasis patients reported lost time from work in the
previous year due to psoriasis [16].

Complications arising from psoriasis cause numerous deaths in the United States each year [17]. Furthermore, psoriasis has been associated with several comorbidities including cardiovascular disease, obesity, diabetes, metabolic syndrome, inflammatory bowel disease and psoriatic arthritis that contribute to decreased longevity among psoriasis patients [18-21].

**II. Clinical presentation of psoriasis**

Psoriasis has multiple cutaneous presentations including plaques, pustular, guttate, erythrodermic and inverse. Plaque psoriasis is the most common manifestation of the disease, affecting approximately 80% of the patient population [22].

![Figure 1. Phenotypic and histological manifestation of plaque psoriasis.](image)

Figure 1. Phenotypic and histological manifestation of plaque psoriasis. A) plaque psoriasis is characterized by demarcated red scaly plaques. As compared to healthy skin (B), psoriatic lesional skin (C) contains thickened cell layers (acanthosis), elongated rete ridges (black arrows), thickened squamous layer (parakeratosis), increased number and size of dermal blood vessels and inflammatory cell infiltrates (Modified from [3]).

Plaque psoriasis is phenotypically characterized by red, scaly, well-
defined, silvery-white, dry plaques (Figure 1A) [3]. These lesions are generally symmetrically distributed and preferentially affect elbows, knees, scalp, and the lumbar area. New lesions may appear after direct cutaneous trauma, known as the Koebner response. Also, when manipulated, lesions can be pruritic and bleed, which is known as Auspitz sign [22]. Plaque psoriasis is characterized by a crosstalk between keratinocyte and dermal vascular endothelial cell and immune cells. The classical histology is described as demarcated epidermal thickening acanthosis), thickening of stratum corneum (hyperkeratosis), squamous cell layer thickening due to retention of nuclei in the upper layers of the epidermis (parakeratosis), elongation of epidermal rete ridges, increased number and size of dermal blood vessels and inflammatory cell infiltrate of neutrophils in the stratum corneum and epidermis, also known as Munro’s microabscesses and Kogoj pustules, as well as significant mononuclear and leukocyte infiltration (mostly T cells and dermal dendritic cells (DCs)) in the dermis (Figure 1C) [3].

III. Psoriasis triggers

a. Genetic susceptibility in psoriasis

Even though psoriasis etiology remains unknown, previous studies have reported that psoriasis results in-part from gene polymorphisms that are related to skin barrier function and immune dysfunction [23]. Genetic studies in twins demonstrated an incidence of approximately 70% probability of monozygotic twins to be affected by psoriasis and
approximately 20% in dizygotic twins [24] [25]. Furthermore, pedigree studies reported that children have a 20% chance of developing psoriasis if only one parent has the disease but 65% if both parents are affected [26]. Also, more than 30 single nucleotide polymorphisms (SNPs) have been associated with increased susceptibility to psoriasis risk [27]. At least nine different psoriasis susceptibility loci (PSORS1-9) have been identified [27]. However, only two gene mutations (IL36RN and CARD14) have been found to independently induce psoriasis by affecting the skin and the immune system [28].

The first susceptibility locus associated with psoriasis (PSORS1) was identified on chromosome 6p21. This region has been shown to have the greatest impact on psoriasis heritability but the identity of its responsible gene remains controversial. PSORS1 is located within the MHC class I and it has been hypothesized that HLA-C is the most likely gene [29]. Altered HLA haplotypes encoded within this susceptibility locus have been shown to be over-represented in psoriasis patients. The most popular and strongest psoriasis associated haplotype is HLACw6 [30]. Even when there is a strong genetic association between HLA-C and HLACw6 and psoriasis risk, the exact role of these loci remains uncertain.

As previously mentioned, even though several psoriasis susceptibility loci have been identified [27], only two gene mutations (IL36RN and CARD14) have been found to independently induce psoriasis by
affecting the skin and the immune system [28]. Genome wide studies identified PSORS2 on chromosome 17q25 [31], [32], which encodes for caspase recruitment domain protein 14 (CARD14). Gain of function mutation in the CARD14 region of this locus has been found in next generation sequencing of patients with familial psoriasis [33, 34]. CARD14 mutations induce increased activation of the NF-κB signaling pathway, which leads to expression of key psoriatic chemokines including; CCL20, CXCL8/IL-8 and IL-36γ/IL-1F9 [28].

Gene sequencing data also identified IL36RN, also known as IL-1F5, to be an important gene associated with psoriasis development [35, 36]. IL36RN encodes for the anti-inflammatory protein IL-36Ra, a natural antagonist of IL-1F9. In plaque psoriasis, levels of IL-36Ra have been shown to be abundant while absence of IL-36Ra, in pustular psoriasis, has been associated with excessive neutrophil accumulation [35, 37].

Since IL-36R is an IL-1 family member associated with NF-kB activation, mutations in IL-36Ra lead to loss of function resulting in unrestrained pro-inflammatory response of IL-36 stimulating cytokines [35, 37].

Large genome-wide association studies (GWAS) have identified significant single nucleotide polymorphisms (SNPs) in psoriasis, many of which are associated with the IL-23/IL-17 axis [38]. Recent meta-GWAS analysis confirmed 21 SNPs and identified 15 new SNPs that had increased frequencies in psoriasis patients when compared to controls.
These SNPs were associated with processes implicated in psoriasis such as keratinocyte differentiation, T and NK cell proliferation, JAK-STAT cascade activation, regulation of Th1 and Th17 cells as well as leukocyte adhesion [39]. Also, a recent study identified a SNP in the \textit{IL23} locus that may have functional relevance due to the role of IL-23 over IL-17 responses and T cell stimulation [40].

In the case of \textit{IL-6} and \textit{STAT3} polymorphisms, their link with predisposition for developing psoriasis is controversial because some have found no significant association [41] while others indicated that genetic variations in IL-6 decreased the risk for developing psoriasis [42]. Also, IL-6 gene promoter polymorphisms have been associated with therapeutic response to TNF-alpha inhibitors [43].

\textbf{b. Epigenetic changes in psoriasis}

Epigenetic modifications comprise heritable and reversible modifications that alter expression of a specific gene without resulting in direct changes in their primary DNA sequence [44], [45]. The most common epigenetic mechanisms are DNA methylation, histone modifications, and genetic regulation by miRNA [46], [47], [48] [49], [50]. Although the full effect of epigenetic changes in psoriasis remain unknown, changes such as these are undoubtedly contributing to psoriasis risk alleles and disease development.

DNA methylation is the transcriptional silencing of a gene by direct covalent modifications of nucleotides, primarily cytosine at cytosine-
guanine (CpG) dinucleotide sequences [51]. DNA methylation can cause transient or irreversible gene modifications. Usually DNA methylation causes repression of gene expression but in some cases it can cause activation if it prevents binding or limits expression of transcriptional repressors. The human epigenome contains more than $10^8$ cytosine nucleotides, of which more than $10^7$ are in CpG [52], [52]. Furthermore, approximately 70% of the human genes are linked to promoter CpG islands [52].

The first study that evaluated tissue-specific DNA methylation status in human disease showed that the tyrosine phosphatase promoter SHP-1, isoform II, is de-methylated in psoriasis [52]. SHP-1 isoform II has also been associated with negative regulation of hematopoietic cell proliferation and differentiation [53], [54]. In addition, abnormal DNA methylation in skin lesions and on PBMCs has been demonstrated in psoriasis vulgaris patients [55]. This study demonstrated that psoriatic PBMCs are hyper-methylated when compared to healthy individuals. Furthermore, tumor suppressor gene $\text{p16}^{\text{INK4a}}$ is repressed in psoriatic plaques and its promoter region is hyper-methylated [56], which causes p53 levels to be reduced in keratinocytes, leading to keratinocyte hyper-proliferation.

The first study of global CpG methylation in psoriasis determined the methylation levels at 27,578 CpG sites in unaffected, involved and uninvolved psoriatic skin. Based on these results, CpG methylation of
involved skin was shown to differ from unaffected skin in 1,108 sites, 12 of which mapped to an epidermal differentiation complex, upstream or within genes that are highly regulated in psoriasis such as KYNU, OAS2, S100A12 and SERPINB3 [52]. Hence, many of the genes observed to have the greatest methylation differences were expressed by keratinocytes. Furthermore, they observed that uninvolved skin has intermediate levels of differential CpG methylations, which might be an indicator of psoriasis predisposition. Also, when they examined the effect of TNF- alpha neutralization on CpG methylation, they observed that after one month, the methylation levels were changing towards levels observed in uninvolved skin [52]. Therefore, CpG methylation changes might account for treatment response as well as disease remission, however this remains to be further confirmed.

DNA methylation also plays an important role in regulation of gene transcription during naïve CD4⁺ T cell polarization. A recent report showed differential DNA methylation changes in CD4⁺ T cells from a pair of monozygotic twins discordant for psoriasis [57]. Another supporting publication examined the genome-wide DNA methylation profile of CD4⁺ T cells between psoriasis, atopic dermatitis and healthy individuals [58]. They showed that only psoriasis naïve CD4⁺ T cells have distinct hypomethylation in 26 regions and increased DNA methylation at the promoter region of genes on the X chromosome [58]. These results may
indicate that epigenetic changes in naïve CD4$^+$ T cells may predispose them to be activated in psoriasis, however further evaluation of the effects of these patterns need to be performed.

Another important epigenetic change is histone modification. Histone modifications can be driven and maintained by CpG methylation. This post-translational modification alters core histone proteins, which pack DNA into nucleosomes. Histones can be modified by phosphorylation, ubiquitination, methylation and acetylation, all of which can alter different processes including binding affinity of proteins and regulation of chromatin structure [59]. The human epigenome consists of more than $10^8$ histone tails that can be potentially modified [60]. In psoriasis, histone deacetylase-1 (HDAC-1) mRNA expression is overexpressed in lesional skin [61]. Also, global histone H4 hypo-acetylation has been shown in psoriatic PBMCs, which had a negative correlation to disease activity [62].

A third epigenetic marker important in psoriasis is Micro-RNA (miRNA). Micro-RNAs have important post-transcriptional roles in regulation of gene expression. To date, more than 100 miRNAs have been identified in the skin [63]. The role of miRNAs in skin morphology has been shown using a keratinocyte-specific Dicer knockout mouse model [64]. Loss of keratinocyte-specific Dicer expression caused distinct skin defects that affected both the epithelium and epithelial-mesenchymal cell signaling causing hyper-proliferation and absence of increased apoptosis.
Expressions of miRNAs differ between normal, involved and uninvolved psoriatic skin [66]. Next-generation sequencing from normal and psoriatic skin miRNA demonstrated 80 known and 18 new miRNAs with differential expression in psoriasis particularly the anti-strand of miR-203 locus, which is important in epithelial differentiation [67]. miR-203 is exclusively expressed in keratinocytes and is upregulated in psoriatic plaques [67]. Up-regulation of miR-203 was consistent with the down-regulation of suppressor of cytokine signaling 3 (SOCS3), a negative regulator of STAT3 signaling. SOCS3 deficiency leads to sustain IL-6 dependent STAT3 activation in keratinocytes, which have been shown to cause spontaneous psoriasis development in transgenic mice [68]. Furthermore, miR-203 has been reported to be related to keratinocyte stem cell differentiation as well as suppressor of p63, a transcription factor that is essential for epidermis formation [69]. Interestingly, miR-203 has been shown to be down-regulated in uninvolved psoriatic skin when compared to both normal and involved skin [69], which may indicate predisposition of uninvolved keratinocytes toward hyperproliferation, however this remains to be confirmed.

Another miRNA that has been identified as important in psoriasis is miR-99a [64]. This microRNA has been proposed to be important in keratinocyte differentiation and has been implicated in regulation of insulin-like growth factor 1 receptor (IGF-1R) signaling in keratinocytes. IGF-1R activation inhibits differentiation and promotes keratinocyte proliferation.
IGF-1R was further confirmed to enhance expression of miR-99a that, in turn, inhibits IGF-1R expression causing a negative feedback between proliferation and differentiation of keratinocytes [64].

Lerman et al. also reported another microRNA, miR-197, to be down-regulated in psoriatic skin lesions when compared to normal or uninvolved skin. miR-197 decreases keratinocyte proliferation and migration in vitro but increases the expression of the keratinocyte differentiation markers keratin 10 and involucrin. They further established the IL-22R subunit IL-22RA1 as a miR-197 target [71]. IL-22 signaling has been identified as necessary for skin hyperplasia, and is thought to play a substantial role in psoriasis pathogenesis. Therefore, miR-197 regulation of IL-22 signaling suggests that this microRNA is a modulator of keratinocyte and the immune system, which might have an important role in psoriasis development.

A recent study identified miR-424 to be important for regulation of the MEK1/cyclin E1 proliferation pathway in keratinocytes. miR-424 was found to be downregulated in psoriatic skin when compared to normal and atopic dermatitis skin [72].

Other differentially expressed miRNAs have been identified in psoriasis including miR-22, miR-24-1, miR-498 and miR-551a, which are upregulated in both involved and uninvolved skin and may be involved in inhibition of metalloprotease activation during epidermal proliferation [59].

Finally, the microRNA, miR-146a, has been suggested to have a
role in psoriasis development because it is involved in regulation of innate responses and TNF-alpha pathway signaling in the skin [73], although recent evidence suggests a plausible role in atopic dermatitis as well [74, 75].

c. Environmental triggers of psoriasis

The exact mechanism for initiation of psoriasis remains unknown. However, several environmental factors in combination with genetic susceptibility and immune response have been associated with a predisposition for psoriasis development as well with exacerbation of the disease. Some of the most well established environmental factors associated with psoriasis include physical trauma, drug induction, obesity, alcohol consumption and smoking.

Physical trauma

Physical trauma as a triggering factor for psoriasis was first described by Heinrich Koebner [76]. He reported that psoriatic lesions could form after a direct cutaneous injury in previously normal-appearing skin. Furthermore, the new psoriatic lesion would have the same morphology as the injury. This phenomenon it not exclusive to psoriasis since it has also been associated with vitiligo and lichen planus, however its frequency is higher among psoriasis patients with a prevalence ranging from 24-51% of patients [77].

Drug- induction
Onset of psoriasis, as well as worsening, has been associated with numerous drugs. The most common drug inducers of psoriasis are lithium, beta- blockers, anti-malarials, tetracyclines and non-steroidal anti-inflammatory medications [78-81].

In recent years, induction or exacerbation of psoriasis has also been associated with the use of neutralizing biologics against TNF-alpha in rheumatoid arthritis [82], [83] [84] [85] and Crohn’s disease patients [86], [87]. This is a paradoxical response because TNF-alpha inhibitors were approved as therapy against psoriasis since TNF-alpha plays an important role in the inflammatory response of the disease [88]. The same phenomenon has also been observed in patients with rheumatoid arthritis treated with IL-6R blockers [89], [90], [91], [92]. Since IL-6 is produced at high levels in critical T cell microenvironments in psoriatic skin [93] causing STAT3 hyper-phosphorylation further causing T effector cells to be refractory to regulatory T cell suppression [94] and because increased IL-6 serum and skin levels are hallmarks of psoriasis [95, 96], the onset of psoriasis after IL-6R inhibition is paradoxical and study it might help to understand how this disease develops.

**Infections**

As with drug-induction, infections, especially among children have been associated with psoriasis development [97]. Recent studies report that streptococcal throat infections can trigger and exacerbate psoriasis [98], [99], [100]. Also, *Staphylococcus aureus*, *Malassezia and*
Candida albicans colonization in the gut and/or skin has been linked to psoriasis exacerbation [98], [99], [100]. The initiation of psoriasis after infections has caused the development of a theory that reports bacterial exotoxins and peptidoglycans as super-antigens that may activate T cells leading to psoriasis development [101-104].

Stress

The immune system is directly influenced by psychological distress and anxiety. Several studies have shown that 40-60% of psoriasis patients consider stress to be the principal factor for worsening of their disease [105], [106], [107], [108], [109]. Furthermore, stress-induced relapse rate among children with psoriasis is up to 90% [110]. However, epidemiologic studies linking stress with psoriasis exacerbation show controversial results since some indicate correlation [111] while others do not [112, 113].

Several studies have suggested a possible mechanism for stress to exacerbate psoriasis through an increase in stress hormone levels due to activation of the hypothalamus-pituitary-adrenal (HPA) axis [114-117]. Corticotrophin-release hormone (CRH) is a central component of the HPA that is important in the coordination of systemic stress responses as well as modulation of inflammatory response. In psoriasis, expression of CRH has been shown to be significantly increased [118], however, CRH effects on skin remain unknown. One possible way CRH may further exacerbate psoriasis is through stimulation of IL-6 or IL-11 production in
keratinocytes during cutaneous stress [119].

**Alcohol consumption**

The association between alcohol consumption and psoriasis is complex and controversial. Meta-analysis data of case controlled studies showed that alcohol consumption is associated with increased risk of psoriasis. Furthermore, epidemiological studies suggest that patients with moderate-to-severe psoriasis have a higher incidence of alcohol-related diseases and mortality [120-122]. However, the mechanism by which alcohol consumption might trigger psoriasis remains unknown. *In vitro* studies have shown that 0.05% ethanol can activate T cells as well as cause keratinocyte hyper-proliferation by directly stimulating TGF-alpha, IL-6 and IFN-alpha production [123].

An epidemiologic article studying atherogenesis in apparently healthy men demonstrated that alcohol consumption appears to affect IL-6 plasma levels because daily drinkers had significantly higher levels of IL-6 than individuals who did not consume alcohol regularly [124]. This might be particularly important because psoriasis patients have higher risk of developing cardiovascular diseases [125].

**Obesity**

Several reports have shown a correlation between obesity and psoriasis severity but the mechanism by which obesity promotes psoriasis is not well understood [126, 127]. One possible mechanism involves the adipocyte-derived cytokines, leptin and resistin since
numerous publications have reported high concentrations of these cytokines in psoriasis patients [128, 129]. These adipokines can induce monocytes to produce pro-inflammatory cytokines such as IL-8, TNF-α and IL-1β [129]. Another possible mechanism by which obesity triggers psoriasis is through enhancement of IL-6 and TNF-α production. In other diseases such as hepatocellular carcinoma, obesity has been shown to promote development of the disease through enhancement of IL-6 and TNF production causing liver inflammation and activation of STAT3 [130]. Therefore, since IL-6 dependent STAT3 has been shown to be important for T cell proliferation and regulatory T cells dysfunction in psoriasis [93, 94], it is possible that this mechanism may mediate psoriasis development. However, more studies are needed to clarify our understanding of the effect of obesity on psoriasis development.

IV. Immune-pathogenesis of psoriasis

The majority of the knowledge we have of psoriasis is based on therapeutic trials and immune targeting drugs used for other inflammatory conditions such as rheumatoid arthritis and inflammatory bowel diseases. Before having a better understanding of the role of the immune system in psoriasis, the primary therapies such as cyclosporine and methotrexate focused on inhibiting epidermal cell hyperproliferation observed in the patients [131],[132]. However, it was then discovered that cyclosporine could also inhibit T cell cytokine mRNA transcription implicated in T cell hyperactivity raising the question of whether keratinocytes or lymphocytes
may be driving psoriatic plaque formation [133].

Early studies using an IL-2 diphtheria toxin-infusion protein specific for activated T cells with high IL-2 receptor affinity and non-reactive to keratinocytes provided evidence that T lymphocytes played an important role in plaque formation [134]. Another piece of early evidence that T lymphocytes play an important role in psoriasis development was the decrease in T cell numbers in psoriatic plaques by up to 90% after continuous therapy with psoralen plus ultraviolet A irradiation (PUVA) which induced prolonged clearing of psoriatic lesions [135].

Histological studies determined that psoriatic lesions contain inflammatory infiltrates composed of innate immune cells such as neutrophils, mast cells, dendritic cells as well as adaptive immune cells such as T cells including Th1 and Th17 cells in crosstalk with keratinocytes and endothelial cells.

Neutrophils have been shown to be enriched in psoriatic lesional skin, especially within the stratum corneum where they assemble into Munro’s microabscesses and within the stratum spinosum where they aggregate as spongiform pustules of Kogoj [136]. Neutrophils are constantly recruited into the dermis by the release of chemokines such as CXCL1, CXCL2 and CXCL8 from the keratinocytes [137]. Likewise, IL-17 producing T cell activation causes CCL20, CXCL1, CXCL2 and CXCL8 synthesis, which leads to the recruitment of more IL-17 producing cells and neutrophils into
the lesion [138]. Neutrophils are the first line of defense and contain many intracellular antimicrobial proteins released during neutrophil extracellular trap-induced apoptosis (NETosis) [139]. NETs have been reported in psoriasis via staining of nucleic acid by DAPI and neutrophil elastase [140], [141] in psoriatic lesions. Furthermore, neutrophils in psoriasis have also been shown to be IL-17 positive [140], further exacerbating a constant inflammatory loop.

Early psoriatic skin lesions have been reported to typically include degranulated mast cells [142, 143]. The number of mast cells [144-147] as well as of histamine concentration [148, 149] increased in psoriatic skin. A particular subset of mast cells, tryptase and chymase positive, have been shown to be enriched in the papillary dermis of psoriasis skin [145], [150]. Mast cells have been termed “ghost cells” in early psoriasis lesions because they are frequently activated and degranulated. Furthermore, approximately 70% of mast cells in psoriatic skin are IFN-γ positive, which suggests they play an important role in triggering psoriasis [150].

In an early study, initial phases of psoriasis triggered by Koebner phenomenon showed that mast cells were significantly increased at day 4 when compared to control skin. Mast cells increase peaked at day 14, which was simultaneous with the peak manifestation of psoriasis [151]. In addition, mast cells numbers decrease in psoriasis lesions after successful therapy with athralin, psoralen plus UVA light therapy and
cyclosporine treatment [147, 152,153].

A recent article demonstrated that the increased number of mast cells and neutrophils in psoriasis contributed to the release of IL-17 through the formation of neutrophil extracellular traps, which may be trigger by IL-23 and IL-1β [140]. More recently, mast cells have been shown to be major producers of IL-22 in psoriasis and atomic dermatitis [154]. Therefore, innate immune cell release of IL-17 is a new and exciting topic that needs further evaluation for determining how it may be triggering psoriasis.

Psoriatic lesions contain different T cell populations of which CD8\(^+\) cells seem to predominate in the epidermis while CD4\(^+\) T cells are mainly observed within the dermis [155]. Among the CD4\(^+\) T cells, the predominating T cell subsets are Th1, Th17 and Th22 cells that produce IFN-γ, IL-17 and IL-22, respectively.

Th17 cells are considered to be one of the main triggers for psoriasis. Th17 cells interact with skin resident cells to contribute to the formation of psoriatic plaque. Th17 cells are induced by the production of IL-23, which has been shown to be highly produced within the psoriatic plaque. Upon activation of Th17 cells, pro-inflammatory cytokines such as IL-17A, IL-17F, IL-21 and IL-22 are released and act on keratinocytes leading to skin hyperplasia, acanthosis and parakeratosis. Furthermore, Th17 cells can induce the production of antimicrobial proteins (S100A8, S100A9 and human beta defensin 1 and 2) that further recruit neutrophils, as well as the cathelicidin LL37 resulting in activation of pDCs and vascular
endothelial growth factor which leads to increased angiogenesis [156].

T cell recruitment into psoriatic skin is thought to occur as a result of the release of cytokines and chemokines from activated keratinocytes [157, 158], macrophages [159, 160] and endothelial cells [161, 162].

Regulatory T cells are important regulators of T effector proliferation and maintenance of immunological tolerance. However in psoriasis, impaired activity and/or reduction in regulatory T cells have been observed [163, 164]. Our laboratory previously showed that the proliferative capacity of T effector cells in psoriasis is enhanced due to IL-6 dependent hyperphosphorylation of STAT3 [93]. Furthermore, a recent study showed that regulatory T cells in psoriasis patients could differentiate in vivo into Th17 cells under pro-inflammatory conditions. In this study, regulatory T cells from severe psoriasis patients had an enhanced propensity to differentiate into IL-17A-producing cells upon ex vivo stimulation when compared to healthy controls [165].

T cell activation requires antigen presentation. Although the specific antigen that T cells recognize in psoriasis patients has not been elucidated, different stimuli including self-proteins, microbial pathogens and super-antigens have been proposed. In psoriasis, the epidermis contains high levels of allo-antigen presenting activity of non-Langerhans cell antigen presenting cells (APCs) population [166] that correlates with abnormal stimulation of autologous T cells in the absence of exogenous antigen [167].
Dermal dendritic cells (dDCs) have been recognized as APCs crucial for antigen presentation to T and B cells [168]. Dermal DCs may be responsible for activation of Th1 and Th17 cells via secretion of IL-2 and IL-23, respectively [156]. There are 3 main dDCs populations in the skin: epidermal Langerhans Cells (LCs), resident dermal myeloid DCs and plasmacytoid dendritic cells (pDCs). Also, during inflammatory conditions such as psoriasis, a fourth population of dendritic cells, inflammatory DCs, can be found in the skin [169]. Therefore, the overall increased DCs observed in psoriasis is due to high numbers of pDCs, immature and mature DCs, iNOS-and-TNF-producing DCs (Tip-DCs) and inflammatory DCs [170-172].

Different DCs exert different functions during the initiation and progression of psoriasis. Based on recent studies, LCs are significantly decreased in human psoriatic plaques, while pDCs are highly increased [173].

LCs are skin resident DCs present in the suprabasal layers of the epidermis, in close contact with keratinocytes [169]. LCs represent approximately 3% of the epidermal cells [174] and are characterized by expression of langerin (CD207), CD1a, e-cadherin and epithelial-cell adhesion molecule (EpCAM) [169]. They are traditionally considered the first line of defense against pathogen invasion [175].

The functional role of LCs in psoriasis is not fully understood, however, previous studies have shown LC reduction in psoriatic skin,
which can be restored to normal levels following therapy [176]. A recent publication demonstrated that LCs are reduced in lesional psoriatic skin of patients as well as the skin of the keratin 5 specific deletion of Jun and JunB (DKO) psoriatic mouse model [173]. LC depletion can aggravate psoriatic-like inflammation in DKO mice in an IL-10- and PD-L1-dependent manner [173].

Also, human LCs have been demonstrated to be the most efficient DCs to induce in vitro differentiation and polarization of naïve CD4+ T cells into either Th2 [177], Th17 cells [178] or Th22 cells [179] as well as priming and cross-priming of naïve CD8+ T cells [180]. Furthermore, LCs have been shown to directly interact with resident skin memory T cells and to induce activation and proliferation of skin resident regulatory T cells under in vitro homeostasis conditions [181]. These results of LC function might be particularly important in psoriasis because the reduction observed in LCs could potentially cause regulatory T cell dysfunction as well as induction of skin resident effector memory T cells.

As previously mentioned, pDCs are found in higher numbers in psoriatic plaques [173]. Even though pDCs account for less that 0.1% of total PBMCs, they are the primary source of interferon alpha (IFNα) [182]. In humans, pDCs express BDCA-2, a cell-specific type II C-type lectin, which is a potent interferon α/β modulator and mediates antigen capture [183]. These DCs are unique because they express endosomal
toll-like receptor (TLR)-7 and -9, which respond to single stranded RNA and unmethylated CpG, respectively [184]. This is particularly important in the context of psoriasis because CpG methylation in psoriatic involved skin have been shown to differ from normal skin in 1,108 CpG sites [52].

Also, it have been shown that in psoriasis, keratinocytes produce elevated levels of the cathelicidin LL-37, an antimicrobial peptide that forms complexes with self DNA/RNA released by damaged cells, which activate pDCs [185, 186]. pDC activation releases IFNα which, in combination with IL-1β, IL-6 and TNFα, is thought to stimulate conventional DCs (cDCs) causing their migration to draining lymph nodes where they can prime Th17 and Th22 differentiation [185].

Another important role of pDCs may be their ability to trigger Th17 cell differentiation. A recent study using the collagen-induced arthritis mouse model demonstrated that in a TGF-β-rich environment, pDCs can produce IL-6 which leads to Th17 commitment [187]. Additionally, these TGF-β exposed pDCs-induced Th17 cells can also secrete IL-22, a cytokine required for cutaneous inflammation [187]. These results support a role for pDCs in controlling Th17 cell polarization.

However, even though pDCs have been demonstrated to be required for psoriasis initiation, they are dispensable for maintaining chronic inflammation in mouse models. This might be a reason why IFN-α therapies have not been successful for treating psoriasis [173]. Therefore, pDCs are thought to be initiators of psoriatic skin development.
while other types of DCs such as myeloid DCs (mDCs) are believed to play an important role in maintaining and amplifying the disease [188].

Dermal mDCs are identified by CD11c and BDCA expression [189]. BDCA-1 or CD1-c is a MHC complex molecule that participates in lipid antigen presentation to T cells [190]. Two populations of mDCs in skin have been identified: classical mDCs (CD11c−BDCA-1+) and inflammatory mDCs (CD11c+BDCA-1−). The first report of mDCs in psoriasis showed that lesion-derived mDCs could stimulate T cells responses through the production of IL-2 and IFNγ [191]. Also, in psoriasis, CD11c+BDCA−neg DCs are 30-fold higher in the dermis, which is nearly the same number as lesional T cells found in the psoriatic skin [169], [192]. Some of these CD11c+BDCA−neg DCs include tumor-necrosis- factor (TNF) and inducible nitric oxide synthase (iNOS)-producing DCs (TipDCs) [171] as well as IL-20- and IL-23-producing DCs [188]. Tip-DCs may be particularly important in psoriasis because not only the produce TNF, iNOS, IL-20 and IL-23 [193] but also they are capable of Th17 polarization as well as production of IL-12p40, IL-23p19 in the psoriatic lesion [194]. Another mDCs subtype that appears to be important for psoriasis progression is a DC expressing 6-sulfo-LacNac (Slan DC), which are the inflammatory DC precursors that might be driving Th1 and Th17 responses in psoriasis [195].

V. Role of IL-6 in psoriasis
a. IL-6 signaling

IL-6 is a pleiotropic cytokine mainly involved in inflammation by controlling differentiation, proliferation, migration and apoptosis of target cells such as T effector cells. It is crucial for immune response regulation, hematopoiesis and inflammation but excessive production and dysregulation causes the development of various chronic immune diseases including rheumatoid arthritis (RA) and psoriasis.

Almost every stromal and immune cell can produce IL-6 and several activators can cause its expression including IL-1β, TNF-α, Toll-like receptors, adipokines, prostaglandins, stress responses and other cytokines. IL-6 concentration in human serum is relatively low (1-5 pg/ml) under physiological conditions but a rapid increase is observed during extreme circumstances including septic shock and inflammatory conditions [196, 197]. Therefore, while IL-6 confers protection during many infections, it is a key factor in the maintenance of chronic inflammation as well.

IL-6 receptor is composed of an 80kDa type 1 cytokine α-receptor subunit named IL-6R or CD126 and an ubiquitously expressed 130kDa glycoprotein signal-transducing β-receptor subunit named gp130 or CD130 [198-201]. Even when IL-6 binds the α-receptor subunit, in order to have a fully competent IL-6R, both subunits are required [202].

Signaling via gp130 is essential for development, hematopoiesis, cell survival and growth [203], [5]. Gp130 functions as the β-receptor subunit for
other IL-6 related cytokines such as IL-11, IL-27, oncostatin-M, ciliary neutrophic factor and leukemia-inhibitory factor, among others [5, 203, 204]. While gp130 is ubiquitously expressed, IL-6R expression is cell specific and restricts cell responsiveness to IL-6 because it is only expressed in hepatocytes, neutrophils, monocytes, macrophages and some lymphocytes [5]. IL-6R signaling can mediate diverse effects through the engagement of several pathways including GTPase, Ras and its effector Raf, MAPK cascade (mitogen-activated protein kinase) and Jak/Stat pathway signaling [203], which is the best understood of the signaling pathways.

Gp130 dimerization causes activation of kinases Jak1, Jak2 and Tyk2 that promote recruitment and phosphorylation of STAT1, STAT3 and STAT5 transcription factors. STAT2 has been shown to play a role in growth arrest by promoting apoptosis and tumor suppression, while STAT3 and STAT5 have been

Figure 2. IL-6 signaling. During the classical IL-6 signaling, IL-6 binds the membrane-bound IL-6 receptor composed of the IL-6R and gp130 subunits. During IL-6 trans-signaling, limited proteolysis and/or alternative splicing leads to the formation of a soluble form of IL-6R (sIL-6R). IL-6 binds sIL-6R forming a complex that can stimulate cells, which only express gp130 but not IL-6R. Modified from [5].
shown to be involved in promoting cell cycle progression, preventing apoptosis and cellular transformation [205].

Regulation of IL-6 dependent JAK/STAT signaling occurs through the activation of suppressors of cytokine signaling, SOCS1 and SOCS3, and by members of cytokine-inducible SH2-domain containing family of cytokine receptor inhibitors [203]. Mice expressing mutant gp130, which is unable to bind SOCS3, have more sustained IL-6 signaling through STAT1 and STAT3, which cause them to have exacerbated inflammation, chronic disease and cancer [206-210].

IL-6 uses two signaling pathways to mediate biological effects (Figure 2). The classical IL-6 receptor signaling occurs when IL-6 binds the membrane-bound IL-6R, hence it is only relevant in cells that express both receptor subunits [211]. The trans-signaling occurs when IL-6 binds the soluble form of the IL-6R (sIL-6R) forming a complex that increases IL-6 circulating half-life and promotes bioavailability [212-214]. sIL-6R is generated by alternative splicing and ectodomain shedding mediated by adamalysin proteases ADAM17 and ADAM 10; it is released by monocytes and activated T cells [210, 215-217]. Furthermore, recent studies with human neutrophils have shown that C-reactive protein, inflammatory chemokines, bradykinin, N-formyl peptides, complement regulators, lipid mediators (platelet-activating factor and leukotrienes) can activate sIL-6R shedding [218-223]. sIL-6R acts as an agonist activating target cells that only express gp130 but not IL-6R [5, 224]. IL-6/sIL-6R binding to a
homodimer of gp130 on the cell surface activates intracellular signaling such as JAK/STAT and MAPK pathways. Furthermore, IL-6 signaling has important functions in influencing T-cell recruitment, activation/differentiation and apoptosis. Classical IL-6R signaling has been shown to be important for acute-phase immune response, glucose metabolism, fatigue and appetite loss [225]. Also, classical IL-6R signaling has been demonstrated to be required for differentiation of Th17 cells in experimental autoimmune encephalomyelitis (EAE) mouse model [226]. On the other hand, IL-6R trans-signaling has been shown to lead to Th17 cell differentiation while also suppressing Treg development in mice [227].

While IL-6 controls proliferation and survival of Th1 and Th2 cells in a context dependent manner, it has been shown to be required for Th17 commitment. While IL-1β, IL-21 and IL-23 are required for the generation of Th17, IL-6 is the key mediator for IL-17 secretion in CD4+ and CD8+ T cells [228-232]. IL-6-dependent STAT3 activation, in the presence of TGF-β, is essential for Th17 differentiation by inducing transcription of the transcription factor retinoid-related orphan receptor gamma t (ROR γt), IL-17 and IL-23R genes and for expansion of differentiated memory Th17 cells [208, 210, 233-238]. Even when TGF-β and IL-6 drive Th17 cell differentiation, their functionality depends on further exposure to IL-23. Therefore, IL-23 is crucial for T cell differentiation into effector T cells as well as for lineage stabilization and full maturation of Th17 cells [40, 156, 233, 234, 239].
b. IL-6 in psoriasis pathogenesis

IL-6 has been associated with psoriasis [95, 96, 240] and other immune diseases such as rheumatoid arthritis [241], Crohn’s disease [242], [201] and systemic lupus erythematosus (SLE) [243-245].

IL-6 and STAT3 polymorphisms have been linked with hereditary predisposition to psoriasis development and response to TNF-α blockers [41, 42, 246]. IL-6 levels have been demonstrated to be elevated in psoriasis tissue [95, 96, 247-249], which correlates to psoriasis severity [250, 251]. Furthermore, it was demonstrated by our laboratory that IL-6 co-localized with CD45, CD11C and CD3+ cells in lesional skin in the microenvironment where T cells first enter and accumulate in the skin [93].

IL-6 has been shown to be produce by keratinocytes, fibroblasts, endothelial cells, DCs and macrophages in psoriatic plaques in response to IL-1, TNF-α, IL-17 and IL-36 [96, 195, 252-254]. Furthermore, synergistic effects of IL-17 and TNF-α have been shown to further upregulate IL-6 in psoriatic skin lesions [252-255]. Furthermore, increased activation of STAT3 has been detected in lesional skin [94] and [68] and observed to be constitutively active in keratinocytes of psoriasis-like mouse models [68, 256, 257]. IL-6 produced by DCs, macrophages, T cells, keratinocytes, fibroblasts and other cells in the psoriatic skin lesions create a IL-6-rich environment that results in robust STAT3 phosphorylation in T effector and Th17 cells [258]. Our laboratory previously demonstrated that psoriatic regulatory T cells are deficient in their ability to suppress T
cell proliferation [164] possibly due to the excessive of IL-6 levels in psoriatic tissue that causes hyper-phosphorylation of STAT3 in T effector cells making them refractory towards regulatory T cell suppression [94]. Persistent STAT3 phosphorylation on T cells enables resistance to suppression and has also been shown to be required for initial Th17 differentiation and promotion of Th17 derived cytokine production [258]. Furthermore, IL-6 mediated STAT3 phosphorylation has been shown to enhance keratinocytes growth and differentiation further causing epidermal hyperplasia [68, 254].

As previously mentioned IL-23 is crucial for T cell differentiation into effector T cells as well as for lineage stabilization and full maturation of Th17 cells [40, 156, 233, 234, 239]. In psoriasis, IL-23 has been shown to be primarily produced by inflammatory DCs and activated keratinocytes. IL-23 stimulates Th17 cells, within the dermis, to release pro-inflammatory cytokines such as IL-17A, IL-17F, IL-21 and IL-22. IL-22 has been further implicated to activate skin resident cells such as keratinocytes causing hyperproliferation via STAT3 activation, which promotes hyperplasia [259]. Furthermore, IL-17 has been implicated in the induction of IL-6, IL-8 and CXCL5 in human keratinocytes, which indirectly promotes neutrophil differentiation, activation and migration [40, 156].

c. **IL-6R blockade as a possible trigger for psoriasis**
IL-6R blockade using a humanized monoclonal antibody that targets the IL-6 receptor (tocilizumab) was initially developed to treat Castleman’s disease [260], but it is now also in use for treating rheumatoid arthritis [9], juvenile idiopathic arthritis [261] and Crohn’s disease [262]. Given the known role of IL-6 in psoriasis, IL-6R blockade might be a good therapy for psoriasis. However, psoriasis onset has been observed in RA patients treated with tocilizumab [90-92].

Tocilizumab is a humanized anti-IL-6R monoclonal IgG1 antibody that was genetically engineered by grafting complementary determining regions (CDR) of a mouse anti human IL-6R antibody onto human IgG1, which reduces the antigenicity in humans [263] (Figure 3a).

Figure 3. anti IL-6 receptor (Tocilizumab). A) Tocilizumab is a humanized antibody engineered by grafting complementary determining regions (CDR) in a mouse antihuman IL-6R antibody. Modified from [4] B) Tocilizumab inhibits both the classical and trans-signaling pathways of IL-6 by binding to the membrane-bound IL-6R (mIL-6R) and soluble IL-6R (sIL-6R). Modified from [9].
Tocilizumab binds to membrane bound and soluble IL-6R (mIL-6R and sIL-6R, respectively) causing inhibition of both classic and trans-signaling pathways of IL-6 by strong inhibition of IL-6 dependent STAT1/STAT3 activation [264] (Figure 3b). However, tocilizumab does not inhibit signal transduction by the receptors of other members of the IL-6 cytokine family such as IL-11, oncostatin M (OSM), leukemia inhibitor factor (LIF) and ciliary neutrophic factor (CNTF) [264]. Furthermore, the effect of anti-IL-6R antibody binding to cells is currently unknown. Some suggested

Figure 4. IL-6 Receptor blockade causes skin hyperplasia. Inflammation induced in rheumatoid arthritis is treated with anti-IL-6R (tocilizumab). Inhibition of IL-6R may cause IL-6 to be free in the periphery where it can bind alternative targets that in combination with TGFβ and IL-23, produced by dDCs, activated keratinocytes and macrophages results in off-target effects shifting naïve CD4 cells to differentiate towards a) Th17 pro-inflammatory phenotype and b) less of the suppressive Treg phenotype. c) Th17 cells can produce IL-17 and IL-22, which will further cause skin hyperplasia.
possibilities include apoptosis, phagocytosis or release to the circulation following receptor blocking [265].

Given the paradoxical onset of psoriasis observed after IL-6R blockade, we hypothesized that blocking IL-6R may initiate IL-6 binding to alternative receptors (i.e., OSMR) that, in combination with TGFβ and/or IL-23, results in off-target effects and a shift of naïve CD4\(^+\) T cells towards a Th17 phenotype as well as decreased suppressive Treg function. As a corollary, Th17 cells may secrete enhanced levels of IL-22, which drives skin hyperplasia (Figure 4).

d. **Immunological effects of IL-6R blockade treatment**

A recent study using blood samples of 8 RA patients treated with tocilizumab evaluated its effect on the balance between regulatory T cells and Th17 cells. No change in the frequency of Th1 or Th17 cells was observed, but the percentage of regulatory T cells was increased after therapy [266]. However, these investigators observed an increase in the percentage of Th17/Th1 cells in almost all the patients after IL-6 was inhibited. A possible explanation is that IL-6R blockade may be causing accumulation of Th17 and Th17/Th1 cells in blood by impairing their recruitment to inflamed joints by decreasing serum levels of the CCR6 ligand, CCL20 [267]. Another study found that tocilizumab treatment induced a significant decrease in the percentage of Th17 cells and increased the percentage of regulatory T cells [268].

An additional study found that tocilizumab reduced IL-21
production by memory and activated CD4\(^+\) T cells in treated RA patients. However, this IL-21 decrease was observed in combination with reduction of IgG4 anti-cyclic citrullinated peptide antibodies, which led them to believe that there is a pathway involving IL-6, IL-21 and IgG4 autoantibodies in RA [269].

A previous report determined that the levels of IL-6 in serum after tocilizumab treatment were elevated. Given that tocilizumab is a competitive inhibitor for IL-6R, the observed increased of IL-6 in serum after its administration might be caused by the inhibition of IL-6R mediated clearance [270]. Therefore, free IL-6 is available for signaling, possibly through alternative receptors. Alternatively, since tocilizumab does not inhibit formation of gp130 dimerization, it is possible that during IL-6R blockade, alternative cytokines such as oncostatin- M (OSM) are signaling through gp130 complex leading to an alternative STAT3 activation resulting in Th17 differentiation from naïve CD4 cells.

VI. Material and Methods

a. Patients

All studies involving human peripheral blood were approved by the Institutional Review Boards of Case Western reserve University, University Hospitals Case Medical Center and the Veterans Affairs Medical Center. Peripheral blood samples were obtained from healthy adult volunteers and patients with moderate to chronic plaque psoriasis following informed consent.
b. **Human peripheral blood mononuclear cells (PBMCs) isolation**

Human PBMCs from heparinized healthy and psoriatic peripheral blood were prepared using Histopaque (Sigma-Aldrich) density gradient centrifugation according to manufacturer’s instructions. For the experiments using CD4\(^+\) T cells, CD4\(^+\) T cells were separated from PBMCs by negative selection MACS columns (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer’s directions. In those experiments using non-CD4 cells, non-CD4 cells were collected by washing the column after collecting the enriched CD4\(^+\) T cells.

c. **Flow Cytometric analysis of activated regulatory T cells**

CD4\(^+\) T cells were stimulated overnight with a 1:2 ratio of CD2/CD3/CD28 T cell activation beads (Miltenyi Biotec Inc., Auburn, CA). Changes in expression levels of key regulatory T cell activation markers including LRRC32 (GARP), LAP/TGF-\(\beta\)1, CD4, CD25\(^{hi}\), FoxP3 and CD127\(^{neg}\) were monitored by flow cytometry and analyzed using a BD LSRII flow cytometer.

d. **Evaluation of IL-17 production by ELISA**

IL-17 levels were determined in supernatants released from stimulated normal and psoriatic PBMCs and CD4\(^+\) T cell cultures following incubation of 1:2 CD2/CD3/CD28 T cell activation beads as well as a)
normal goat IgG control (5μg/ml), b) recombinant human IL-6 (25ng/ml), c) soluble IL-6R (50ng/ml), and d) anti-IL-6Rα (5μg/ml) by ELISA assay (Ray Biotech) according to the manufacturer’s instructions. Mean absorbance (450nm) for each set of duplicate standards and triplicate sample supernatants were calculated and subtracted from the baseline standard optical density. Standard curves were calculated and used to determine the IL-17 concentration.

VII. Results

Anti-IL-6R did not affect IL-17 levels in activated psoriatic CD4⁺ T cells

Since IL-6 is a key mediator for IL-17 secretion in CD4⁺ and CD8⁺ T cells [228-232], we evaluated the effect of IL-6R blockade on IL-17 secretion by psoriatic CD4⁺ T cells (Figure 5). Based on our results, blockade of IL-6R did not affect IL-17 production by psoriatic activated CD4⁺ T cells.

Figure 5. IL-17 production in psoriasis after anti-IL-6R treatment. Psoriatic CD4⁺ T cells were negatively isolated from PBMCs. Then they were incubated overnight with 1:2 CD2/CD3/CD28 activation and expansion beads and treated with rhIL-6 (25ng/ml), sIL-6R (50ng/ml), anti-IL-6R (5μg/ml) and/or normal goat IgG control (5μg/ml) and IL-17 levels were measured by ELISA. (n=2)
Activated CD4+ T cells treated with recombinant human IL-6 did not have a statistically increased production of IL-17 when compared to activated CD4+ T cells alone. This may indicate that IL-6 was not sufficient for causing production of IL-17 in psoriatic CD4+ T cells. Also, when IL-6R blocker was added in presence or absence of rhIL-6/sIL-6R, no statistical difference was observed when compared with activated CD4+ T cells alone. This result suggests that the IL-6R inhibitor was unable to diminish production of IL-17. However, since peripheral blood cells in psoriasis expresses high levels of IL-6, it is surprising that the levels of IL-17 did not increase when rhIL-6 was added.

Anti-IL-6R did not affect IL-17 production by healthy PBMCs

Given that anti-IL-6R treatments did not affect the IL-17 production levels on psoriatic CD4+T cells, we next decided to evaluate

![Figure 6. IL-17 production by healthy PBMCs after anti-IL6R treatment.](image)

Healthy PBMCs were isolated and incubated with 1:2 CD2/CD3/CD28 beads as well as with or without rhIL-6 (25ng/ml) in presence or absence of anti-IL-6Rα (5ug/ml). 72 hours later, the cells were re-stimulated with the 1:2 CD2/CD3/CD28 beads as well as rhIL-6 and anti-IL-6Rα. Then the cells were incubated for another 72 hours and ELISA was performed for evaluation of IL-17 production. (n=5)
whether anti-IL-6R had an effect on healthy peripheral blood cell production of IL-17 (Figure 6). Based on our results, production of IL-17 among healthy PBMCs without activation was almost very low, which is in agreement with current literature. Furthermore, as expected, addition of T cell activation and expansion beads caused an increase of IL-17 production in the supernatant of healthy PBMCs, which was further augmented by the addition of rhIL-6. However, when anti-IL-6R was added, no statistically significant difference was observed on the production of IL-17. These results might indicate that IL-6R blockade is not sufficient for decreasing IL-17 production in the presence of activated healthy PBMCs.

**Activated healthy and psoriatic CD4+ T cells are the main producers of IL-17**

In order to verify whether different cell types were required for the difference in production of IL-17 observed with healthy PBMCs, we isolated CD4+ T cells as well as non-CD4+ T cells from healthy and psoriatic PBMCs and stimulated them using T cell activation and expansion beads in presence or absence of rhIL-6 (Figure 7). CD4+ T cells from both healthy and psoriasis patients did not produce IL-17 in the untreated condition. Based on our preliminary results, there was a trend of higher IL-17 production by psoriatic CD4+ T cells when activated with CD2/CD3/CD28 beads alone as well as in the presence of rhIL-6.
However, rhIL-6 did not augment the IL-17 production in either normal or psoriatic T cells. This result further confirms that IL-6 did not augment IL-117 production in CD4$^+$ T cells. Therefore, based on our results, the pooled CD4$^+$ T cells produce IL-17 and non-CD4$^+$ cells did not produce IL-17 (Figure 7).

![IL-17 production by healthy and psoriatic CD4+ T cells and non-CD4+ cells](image)

**Figure 7.** IL-17 production by healthy and psoriatic CD4+ T cells and non-CD4+ cells. Healthy and psoriatic CD4+ T cells and non-CD4 cells were isolated and incubated with 1:2 CD2/CD3/CD28 beads as well as with or without rhIL-6 (25ng/ml). 72 hours later, the cells were re-stimulated with the 1:2 CD2/CD3/CD28 beads as well as rhIL-6 and anti-IL-6Rα. Then the cells were incubated for another 72 hours and ELISA was performed for evaluation of IL-17 production. (n=2)
Psoriatic regulatory T cells have elevated levels of activation markers

As previously mentioned, the suppressive function of regulatory T cells in psoriasis is impaired due to the STAT3 hyper-phosphorylation of T effector cells making them refractory to regulatory T cells suppression [93]. Furthermore, our laboratory have shown that activated regulatory T cells are potent sources of TGFβ as they use the leucine rich repeat region 32 or glycoprotein A repetitions predominant (LRRC32 or GARP) to tether TGFβ [271]. Based on a previously published model (Figure 8a), upon regulatory T cell activation, TGFβ associated with the latent associated peptide (LAP) is expressed on the cell surface where it is bound to GARP. When activated, it is released locally where it can signal through autocrine or paracrine signals to proximal cells, which further induce FoxP3 expression. Therefore, this model predicts that by blocking TGFβ receptor, FoxP3 expression can also be inhibited [6].

Our results indicate that regulatory T cells isolated from psoriatic patient peripheral blood have elevated levels of the regulatory specific activation markers LRRC32 or GARP and latency associated peptide (LAP/ TGFβ1) (Figure 8b). Therefore, activated regulatory T cells that express GARP/LAP may be tethering TGFβ, which would continuously promote a loop for naïve CD4+ T cells to differentiate towards Th17 phenotype especially under high levels of IL-6. Since TGFβ has a direct suppressive effect on effector T cells [272], the fact that regulatory T cells
in psoriasis may be holding TGFβ on their surface and not liberating it, may be another reason for the dysfunctionality of regulatory T cells observed in psoriasis.

**VIII. Discussion**

Psoriasis is a chronic inflammatory skin disease mediated by a cross-talk between epidermal keratinocytes and activated immune cells such as neutrophils, macrophages, dendritic cells and T cells [28]. Although the etiology of psoriasis remains unknown, genetic susceptibility and environmental triggers as well as immunological responses have been associated with its development [25-27, 29, 38, 42, 56, 104]. Several pro-inflammatory cytokines have been shown to be involved in psoriatic plaque formation and maintenance including type I interferon, TNF-α, IL-6.
and IL-17. Treatments targeting interferon in psoriasis have not been successful because the presence of this cytokine is involved in the initiation of the diseases but not in its maintenance [173]. Similarly, even when several TNFα inhibitors and fusion proteins are use as therapies in psoriasis, paradoxical onset of psoriasis as well as exacerbation of the disease have been linked with TNF therapy [85]. Furthermore, IL-17 related therapies even given their high success levels, are fairly new treatments which may induce long-term side effects that are not yet know and will remain to be further analyzed [234].

Given the need for better treatments for psoriasis and the accumulated knowledge regarding the role of IL-6 in psoriasis, this cytokine appeared to be a good candidate for psoriasis therapy. However, several reports have now demonstrated the onset and/or worsening of psoriasis after treatment with the humanized anti-IL-6R antibody, tocilizumab [89-92]. This approved medication for Castleman's disease, rheumatoid arthritis and polyarticular juvenile inflammatory arthritis functions as a competitive inhibitor of both types of IL-6R, membrane bound and soluble forms (mIL-6R and sIL-6R, respectively), which blocks the classical and the trans-signaling pathways of IL-6 [4, 264, 266].

Given the paradoxical onset of psoriasis after tocilizumab treatment we proposed that under inflammatory conditions such as rheumatoid arthritis, the blockade of IL-6R causes free IL-6 to be liberated into the periphery where it can bind alternative receptors causing off-target effects
that, in the presence of TGF-β and IL-23, cytokines required for the maintenance of Th17 cells, can cause the differentiation of naïve CD4 cells towards a Th17 phenotype and away from the suppressive regulatory T cell phenotype. Th17 cells can then produce other pro-inflammatory cytokines such as IL-17 and IL-22, which have been shown to be required for initiating and sustaining epidermal hyperplasia and therefore psoriasis. We wanted to determine if anti-IL-6R increased Th17 cell differentiation and if anti-IL-6R altered the phenotype and/or function of regulatory T cells.

IL-6 is a key mediator of IL-17 secretion in CD4⁺ cells [228-232], therefore, we evaluated the effect of IL-6R blockade on the production of IL-17 from psoriatic CD4⁺ T cells. However, when we blocked IL-6R, we observed no statistical difference between activated psoriatic CD4 T cells and those treated with anti-IL-6R (Figure 5). This result suggested that the IL-6R inhibitor was unable to diminish production of IL-17. However, since peripheral blood cells in

![Figure 9. IL-17 production in psoriasis. The percentage of IL-17A producing T cells in healthy controls and psoriasis blood and lesional skin biopsies. (Modified from [1].)](image)
psoriasis expresses high levels of IL-6, it was surprising that the levels of IL-17 did not increase when rhIL-6 was added. It is possible that in order to see an effect, the concentration of rhIL-6 added to the system needs to be modified. Furthermore, it is possible that in order to see augmentation of IL-17 production, other cytokines such as TGF-β in combination with IL-6 will be required [273].

Since anti-IL-6R treatments did not affect the IL-17 production levels on psoriatic CD4⁺ T cells, we evaluated if anti-IL-6R had an effect on the levels of IL-17 produced in cultures of healthy peripheral blood cells. Unstimulated PBMCs isolated from healthy individuals did not produce quantifiable levels of IL-17, although stimulation of T cells through anti-CD2/CD3/CD28 stimulation upregulated IL-17 production, which was further augmented by the addition of recombinant human IL-6 (Figure 6). Interestingly, the addition of anti-IL-6R did not decrease the levels of IL-17 production in this system. Several explanations can be given based on these results. First, it is possible that IL-6R blockade was insufficient to overcome the IL-17 production by these cells. Second, it is possible that the free IL-6 is binding alternative targets and therefore the anti-IL-6R treatment cannot overcome this effect. Even when the latter explanation confirms our hypothesis of an IL-6R independent IL-6 signaling, in order to make further conclusions, we need to determine whether phosphorylation of STAT3 occurs under anti-IL-6R and IL-6 conditions.

In order to clarify which cell type was required for the differences observed
in the production of IL-17 in PBMC cultures, we compared IL-17 production in CD4\(^+\) T cells and non-CD4 cells from both healthy and psoriatic PBMCs (Figure 7). In accordance with our previous result, unstimulated healthy CD4 T cells did not produce quantifiable levels of IL-17; interestingly the same trend was observed with psoriatic CD4 T cells. This result agrees with a recent report demonstrating that the only statistically difference in the IL-17 levels was in lesional tissue and not between psoriasis and healthy peripheral blood (Figure 9) [1]. Activated healthy and psoriatic CD4 T cells did produce higher levels of IL-17 and there is a trend of higher production of IL-17 by activated psoriatic CD4 T cells. Surprisingly, addition of IL-6 did not augment the production of IL-17 between the groups, indicating that the addition of IL-6 was not enough to increase production of IL-17 either because the actual concentration used was not enough or because synergistic effects with other cytokines such as TGF-\(\beta\) are required in order for CD4 cells to produce IL-17. However, we can speculate that only CD4\(^+\) cells are required to produce IL-17 because a. non-CD4 cells didn’t produce quantifiable IL-17 levels and b. when comparing the IL-17 production by activated PBMC cultures and activated CD4\(^+\) T cells, the levels of IL-17 are similar (Figures 6 and 7). Given that we did not treat these cells with anti-IL-6R, we cannot make any conclusions on how anti-IL-6R may affect the production of IL-17 by psoriatic and healthy CD4\(^+\) T cells.

Since we also wanted to elucidate how IL-6R blockade affects
regulatory T cells, we first wanted to evaluate if there was a difference in the activation profile in healthy and psoriatic regulatory T cells. Using the activation markers GARP and LAP/ TGFβ1 we analyzed the activation of regulatory T cells (CD4\(^+\) CD25\(^{hi}\) CD127\(^-\)) by flow cytometry. Based on our results, psoriatic regulatory T cells have higher levels of the activation markers GARP and LAP/ TGFβ1 on their surface, which may indicate higher activation of these cells in psoriasis (Figure 8b). This is particularly important in the context of psoriasis because activated regulatory T cells that express GARP/LAP may be tethering TGFβ causing a continuous loop for naïve CD4\(^+\) T cells to differentiate into IL-17-producing cells especially under high levels of IL-6. Furthermore, given that TGFβ has direct suppressive effects on effector T cells [272], psoriatic regulatory T cells dysfunction might be associated with TGFβ tethered to their surface.

However, further studies are needed to evaluate the suppressive function of GARP\(^+\)LAP\(^+\) regulatory T cells, as well as to evaluate how anti-IL-6R may be modifying regulatory T cell function and differentiation.

Lastly, further work is necessary before we can make strong conclusions regarding how anti-IL-6R may be modulating the differentiation of naïve CD4 cells towards a Th17 pro-inflammatory phenotype and less of a regulatory cell suppressive phenotype in psoriasis. This work opens a new line of research regarding the role of IL-6 and IL-6R signaling in combination with activated regulatory T cell markers for potential Th17 differentiation signaling. Further clarification of
how these signals work in the context of psoriasis is necessary in order to elucidate what this means in the context of development of psoriasis.
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