

2008

Dendritic Cells in Normal and Inflamed Human Skin

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Dendritic Cells in Normal and Inflamed Human Skin

A Thesis Presented to the Faculty of
The Rockefeller University
in Partial Fulfillment of the Requirements for
the degree of Doctor of Philosophy

by
Lisa C. Zaba
June 2008

Dendritic Cells in Normal and Inflamed Human Skin

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The Rockefeller University 2008

Psoriasis is a skin disease originally thought to be a primary keratinocyte differentiation and maturation disease. Several T cell targeted therapeutics were found to reverse disease, and thus subsequent research has focussed on the adaptive immune system, particularly effector CD8⁺ T cells infiltrating the epidermis. Recent studies, however, show that inhibitors of tumor necrosis factor (TNF) are also effective therapeutics. Activated dendritic cells (DCs) produce large amounts of TNF which acts in an autocrine loop to increase DC maturation. Thus, TNF inhibitors may inhibit DC maturation and downstream T cell activation. This thesis elucidates DC subsets present in normal human skin and psoriasis lesional skin, and the mechanisms by which psoriatic inflammatory DCs activate Th17 T cells and downstream mediators to maintain psoriatic cutaneous inflammation.

In normal blood, there exists 3 non-overlapping subsets of myeloid dendritic all of which are Lin⁻CD11c⁺HLA-DR⁺ and either BDCA-1⁺ (24 ± 2%), CD16⁺ (70 ± 4%), or BDCA-3⁺ (5 ± 1%), in order of immunostimulatory capacity. Only two myeloid dendritic cell populations exist in normal human dermis: Lin⁻CD11c⁺HLA-DR⁺CD16⁺ and either BDCA-1⁺ (≈ 90%) or BDCA-3⁺ (≈ 10%). In situ double-label immunofluorescence showed that approximately 10-15% of CD11c⁺ dermal cells cluster together in lymphoid-like structures and are BDCA-1⁺CD205⁺DC-LAMP⁺. Upon emigration from the dermis,

90-95% of BDCA-1⁺ cells expressed these mature antigens, stimulated allogeneic T cells, and increased immunostimulatory capacity after the addition of TNF, PGE₂, IL-1 β , and IL-6. Functional studies were not performed on BDCA-3⁺ cells because of limited cell numbers. In normal dermis there also exists a large population of FXIII⁺CD163⁺ macrophages that are not immunostimulatory and phagocytose large particles.

As in normal blood, psoriasis patient blood contained 3 non-overlapping subsets of myeloid DCs (BDCA-1⁺, CD16⁺, or BDCA-3⁺). In psoriatic skin the frequency and distribution of BDCA-1⁺ and BDCA-3⁺ cells is similar to normal, however, there was a 30-fold increase in “inflammatory” CD11c⁺ cells that did not express either marker. Most BDCA-1⁺ cells expressed maturation markers CD205 and DC-LAMP, while most BDCA-1⁻ inflammatory cells expressed CD209 immature DC/ macrophage marker. Some myeloid cells expressed TNF and inducible nitric oxide synthase (iNOS).

Treatment of psoriasis patients with the TNF neutralizing antibody etanercept not only inhibited dendritic cells as expected, but also had inhibitory effects on a newly appreciated type of T cell – Th17 cells. Etanercept reduced inflammatory DC products that drive Th17 cell proliferation (IL-23) as well as Th17 products and downstream effector molecules (IL-17, IL-22, CCL20, and DEFB4). In contrast, Th1 cellular products and effector molecules (IFN γ , LTA-1, and MX-1) were reduced late in disease resolution. Using affymetrix gene array we characterized a global set of 4 gene clusters modulated temporally over the course of etanercept treatment. TNF and IL-17 pathway genes were downmodulated with a similar velocity, while IFN γ pathway genes were downmodulated later.

ACKNOWLEDGEMENTS

Clinical lab research is a team effort, and were it not for my friends and colleagues in the Krueger lab, this work would not have been possible.

Thank you Dr. Jim Krueger for investing your time and money in me – in particular, thank you for buying the FACS machine. My favorite PhD memories are of us drinking coffee and going over data in the mornings. Thank you Michelle Lowes who has taught me a lot about how to successfully juggle motherhood and science, and for being a friend. Thank you Dr. John Carucci, Dr. Sarah Schlesinger, and Dr. Olaf Andersen for being my support team and advisors. Also John, thank you for encouraging me to do dermatology and follow my dreams. Thank you Dr. Munz for being on my thesis committee, and Dr. Nestle for flying in from England for the defense. I especially thank Dr. Steinman for being a mentor and a role model. Thank you MD-PhD program for funding me, and thank you Rockefeller University for providing me with really really nice housing.

Of course, I thank my husband Derek who supports me like no other, and who keeps me laughing at every situation.

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ABSTRACT

Psoriasis is a skin disease originally thought to be a primary keratinocyte differentiation and maturation disease. Several T cell targeted therapeutics were found to reverse disease, and thus subsequent research has focussed on the adaptive immune system, particularly effector CD8⁺ T cells infiltrating the epidermis. Recent studies, however, show that inhibitors of tumor necrosis factor (TNF) are also effective therapeutics. Activated dendritic cells (DCs) produce large amounts of TNF which acts in an autocrine loop to increase DC maturation. Thus, TNF inhibitors may inhibit DC maturation and downstream T cell activation. This thesis elucidates DC subsets present in normal human skin and psoriasis lesional skin, and the mechanisms by which psoriatic inflammatory DCs activate Th17 T cells and downstream mediators to maintain psoriatic cutaneous inflammation.

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IL-6. Functional studies were not performed on BDCA-3⁺ cells because of limited cell numbers. In normal dermis there also exists a large population of FXIII⁺CD163⁺ macrophages that are not immunostimulatory and phagocytose large particles.

As in normal blood, psoriasis patient blood contained 3 non-overlapping subsets of myeloid DCs (BDCA-1⁺, CD16⁺, or BDCA-3⁺). In psoriatic skin the frequency and distribution of BDCA-1⁺ and BDCA-3⁺ cells is similar to normal, however, there was a 30-fold increase in “inflammatory“ CD11c⁺ cells that did not express either marker. Most BDCA-1⁺ cells expressed maturation markers CD205 and DC-LAMP, while most BDCA-1⁺ inflammatory cells expressed CD209 immature DC/ macrophage marker. Some myeloid cells expressed TNF and inducible nitric oxide synthase (iNOS).

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CHAPTER 1: Introduction to psoriasis immunopathogenesis and dermal dendritic cell subsets

Psoriasis vulgaris

Psoriasis epidemiology and phenotype

Psoriasis vulgaris affects 2-3% of the population (Lebwohl, 2003). Most people with psoriasis have a mild form of the disease that affects less than 20% of their body surface area, typically appearing as itchy red patches behind the ears, around the eyebrows or scalp, or on extensor areas (elbows, knee caps). Approximately 5% of psoriatic patients have severe disease covering more than 80% of their total body surface area. Affected areas are clearly demarcated, red, and scaly. Plaques typically remain stationary over time (if untreated) and can appear in areas after injury such as a severe sunburn or trauma to the skin– a sign known as the Koebner phenomenon (Barisic-Drusko and Rucevic, 2004). Psoriasis has a genetic component as evidenced by 80% penetrance between monozygotic twins and 30% between dizygotic twins (Bowcock, 2004; Brandrup *et al.*, 1978; Brandrup *et al.*, 1982). Approximately 20% of psoriatic patients also have arthritis.

Psoriasis histology

Normal epidermis has four discrete layers: The *basal layer* attached to the dermis by hemidesmosomes, the *spiny layer* which gets its spiny appearance from solid intercellular desmosome attachments, the *granular layer* due to keratohyaline granules

forming in the keratinocytes (KCs), and the *stratum corneum* consisting of old granular cells that have lost their nuclei and form a waxy outer barrier that protects the skin from dehydration and mechanical damage. Psoriasis skin is flaky, red, and itchy – all of which can be explained by looking at psoriatic plaque histology. The most glaring difference between psoriatic and normal skin is a greatly thickened epidermis with elongated rete ridges extending deep into the dermis. The thickening is a result of an increase in epidermal proliferative rate by 20X. In addition, there is a reduced transit time of KCs migrating outward through the epidermis, from 28 days to 4 days. This leads to nuclei being retained in the stratum corneum (parakeratosis) because cells have not had a chance to mature and differentiate. For similar reasons, the stratum corneum in psoriatic tissue is abnormal because immature granular cells are not able to form strong intercellular connections. In the dermis, psoriatic blood vessels are also elongated and dilated, surrounded by leukocytes, resulting in redness and itch (from cytokine release). Notably, follicular epithelial lining is unaffected by the disease (Bowcock and Krueger, 2005).

Psoriasis-like animal models

It should be emphasized at the outset that there is no spontaneous model of psoriasis in animals. However, the use of genetically engineered xenograft transplant mice has aided greatly in understanding some of the basic mechanisms of skin inflammation as they apply to psoriasis and other inflammatory diseases. Several murine models of “psoriasis” are now described.

Constitutive p40 expressing keratinocyte model: IL-12 and IL-23 (related cytokines that share a p40 subunit) stimulate T cells to differentiate and produce IFN γ and IL-17/IL-22, respectively. Mice were genetically engineered to constitutively express p40 in the skin by joining the keratin-14 promoter with the p40 gene (Kopp *et al.*, 2001). Although it was expected that these mice would overproduce both IL-12 and IL-23, basal keratinocytes co-secrete transgenic p40 only with endogenous p19 to constitutively produce IL-23 (p19/p40) (Kopp *et al.*, 2003). These animals developed inflammatory skin lesions with marked epidermal hyperplasia and increased levels of DC and T cell derived cytokines similar to those found in psoriasis lesions. Thus, IL-23 appears to be an important “up stream” inflammatory product leading to synthesis of “down stream” genes controlled by IL-17 (STAT3 and neutrophil chemokines) and IL-22 (upregulation of antimicrobial peptides and downregulation of keratinocyte maturation genes) (Nogales *et. al.*, submitted).

Interferon regulatory factor-2 knockout model: Interferon (IFN)-induced genes are transcribed by several types of transcription factors, including STAT1 and IFN regulatory factors (IRFs). IRF-1 is a transcriptional activator whereas IRF-2 suppresses IRF-1 activity. Hida *et al* have engineered an IRF-2 null mouse that chronically over expresses IRF-1 (Hida *et al.*, 2000). These mice spontaneously develop inflammatory skin lesions similar to psoriasis including CD8⁺ T cell infiltration in the epidermis, CD4⁺ T cells in the dermis, and marked epidermal hyperplasia, implicating IFN γ as another important mediator of psoriasis.

Xenograft models: Another approach that has yielded useful information has been to transplant unaffected (non-lesional) skin or lesional skin from a psoriasis patient on severe combined immunodeficiency (SCID) mice (Boehncke *et al.*, 1994). Injection of cytokines into the uninvolved tissue grafts induces some mild hyperplasia whereas injection of superantigen-activated mononuclear leucocytes obtained from peripheral blood of the same patient induces a full psoriasis phenotype (but without neutrophil infiltration), suggesting that the psoriasis phenotype can be induced in genetically predisposed skin by bacterial antigen primed leukocytes (Wrone-Smith and Nickoloff, 1996). When psoriasis lesional skin is grafted, long-term grafts continue to show viable T cells and other mononuclear leukocytes. Thus, in this model it appears that T cells can continue to expand in situ in skin lesions at a rate that matches the rate of programmed cell death, and one does not need new T cells from the peripheral circulation to perpetuate the lesion.

Perhaps of greater importance has been the observation that when non-lesional “normal” skin from psoriasis patients are grafted on to highly immunodeficient AGR129 mice, the bystander T cells in the graft expand in situ and cause a full blown psoriatic lesion (Boyman *et al.*, 2004). Unlike SCID mice, AGR129 mice lack both NK cells and IFN receptors, which may leave them unable to reject graft T cells and/or create a cytokine environment more conducive to T cell activation. Also of interest is the fact that either anti-TNF or anti-CD3 antibodies can block the onset of these lesions. More recently, this model was used to show dependence of psoriatic phenotype on invasion of T cells into the epidermis (Conrad *et al.*, 2007).

Pathogenic insights provided by genetics

Twin studies suggest that there is a strong genetic component to psoriasis. Over ten gene mapping studies have been performed on families with high prevalence of psoriasis in the USA and Netherlands, indicating there are at least 17 susceptibility genes (Bowcock, 2004). One of the most compelling susceptibility genes is human leukocyte antigen (HLA)-Cw*0602 which was found in 10% of healthy controls and 50% of psoriasis patients (Nair *et al.*, 2000). HLA-Cw is a likely convergence between genetic linkage studies and psoriasis pathophysiology because it is an HLA class I molecule that can bind type A strep and has a strong association with psoriasis triggered by streptococcal pharyngitis. T cells in the tonsil are almost exclusively skin homing, thus it is possible that HLA-Cw T cells reacting to strep have antigenic mimicry with an epidermal self-antigen (Elder, 2005, 2006). Finally, a susceptibility region (PSORS2) has been mapped to a discrete DNA sequence. PSORS2 is shown to encode a mutated binding site for the transcription factor RUNX1. Two adjacent genes, SCL9A3R1/EBP50 and RAPTOR, each associated with activation-related signal transduction, may be affected in a way that leads to T cell activation or keratinocyte hyperplasia, but more work is needed to fully understand the functional consequences of this mutation. Other susceptibility loci for psoriasis are found on chromosomes 1q21, 3q21, 4q, 7p, 8, 11, 16q, 17q, and 20p (Bowcock, 2005).

More recently, large linkage studies from multiple groups have shown that psoriatic patients are more likely to carry certain polymorphisms in their IL-23/IL-12 p40 subunit and IL-23R genes compared to controls (Capon *et al.*, 2007; Cargill *et al.*, 2007;

Liu *et al.*, 2008). Researchers speculate that the alternative allele may be protective against psoriasis, and that the psoriatic allele is permissive. Indeed, the IL-23 pathway is important for psoriasis pathogenesis as described in detail throughout this thesis.

Psoriasis and autoimmunity

While psoriasis is commonly referred to as an autoimmune disease, the strict definition of autoimmunity requires that the immune system react against host antigen(s). Although these autoimmune antigens have not yet been identified, it is clear that the psoriatic cellular immune response is overactive either due to auto-antigen mimicry or innate immune dysregulation that does not require a host antigen catalyst. The existence of an auto-antigen may be inferred from clonal T cells isolated from psoriatic plaques (Diluvio *et al.*, 2006). This theory is also appealing because it fits with the model of other common chronic inflammatory diseases, particularly arthritis where there is mimicry of bacterial peptides or lupus where dsDNA appears to be an immunogen. It does not fit, however, with the immunohistochemistry staining of psoriasis lesions that reveal very few B cells in lesional skin compared with a vast influx of dendritic cells and T cells. Autoimmune diseases like arthritis or lupus with isolated antigens have a large B cell component at the site of injury (Yurasov *et al.*, 2005).

Evolving theory of immunopathology

The earliest studies on psoriasis concluded from the histology that it was a disease caused by hyperproliferative keratinocytes (Farber and McClintock, 1968). Only when immunomodulating therapies targeting T cell influx into the tissue proved effective did

researchers focus on an overactive cellular immunity as the primary cause of disease (Gottlieb *et al.*, 1995). Recently it has been noted that psoriatic plaques contain an equal number of DCs as T cells distributed throughout the epidermis and dermis. Moreover, as with T cells, decrease in DC numbers corresponds with plaque clearance during treatment (Nickoloff and Stevens, 2006). Affymetrix expression data shows that more dendritic cell genes are dysregulated in psoriasis lesions than T cell lineage genes (Haider *et al.*, 2007). Another indication that psoriasis may be in part a DC-mediated disease, is that TNF inhibitors reliably clear psoriatic skin plaques. TNF and TNF receptors are not found at high levels on T cells but are found on monocyte derived dendritic cells (Gottlieb *et al.*, 2005). Recent work on mouse models has identified a type of DC termed the TNF and inducible nitric oxide synthase (iNOS)-producing dendritic cell (Tip-DC) that has activated NF κ B and STAT transcription factor signal transduction pathways (Serbina *et al.*, 2003). The upstream regulators of these transcription factors are TNF and IFN γ /IL-17 respectively. Thus, TNF inhibitors may inhibit TIP-DC function or maturation, shifting the immune response away from the pathogenic T cell response. An in depth description of DC and T cell subsets in normal skin is now presented followed by a description of DC and T cell subsets in inflammatory psoriatic lesions.

Human cutaneous DC subsets during-steady state and inflammation

Cells with long dendrites were first described in the epidermis of the skin by medical student Paul Langerhans nearly 140 years ago (Langerhans, 1868). It was not until 1973 that the term “dendritic cell” was coined as these cells were effectively re-

discovered by Ralph Steinman and Zanvil Cohn (Steinman and Cohn, 1973). These DCs were identified as potent antigen presenting cells (APCs) in the mixed leukocyte reaction (MLR). However, it took a number of years for scientists to appreciate the significance and potential roles of these cells, and we now know that they are central in generating and regulating immune responses.

DCs are a heterogeneous population of cells in the immune system, defined initially by their appearance, but more specifically by their potent ability to present antigen to T cell. Standardized characterization of human cutaneous DC populations is complicated by pleomorphic phenotype and function during emigration from the skin for *ex vivo* study, and by the great number of potential surface and intracellular antigens that are present on these leukocytes. In addition, there are differences between human and murine DC networks. DC populations have been historically classified either spatially (circulating blood DCs, draining lymph node DCs, epidermal DCs, and dermal DCs), by their presumed origin (myeloid DCs, plasmacytoid DCs [pDCs]), by physiological or pathophysiological state (steady-state DCs, inflammatory DCs), or by antigen expression (Langerin, DEC-205 etc.). There are three cutaneous DC populations in the steady state – epidermal Langerhans cells (LCs), resident dermal myeloid DCs, and pDCs – and during inflammation there appears to be an additional population of myeloid dermal “inflammatory DCs”.

Langerhans cells

LCs were the first DC subset described (Langerhans, 1868). They reside in the suprabasal layers of the epidermis wedged in between, and in close contact with, keratinocytes. The stellate appearance of these cells led researchers to believe that they were of neural origin until over 100 years later, when their role in antigen presentation was elucidated (Braathen and Thorsby, 1980). LCs were initially identified by the electron-dense organelle, the Birbeck granule, which has a unique tennis-racket appearance. The function of Birbeck granules is still unclear, but likely includes receptor-mediated endocytosis and transport of cellular materials into the extracellular space (Mc Dermott *et al.*, 2002). The first monoclonal antibody that clearly identified LCs was CD1a, an MHC-I-like molecule that presents microbial lipids to T cells (Barral and Brenner, 2007). More recently, the monoclonal antibody Langerin/CD207 has been used to specifically recognize LCs (Figure 2). Langerin/CD207 is a membranous C-type lectin that recognizes mannosylated ligands found on the surface of a wide range of pathogens including viruses, bacteria, fungi, and protozoa (Figdor *et al.*, 2002). Following receptor mediated endocytosis, CD1a and Langerin/CD207 traffic to the Birbeck granule where they may participate in antigen processing (Stossel *et al.*, 1990).

Elegant studies by Merad *et al.* have shed light on the origin and trafficking of LCs during steady state and inflammation (Merad *et al.*, 2004; Merad *et al.*, 2002). It now seems clear that epidermal LCs are continuously replaced from a resident precursor pool (perhaps by self-renewal) throughout life under steady-state conditions (Merad *et al.*, 2002). However, LCs are repopulated by blood precursors, most likely monocytes, following inflammation (Ginhoux *et al.*, 2006). In human psoriasis vulgaris, there are variable reports of the number and

arrangement of LCs in the epidermis adjacent to non-inflamed epidermis. This may be due to different staining and counting methods used for analysis, or non-equivalent patient populations. In our studies on stable plaque psoriasis, we have found that LCs in lesional and non-lesional epidermis are of similar number per linear surface, with a redistribution to the upper layers of the thickened psoriatic epidermis, and express comparable antigen markers (unpublished data). These observations are supported by studies showing that LC migration in psoriasis is impaired, leading to retention of LCs in psoriatic inflammation (Cumberbatch *et al.*, 2006). Further studies need to be performed to evaluate LCs during human disease states using these new markers.

Interest in LCs has increased with two key observations. Firstly, in mice, there is a newly discovered population of Langerin⁺ DCs in the dermis and skin draining lymph nodes (Bursch *et al.*, 2007; Douillard *et al.*, 2005; Ginhoux *et al.*, 2007; Poulin *et al.*, 2007; Stoitzner *et al.*, 2003; Stoitzner *et al.*, 2005). These epidermal and dermal LC populations have distinct phenotypic and mitotic capacities, and the dermal Langerin⁺ LCs do not appear to be simply epidermal LCs en route to the draining lymph node. Epidermal LCs are Langerin⁺CCR6⁺CCR7^{+/-}, radioresistant, slowly self-replicating (Merad *et al.*, 2002), and are highly responsive to CCR6 agonist macrophage inflammatory protein (MIP)-3alpha (CCL20). In contrast, dermal LCs are Langerin⁺CCR2⁺CCR7^{+/-}, radiosensitive, rapidly self-replicating, and are responsive to CCR2 agonist monocyte chemoattractant protein (MCP)-1 (CCL2) (Bursch *et al.*, 2007; Ginhoux *et al.*, 2007; Poulin *et al.*, 2007). Langerin⁺CD1a⁺ dermal LCs may also exist in human skin (Angel *et al.*, 2006), although we have not yet been able to identify many dermal LCs in normal or psoriatic skin *in situ* (Lowes *et al.*, 2005a). Secondly, there is much debate concerning the pathophysiological role of LCs. Until recently it was assumed that cutaneous antigens were

locally processed by epidermal LCs which then migrate out of the skin into the draining lymph node for efficient antigen presentation to T cells. During this journey to the lymph node, the LCs change their surface phenotype and “mature”, simultaneously down-regulating antigen-processing and acquiring improved ability for T cell co-stimulation (Larregina and Falo, 2005). Recent studies have questioned the biological significance of this pathway as antigen-specific T cell activation remains intact in LC deficient murine models (Bennett *et al.*, 2005; Kaplan *et al.*, 2005; Kissenpfennig *et al.*, 2005a; Mommaas *et al.*, 1994; Ritter *et al.*, 2004; Zhao *et al.*, 2003). These new observations suggest that there is steady-state migration of LCs to skin-draining lymph nodes, perhaps to induce and maintain tolerance to cutaneous antigens (Steinman and Nussenzweig, 2002). Thus a working hypothesis is emerging whereby DCs that reside in the dermis may be essential for the process of cutaneous immune activation while LCs may play a more important role in sustaining cutaneous immunological tolerance.

Dermal dendritic cells

The population of DCs that reside in the dermis of the skin are known as dermal DCs, and these are considered analogous to “interstitial DCs” found in the connective tissue and stroma of other organs (Shortman and Naik, 2007). As the potential redundancy of LCs for immune stimulation has emerged, there has been more interest in dermal DCs. These important cells have the capacity to take up cutaneous antigens, mature and migrate to draining local lymph nodes, and present these antigens to T cells and B cells (Dubois *et al.*, 1998; Kissenpfennig *et al.*, 2005b). Because culture methods change the surface cellular phenotype, the purest phenotypic classification of these DCs is best performed *in situ*, followed by *ex vivo* functional verification of antigen presenting

capacity. Unfortunately, there is no single or specific marker for these cells, although the integrin CD11c is probably the best tool we currently have to identify them. For many years, antibodies to Factor XIIIa (FXIIIa) clotting factor were used to identify a dermal population of cells which have multiple dendritic processes protruding from a stellate-shaped cell body, and were thus called “dermal dendrocytes” (Headington, 1986). In 1993 Meunier et al. described a small population of HLA-DR⁺CD11c⁺CD1c⁺FXIIIa⁺ cells from cultured normal human dermis that had the capacity to stimulate T cells in a MLR (Meunier *et al.*, 1993). In addition, Nestle et al showed that immunostimulatory dermal DC populations were present from psoriatic skin (Nestle *et al.*, 1998; Nestle *et al.*, 1993). However, it has recently been demonstrated that Factor XIIIa is induced with culture and is actually a macrophage marker, rather than a specific DC marker (D. Torocsik, 2005). Thus, more work needs to be performed to re-evaluate the body of literature classifying dermal APCs as FXIIIa⁺.

Resident dermal DCs

In order to understand dermal DCs and to develop working models to study these cells, we and others have developed the concept that there is a resident population of dermal DCs, as well as an additional group of DCs that appear or develop during inflammation (“inflammatory dermal DCs”) (Shortman and Naik, 2007). Resident murine steady-state dermal DCs (CD11c⁺) appear to be able to proliferate in situ to maintain this population (Bogunovic *et al.*, 2006). The major resident population in normal dermis is identified phenotypically with a single monoclonal antibody, CD1c, which is also known as blood dendritic cell antigen (BDCA)-1. BDCA antibodies -1, -2, -3, and -4 form a

group of proteins that were first used to identify circulating DCs (MacDonald *et al.*, 2002). In steady-state, BDCA-1⁺ DCs are relatively immature with modest T cell stimulatory ability, but their immunostimulatory capacity can be greatly increased with DC maturing stimuli (Zaba *et al.*, 2007b). These BDCA-1⁺ DCs are also CD11c⁺HLA-DR⁺CD45⁺CD14^{lo}, are mostly dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN)/CD209⁺, and by flow cytometric analysis, are approximately 50% CD1a⁺. CD1c⁺CD14⁻CD1a⁺ dermal DCs from normal skin are also CCR7⁺ and are responsive to the lymph node chemokine CCL19, suggesting that these cells can migrate to draining lymph nodes for antigen presentation (Angel *et al.*, 2006). It is possible that these immature BDCA-1⁺ resident DCs are tolerogenic in steady state, analogous to epidermal Langerhans cells.

While most of these BDCA-1⁺ cells are relatively immature, there is a small subgroup (~5%) expressing mature DC markers dendritic cell-lysosomal-membrane associated protein (DC-LAMP)/CD208 and DEC-205/CD205 (Zaba *et al.*, 2007b). These rare, phenotypically mature cells aggregate together in dermal clusters, but their scarcity prevents detailed functional analysis. Perhaps normal skin requires a small population of mature DCs for fast antigen presentation to local T cells.

In normal skin, there is also a population of dermal DCs identified by BDCA-3. These BDCA-3⁺ cells constitute approximately 10% of all CD11c⁺ dermal DCs and do not overlap with BDCA-1⁺ DCs. Again, functional analysis has not been performed on dermal BDCA-3⁺ cells due to their low frequency; however, it has been established that

BDCA-3⁺ DCs in blood are non-overlapping with blood BDCA-1⁺ DCs and are the least immunostimulatory myeloid blood DC population (MacDonald *et al.*, 2002).

Inflammatory dermal DCs

Inflammation induces dramatic changes in dermal DC populations, most obviously a 30-fold increase in CD11c⁺ DCs in the dermis (approximately equal to T cell numbers) (Zaba *et al.*, 2007a). These DCs return to normal or non-lesional levels with effective treatment (Lowes *et al.*, 2005b; Zaba *et al.*, 2007a). Characterization of these cells during inflammation is not yet complete, but our preliminary studies suggest that they are not BDCA-1⁺. This indicates that they may be derived from circulating DC-precursors migrating into the skin due to inflammatory and chemotactic signals. Potential precursors include circulating hematopoietic precursor cells (Massberg *et al.*, 2007; Svensson and Kaye, 2006), circulating “pre-DCs” (CD11c⁺ HLA-DR^{hi}, CD16⁺) (Piccioli *et al.*, 2007; Randolph *et al.*, 2002; Tacke and Randolph, 2006) monocytes (Serbina *et al.*, 2003), or resident DCs.

In psoriasis, we have been able to determine that dermal DCs produce mediators TNF and intracellular nitric oxide synthase (iNOS), and these have been termed TNF and iNOS-producing DCs (Tip-DCs) (Lowes *et al.*, 2005a). Tip-DCs were first described in a murine model of *Listeria monocytogenes* infection (Serbina *et al.*, 2003) and have also been found in murine E.coli bladder infection (Engel *et al.*, 2006). In humans, the location and functions of Tip-DCs are emerging: they are present in the lamina propria of the gut where they may be important for IgA production (Tezuka *et al.*, 2007), and they

appear to be induced by topical imiquimod treatment of basal cell carcinoma (Stary *et al.*, 2007), so they may participate in tumor rejection. Pathogenicity of these Tip-DCs in psoriasis is suggested by the rapid downmodulation of Tip-DC products TNF, iNOS, IL-20, and IL-23 during effective treatment with effective therapies (Haider *et al.*, 2008; Zaba *et al.*, 2007a). We now hypothesize that the Tip-DC is a novel type of DC associated with inflammation that can directly mediate inflammatory processes, through innate immune pathways and associated cytokines such as IL-20 (Wang *et al.*, 2006) and IL-23 (Zaba *et al.*, 2007a). Tip-DCs can also stimulate the differentiation and activation of Th17 T cells (unpublished data), which are a new set of T cells associated with autoimmune inflammation in many disease models (Lowes *et al.*, 2008). The potential role of these DCs as sources of inflammatory mediators is in contrast to the classic role of DCs as antigen-presenting cells, and warrants further attention. Because these DCs may actually be the key target of a variety of anti-inflammatory therapies, and may be broadly involved in "autoimmune" inflammation of many different human diseases, we need to better understand the development and activation of these cells.

In another common skin disease, atopic dermatitis, a population of inflammatory DCs have also been described. These cells were initially termed inflammatory dendritic epidermal cells (IDECs) based on flow cytometric analysis of cells from epidermal cell suspensions (Novak and Bieber, 2005; Wollenberg *et al.*, 1996; Wollenberg *et al.*, 2002). IDECs were defined by the following: HLA-DR⁺Lin⁻CD11c⁺CD1a⁺ and these DCs co-express CD206/macrophage mannose receptor (MMR), CD36, FcεRI, IgE, CD1b/c, CD11b, as well as DC-SIGN/CD209 (Guttman-Yassky *et al.*, 2007). There is now

appreciation that these DCs are dermal and appear to produce a different array of cytokines and chemokines (Th2 chemokines CCL17 and CCL18) than do psoriatic inflammatory DCs, and do not have an iNOS signature (Guttman-Yassky *et al.*, 2007). Thus the cutaneous inflammatory milieu may drive the differentiation of different types of inflammatory DCs that contribute specifically to the disease specific process and even clinical phenotype.

Plasmacytoid DCs

PDCs are an additional unique population of resident cutaneous DCs initially described by their morphology, which is similar to a plasma cell (Corcoran *et al.*, 2003). PDCs share many characteristics with B cells, including dependence on a B cell transcription factor (SPI-B), and immunoglobulin gene rearrangements. It now appears that both PDCs and myeloid DCs may actually arise from a bone marrow-derived common DC precursor (Naik *et al.*, 2007; Onai *et al.*, 2007). While both myeloid and plasmacytoid DCs express high levels of HLA-DR and have the capacity to present antigen, pDCs are characterized by their ability to produce large amounts of type 1 interferon (IFN) (IFN- α , β , ω) during viral infection – 10,000 fold more IFN than any other cell type (Ito *et al.*, 2004; Kadowaki *et al.*, 2000). This potent IFN production is induced by viral RNA and DNA containing repeated unmethylated CG nucleotides binding to Toll-like receptor (TLR) 7 and TLR9 endosomal receptors. IFN- α , the most studied of the Type I IFNs, modulates development and maturation of many immune cells, including T cells and myeloid DCs, by binding to abundantly expressed IFN

receptor (Theofilopoulos *et al.*, 2005). The pleiotropic effects of pDC activation are complicated and not fully understood.

Monoclonal antibodies for pDC identification are well developed and include CD123/IL-3R, BDCA-2, and BDCA-4. BDCA-2 is the only marker that is exclusive to pDCs, as myeloid DCs can express low levels of CD123 and BDCA-4 (Liu, 2005). Thus, a common phenotypic definition of blood and tissue pDCs is HLA-DR⁺CD11c⁻CD123^{hi}BDCA-2⁺. Research on the function of human pDCs during steady-state and disease is limited by low frequency and transitory expression of IFN α (Nestle and Gilliet, 2005).

In our studies of normal skin, we find a small number of pDCs (Zaba *et al.*, 2007b), and there does not appear to be any marked increase in the frequency of these cells in chronic large plaque psoriasis (Guttman-Yassky *et al.*, 2007), although others have found an increase of these cells in psoriasis (Nestle *et al.*, 2005). However, two recent observations support a role for pDCs during the initiation phase of psoriasis. Firstly, in xenotransplant experiments of non-lesional skin from patients with psoriasis grafted onto immunosuppressed mice, the stress of transplantation is sufficient for psoriasis to develop spontaneously in the graft. Blockade of IFN- α prevented development of psoriasis, implicating pDCs as they are the primary source of IFN- α in the skin (Nestle and Gilliet, 2005). Secondly, keratinocyte peptide LL37 (also known as cathelicidin or cathelicidin antimicrobial peptide [CAMP]), binds to self-DNA and TLR9 in pDCs, and induces IFN- α (Lande *et al.*, 2007). Expression of this antimicrobial

peptide is increased during inflammation such as psoriasis, and down-stream effect of this increased IFN- α production may be activation of myeloid DCs and initiation of psoriasis (Farkas *et al.*, 2001; Nestle and Gilliet, 2005). Patients with psoriasis may have pDCs that are particularly sensitive to LL37 and respond in an exaggerated manner with increased IFN- α induction, thus setting in motion psoriatic inflammation.

Th17 T cells as mediators of psoriasis pathogenesis

Until recently, IFN γ -producing Th1 T cells were implicated as the main pathogenic cells (Blauvelt, 2007) as certain T cell-targeted therapies were successful in clearing psoriasis (Lowes *et al.*, 2007), and clonal T cells have been found in psoriatic skin (Prinz *et al.*, 1994). However, we are beginning to appreciate that there may be an important pathogenic contribution from a recently-recognized subset of T cells – Th17 cells producing IL-17 and IL-22 (Bettelli *et al.*, 2007; Blauvelt, 2007). In model systems, IL-17 stimulates keratinocyte production of innate inflammatory “danger signals” such as defensins and S100 proteins as well as IL-8 neutrophil chemokine (Liang *et al.*, 2006), while IL-22 induces S100 innate defense proteins (Wolk *et al.*, 2006) and keratinocyte hyperproliferation (Sa *et al.*, 2007; Zheng *et al.*, 2007). Upstream inducers of Th17 cells are still being understood, as most experiments have been performed in murine model systems. Mediators may include IL-1, IL-6, and TGF β , which stimulate differentiation of naïve CD4⁺ T cells into activated memory Th17 cells (Annunziato *et al.*, 2007; Mangan *et al.*, 2006; Sutton *et al.*, 2006), and IL-23 which drives Th17 proliferation (Vanden Eijnden *et al.*, 2005).

Th17 T cells producing IL-17 and IL-22 have been implicated as pathogenic in murine models of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and inflammatory bowel disease (IBD) (Granelli-Piperno *et al.*, 2005; Komiyama *et al.*, 2006; Nakae *et al.*, 2002; Nakae *et al.*, 2003). IL-17 knockout mice are resistant to both EAE and CIA. Also, mice with EAE have increased numbers of Th17 cells, but are resistant to disease if immunized against IL-17 (Uyttenhove and Van Snick, 2006). The DC product IL-23, a survival factor for Th17 cells, also appears to be necessary for IBD pathogenesis in mice (Yen *et al.*, 2006). Thus a model is emerging of autoimmune inflammation that begins with activated APCs producing IL-23, subsequent Th17 cell proliferation and IL-17/IL-22 release, and downstream inflammatory tissue damage.

Most studies on Th17 cells have been performed in murine models or *in vitro*. However, there is some human data also supporting a similar model of Th17-mediated autoimmune inflammation. Patients with IBD have elevated IL-17 and IL-22 in affected colonic tissue and serum, dependent on disease activity and severity (Fujino *et al.*, 2003; Nielsen *et al.*, 2003; Te Velde *et al.*, 2006), and patients with rheumatoid arthritis (RA) have elevated IL-17 and IL-22 protein in synovial fluid (Ikeuchi *et al.*, 2005; Kotake *et al.*, 1999). In psoriasis patients, IL-17 mRNA has been demonstrated within lesions (Li *et al.*, 2004), but protein levels are not increased in the serum (Arican *et al.*, 2005). IL-22 protein is increased in psoriatic serum compared to normal, and mRNA is increased in lesional tissue (Wolk *et al.*, 2006). High levels of IL-23 have also been detected in

psoriasis lesions (Piskin *et al.*, 2006), and are strongly diminished by effective therapies for psoriasis (Lowes *et al.*, 2005b).

Anti-inflammatory and immune modulating effects of TNF inhibition in psoriasis

Psoriasis drug treatment falls into two basic categories: general immunosuppressive drugs (cyclosporine) and targeted immunomodulating drugs (anti-CD2, anti-CD11a, anti-TNF). The systemic immunosuppressive drugs tend to have major side effects such as general immunosuppression and kidney failure (Andoh and Bennett, 1998; Bennett, 1998), whereas the targeted immunotherapy has a unique side effect profile for each drug. The effectiveness of each drug is also variable and unpredictable. Currently, TNF blockers have become a front-line drug for treatment of moderate to severe psoriasis (Strober, 2005).

Two anti-TNF drugs are currently in use in the clinic: etanercept (Enbrel, Amgen) is a TNF/lymphotoxin antagonist fusion protein consisting of the extracellular domain of the low affinity TNF-RII and immunoglobulin constant region domain, and infliximab (Remicade, Johnson & Johnson) is a chimeric antibody with a mouse Fab fragment raised against human TNF, fused with a human Ig constant region. Etanercept effectively improves psoriasis in 60-70% of patients depending on the drug regimen (Gottlieb, 2004; Gottlieb *et al.*, 2005), and infliximab is slightly more effective for first time users of the drug, although development of human anti-mouse antibodies frequently prevent a second drug course. Mechanism of action of these drugs is complicated, as both the Fab

fragment and Fc portion of the antibodies can modulate immunity and TNF is a global cytokine produced by many cells in abundance. The primary mechanism of action for etanercept is still unknown, although we postulate that a primary effect of etanercept is that it blocks TNF that would otherwise lead to maturation of dermal DCs and downstream activation of Th1 and Th17 cells.

Summary

Psoriasis is a rapidly changing field, facilitated by relatively easy access to the diseased organ (human skin), and by immune modulating drugs effectively creating human knock-out models. Elucidation of the physical and chemical interactions between DCs and T cells is important for understanding psoriasis immunopathogenesis and creation of more specific immune modulating therapies. Effective clinical trials using TNF or IL-12/IL-23 p40 subunit blockers demonstrate that DC activation products and downstream DC-dependent Th1/Th17 T cell activation is important in psoriasis pathogenesis.

CHAPTER 2 : Materials and methods

Skin samples

Normal skin. Skin punch biopsies (6 mm diameter) were obtained from 15 normal volunteers under a Rockefeller University IRB-approved protocol. Informed consent was obtained. Biopsies were frozen in OTC (Sakura) and stored at -80°C for immunohistochemistry and immunofluorescence. Normal samples from abdominoplasty were processed within 4 hours post-surgery. These abdominoplasty samples were also the source of tattoo material. Dermal single cell suspensions from normal skin were obtained using a modified collagenase/dispase method (Angel *et al.*, 2006). Subcutaneous fat was excised and remaining tissue was washed with PBS. The dermal layer was heavily scored with a scalpel and incubated in 1 mg/ml type 1 collagenase (Invitrogen Life Technologies), 1 mg/ml dispase (Invitrogen Life Technologies), and 1% penicillin-streptomycin solution (Sigma) overnight at 37°C . The epidermis was peeled off and discarded, and the dermis was transferred to fresh RPMI 1640 supplemented with 10% pooled human serum (Mediatech Inc.), 0.1% gentamicin reagent solution (Gibco-BRL Life Technologies), and 1% 1M HEPES buffer (Sigma). The dermis was incubated 24-48 hrs at 37°C and the supernatant was collected and filtered with 40- μm cell strainers (BD Biosciences). Cells were then either used immediately for MLR, or frozen in RPMI 1640 (Gibco-BRL Life Technologies) and 10% DMSO (ATCC) for FACS.

Psoriasis skin samples for etanercept clinical trial. Twenty adult patients with moderate to severe psoriasis were treated with etanercept 50mg subcutaneously twice weekly (BIW) for 12 weeks under a Rockefeller University IRB approved protocol. Patients did not receive topical or systemic psoriasis therapy for a minimum of 1 month before dosing. No patient was experiencing flare at the initiation of etanercept treatment. At baseline, 6mm (diameter) punch biopsies were taken from an uninvolved area (non-lesional) and from an index psoriasis lesion. Punch biopsies were obtained again from the index lesion at weeks 1, 2, 4, and 12 of etanercept treatment. All biopsies were cut in half – one piece was frozen in liquid nitrogen for RNA extraction, and the other was frozen in cutting media for immunostaining. 1cm² shave biopsies used for FACS analysis on dermal émigrés were obtained from lesional areas at baseline and week 2. Patients were classified as histological responders based on frozen section epidermal thickness, K16 immunostaining, and Ki67 cell counts decreasing to non-lesional magnitude by week 12 (Gottlieb *et al.*, 2005).

Cell phenotyping assays

Antibodies. All antibodies used for immunohistochemistry, immunofluorescence and FACS are listed in Tables 1 and 2.

Immunohistochemistry. Skin sections were stained with primary antibodies listed in Table 1. Biotin labeled horse anti-mouse, and biotin labeled rabbit anti-sheep antibodies (Vector Laboratories) were used to detect mouse monoclonal and rabbit polyclonal

antibodies, respectively. The staining signal was amplified with avidin-biotin complex (Vector Laboratories) and developed using chromogen 3-amino-9-ethylcarbazole (Sigma Aldrich). Positive cells per mm were counted manually using computer-assisted image analysis (NIH IMAGE 6.1). Appropriate negative controls were used.

Immunofluorescence. Skin sections were fixed with acetone and blocked in 10% normal goat serum (Vector Laboratories) for 30 mins. Primary antibodies (listed in Table 1) were incubated overnight at 4°C, and amplified with the appropriate secondary antibody: goat anti-mouse IgG1 conjugated with Alexa Fluor 488 or 568, or donkey anti-sheep conjugated with Alexa Fluor 488 or 568, respectively. For co-localization, sections were co-stained overnight with a second antibody (listed in Table 1), and amplified with the appropriate goat-anti mouse secondary antibody. Images were acquired using a Zeiss Axioplan 2I microscope with Plan Apochromat 20 X 0.7 numerical aperture lens and a Hagamatsu orca ER-cooled charge-coupled device camera, controlled by METAVUE software (Universal Imaging). Dermal collagen fibers gave green autofluorescence. Antibodies conjugated with a fluorochrome often gave background epidermal fluorescence. Images in each figure are presented both as single color stains (green and red) so that one can easily appreciate the localization of two markers on similar or different cells. Single stains are above the merged images. Cells that co-express the two markers in a similar location are often yellow in color.

FACS. Cells were stained with the antibodies listed in Table 2. Briefly, cells were stained for 20 mins at 4°C, washed with FACSwash (PBS 0.1% sodium azide and 2% FBS) and

resuspended in 1.3% formaldehyde (Fisher Scientific) in FACSwash. For intracellular cytokine staining assays, cells were activated in RPMI for 4 hours using 25 ng/ml phorbol myristate acetate (PMA) and 2 µg/ml ionomycin, in the presence of 10 µg/ml brefeldin A (all Sigma Aldrich) at 37°C. Unactivated controls were treated with brefeldin A only. Ethylenediaminetetraacetic acid (2 mM, Fisher Scientific) was added for 10 min. at 37°C to stop activation. Cells were then incubated in aqua marina live/dead dye (BD Biosciences) for 30 min. on ice, washed, fixed with 4% paraformaldehyde (BD Biosciences) for 20 min. on ice, blocked in 1:100 mouse serum (BD Biosciences), permeabilized in FACSPERM (BD Biosciences), incubated simultaneously with intracellular cytokine and extracellular antibodies, washed, and collected. Samples were acquired using either a FACSCanto or LSR-II (both BD Biosciences) and analyzed with FlowJo (Treestar). Appropriate isotypes and unactivated controls were used.

Electron Microscopy. Skin was fixed in 2.5% glutaraldehyde and processed by routine transmission electron microscopy procedure. Semi-thin plastic sections were stained with toluidine blue for light microscopic evaluation. Ultra-thin (65nm) sections were cut with a diamond knife, collected on copper grids, stained with both uranyl acetate and lead citrate before viewing in a Tecnaispirit electron microscope (FEI Company) equipped with Ultrascan 895 CCD camera (Gatan).

Functional assays

FACS sort and Mixed Leukocyte Reaction (MLR) using BDCA-1⁺ or CD163⁺ cells from normal dermal émigrés. Dermal cells from single cell suspensions of normal skin were

stained with BDCA-1, CD19, and CD163 antibody and sorted on a FACS Aria (BD Biosciences) using a low-pressure setting. Two populations were obtained: BDCA-1⁺CD19⁻ and CD163⁺. A post-sort collection was performed to confirm the purity of each stimulating population. For some experiments, sorted cells were cultured for 2 days with and without cytokines for maturing DCs ex-vivo (IL-1 β , IL-6, TNF- α , PGE₂), then washed and prepared for the MLR.

Responding T cells were obtained from a normal volunteer by density centrifugation over Ficoll-Paque Plus (Amersham Biosciences), followed by T cell purification using a T cell negative selection kit (Dyna). T cells were labeled with 10 μ m CFSE and co-cultured with either BDCA-1⁺ or CD163⁺ sorted cells at 1:10, 1:50, or 1:100, depending on cell yield. T cells without a stimulator population were used as a negative control, and monocyte-derived mature DCs were added to T cells as a positive control. The process for making mature DCs was previously described (Lee *et al.*, 2002). T cell proliferation was analyzed on day 8 post-sort. The cultures were harvested, stained with 250 ng/ml propidium iodide (PI) to label dead cells, and CD3-APC (Becton Dickinson) for 15 mins at room temperature. PI negative cells were gated and then plotted as CFSE versus CD3⁺ cells, where proliferating cells diluted their content of CFSE and move to the left of the non-proliferating cells. The CFSE low cells were quantified as a percentage of live cells in the culture (Parish, 2001).

FACS sort and MLR using BDCA-1⁺, BDCA-1⁻ or CD163⁺ cells from psoriatic dermal émigrés. Dermal cells from single cell suspensions of psoriasis shave biopsies were stained with BDCA-1, CD163, CD11c, and HLA-DR antibodies and sorted on a

FACSAria using a low-pressure setting. Three populations were obtained: CD163⁺, CD11c⁺HLA-DR⁺BDCA-1⁺ and CD11c⁺HLA-DR⁺BDCA-1⁻. A post-sort collection was performed to confirm the purity of each stimulating population. An MLR was performed as described for cells obtained from normal skin.

In vitro TNF neutralization assays. The process for making monocyte derived DCs (moDCs) has been previously described (Dhodapkar *et al.*, 2002). All analysis was performed on “day 5” immature DCs. Etanercept 10µg/mL was added to experimental wells on days 0, 2, and 4. We chose this concentration of etanercept as it approximates the plasma concentration of drug when given 50mg BIW. Three biological replicates of each condition were prepared. For the MLR, responding T cells were obtained from a normal volunteer by density centrifugation over Ficoll-Paque Plus (Amersham Biosciences), followed by T cell purification using RosetteSep (StemCell Technologies). T cells for cytokine FACS staining were purified from peripheral blood using T negative select beads (Dyna), activated using PMA and ionomycin as previously described (Vugmeyster *et al.*, 2004), and cultured for 24 hours +/- etanercept 10µg/mL. Three biological replicates of each condition were prepared.

P40 neutralization and T cell polarization assays. Dermal émigrés from 3 psoriatic patients were cultured with donor T cells (with or without CFDA) (1:10) and IgG isotype or anti-p40 monoclonal antibodies (10ug/mL) for 8 days. Cells were activated using PMA and ionomycin and golgi-stopped using BFA. Non-CFDA loaded cells were stained using live-dead assay, CD3, CD4, CD8, IFN γ , IL-17, and IL-4 and analyzed using

the LSRII FACS machine (BD Biosciences). CFDA cells were stained with PI and CD3 as previously described. T cells alone were used as a negative control, and T cells stimulated with CD3/28 beads (Dyna: 1/2 bead per T cell) were used as a positive control.

Luminex. T cell polarization assay plates were centrifuged and the supernatant collected for measurement of cytokine protein concentration using the human cytokine 25-plex kit (Invitrogen) on a luminex CS1000 autoplex analyzer (Luminex corporation). The assay was performed in duplicate according to the manufacturer's instruction, and results were averaged.

Primary keratinocyte cultures. Primary pooled human keratinocytes (n=3) were obtained from Yale Skin Diseases Research Center core facility and cultured in RPMI 1640 with 10% pooled human serum, 0.1% gentamicin reagent solution, and 1% 1M Hepes buffer at 37°C as above. Once 80% confluent, media was supplemented with or without cytokines rh-IL-17 (R&D) 200ng/ml, rh-IL-22 (Peprotech Inc.) 200 ng/ml, or rh-IFN γ (R&D) 20 ng/ml, for 24 hours before harvesting for other analyses.

RNA assays

RNA extraction and gene array processing. Tissue and cell line RNA was extracted using the RNeasy Mini Kit (Qiagen), DNA was removed with on-column DNase digestion using RNase-free DNase Set (Qiagen), and used for either RT-PCR or gene

array. For each affymetrix genechip, 4µg total RNA was reverse-transcribed, amplified, and labeled as described previously using BioArray High Yield RNA Transcription Labeling Kit (Enzo Biochem Inc.) (Zhou *et al.*, 2003). 15µg of the biotinylated cRNA was then hybridized to Affymetrix Human Genome U133A 2.0 Array (14,500 probe sets) (Affymetrix Inc.). The chips were washed, stained with streptavidin-phycoerythrin, and scanned with an HP GeneArray Scanner (Hewlett-Packard Company). Raw fluorescence intensity values were analyzed using GeneChip Operating software version 1.2 (Affymetrix) and GeneSpringTM. Data for triplicates were averaged. Array files are deposited at: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE9239>

RT-PCR. RT-PCR was performed using EZ PCR core reagents, primers, and probes (Applied Biosystems) as previously published (Chamian *et al.*, 2005). The primers and probes for TaqMan RT-PCR assays for K16, inducible nitric oxide synthase (iNOS), IL-23p19, IL-12/IL-23p40, IFN γ , IL-8, and human acidic ribosomal protein (HARP) were also previously published (Chamian *et al.*, 2005). Sequences of other primers and probes used in these studies were as follows: CCL20 (MIP-3 α) forward GCTTTGATGTCAGTGCTGCTACTC, CCL20 reverse GTATCCAAGACAGCAGTCAAAGTTG, CCL20 probe FAM-TGCGGCGAATCAGAAGC AGCAA-TAMRA, IL-6 forward CCAGGAGCCCAGCTATGAAC, IL-6 reverse CCCAGG GAGAAGGCAACTG, IL-6 probe FAM-CCTTCTCCACAAGCGCCTTCGGT-TAMRA, IL-4 forward CGACTGCACAGCAGTTCCA, IL-4 reverse AGGTTCCCTGTCGAGCCGTTT, IL-4 probe FAM-AGGCACAAGCAGCTGATCCGATTCC-TAMRA, IL-20 (ABI assay #

Hs00218888_m1), IL-12p35 (ABI assay # Hs00168405_m1), LTA-1 (ABI assay # Hs00236874_m1), MX-1 (ABI assay # Hs00182073_m1), IL-17A (ABI assay # Hs00174383_m1), IL-22 (ABI assay # Hs00220924_m1), defensin (ABI assay # Hs00175474_m1), IL-1 β (ABI assay # Hs00174097_m1), and TGF β 1 (ABI assay # Hs00171257_m1). The data were analyzed and samples quantified by software provided with the Applied Biosystems PRISM 7700 (Sequence Detection Systems, ver. 1.7). Data was normalized to HARP housekeeping mRNA.

Statistical analysis

ANOVA. All clinical variables were analyzed using Repeated Measures ANOVA models using the MIXED procedure available in SAS. The within-subjects correlation that best modeled the data was an AR (1) structure that considered each time measurement as dependent on the previous one. Differences between Lesional (Baseline) and weeks 1, 2, 4, and 12 were estimated, and the one-tail p-values are designated as p<0.05 (*), p<0.01 (**), and p<0.001 (***).

Etanercept clinical trial gene array analysis

Array quality control, preprocessing and filtering. GeneChip CEL files were scrutinized for spatial artifacts using Harshlight package

(<http://asterion.rockefeller.edu/Harshlight/index2.html>) (Suarez-Farinas *et al.*, 2005).

Classical microarray quality control report was obtained using *affyQCReport* package

from Bioconductor. Intensity values (CEL files) were pre-processed to obtain expression values using GCRMA algorithm. Expression values were filtered to eliminate probesets with low variation or intensity. Probesets with standard deviations greater than 0.15, and expression values greater than 4 in at least 5 samples, were kept for further analysis. *Affy* control probes were also eliminated. A total of 12,864 probesets passed these filters.

Differential Expression Criteria: The etanercept clinical trial was a repeated measures time course model with a between factor Group (Responders/ Non-Responders). A linear model with Time, Group, Time \times Group and Patient as random effect was considered. Model fitting and hypothesis testing were conducted using the *limma* package from Bioconductor (Wettenhall and Smyth, 2004). *Limma* uses an empirical method to moderate the standard errors of the estimated comparisons, resulting in more stable inference and improved power. All hypotheses tested were derived from this model. For each hypothesis, the p-value of the corresponding statistic (F or t) was adjusted for multiple hypothesis testing, controlling the false discovery rate (FDR) using the Benjamini-Hochberg procedure. Additionally, to determine if individual comparisons were significant, FDR adjustments were made across genes.

To determine disease related genes, a moderated t-test was used between non-lesional (NL) and lesional (LS) measures. To identify genes that varied across time for responders, a moderated F test was used to determine whether any of the expression values across time (weeks 1, 2, 4, and 12) differed from lesional baseline. To determine

genes with different time profiles among responders vs. non-responders (Interaction Term TimexGroup), we used an F test to identify significant interaction terms.

Consensus Clustering: The optimal number of clusters was assessed using Consensus Clustering (CC) algorithm available in GenePattern software (Monti *et al.*, 2003). To assess the stability of the discovered clusters, resampling techniques simulated perturbations of the original data. The clustering algorithm of choice was applied to each of the perturbed data sets, and the agreement (or consensus) among the multiple runs was assessed and summarized in a consensus matrix. A distinct consensus matrix and summary statistics were generated for each cluster scenario. Selection of the optimal number of clusters was done using the consensus matrix and the statistics associated with the empirical cumulative distribution (CDF) of the consensus matrix. The ideal partition had a clear consensus matrix and any further increase in the number of clusters did not lead to an increase of the area under the CDF.

MuSTAT correlations. In order to assess the correlation between IL-17/IL-22 or IFN γ /LTA-1 with epidermal thickness/K16/Ki67, the muStat package available in R (www.r-project.org) was used. U scores were computed for histological response and gene expression taking into account the clustered structure of the data (time points for each patient) as previously described (Wittkowski and Liu, 2002). Variables were normalized within patients in order to make all patients comparable. Correlation between the histological response score and the expression score were calculated and its significance is presented.

Table 1: Antibodies used for immunohistochemistry and immunofluorescence

Antigen	Manufacturer	Clone	Dil
CD11c	BD Pharmingen	B-ly6	1:100
CD11c- A488	BD Pharmingen	B-ly6	1:10
Factor XIIIa	Enzyme Research	AP Polyclonal Sheep	1:100
HLA-DR-FITC	BD	L243	1:100
DEC-205	Dr Steinman	NA	1:100
CD1a-FITC	Pharmingen	HI149	1:10
Langerin	Immunotech	DCGM4	1:100
DC-SIGN-FITC	BD Pharmingen	DCN46	1:50
DC-LAMP-PE	Immunotech	104.G4	1:50
CD14-A488	BD Pharmingen	M5E2	1:100
BDCA-1	Miltenyi	AD5-8E7	1:100
BDCA-2	Miltenyi	AC144	1:100
CD123	BD	9F5	1:20
CD206	GeneTex	15-2	1:100
CD68	BD Pharmingen	Y1/82A	1:100
CD11b	BD Pharmingen	ICRF44	1:10
CD45-FITC	BD Pharmingen	HI30	1:100
FDC	Dako Cytomation	CNA.42	1:25
CD163-FITC	Acris	5C6-FAT	1:100
Vimentin	Lab Vision/ Neomarkers	V9	1:100
CD63-FITC	BD Pharmingen	H5C6	1:100
RFD7	Abcam	RFD7	1:100
INOS-FITC	BD Biosciences	6	1:50
INOS	Santa Cruz	N-20	1: 20
TNF- α -FITC	BD Biosciences	6401.1111	1:10
K16	Sigma Aldrich	K8.12	1:1000
Ki67	Immunotech	Mib-1	1:100
CD3	BD Pharmingen	Sk7	1:100
CD83	BD Pharmingen	HB15e	1:100
IL-20	R&D Systems	158609	1:10
IL-23/IL-12p40	R&D Systems	31052.11	1:50
TNF α -FITC	BD Pharmingen	6401.111	1:25

Notes:

All are murine monoclonals unless stated

NA = not available for purchase

All amplification/ detection antibodies are from Molecular Probes unless stated

CD11c conjugated to A488 gives high non-specific epidermal staining. This was used where there were two IgG1 antibodies.

Table 2: Antibodies used for flow cytometry

Antigen-fluorophor	Manufacturer	Clone	Iso	Dil
BDCA-1 (CD1c)-FITC or PE	Miltenyi	AD5-8E7	IgG2a	1:50
CD11c-PE-Cy7	BD Pharmingen	B-ly6	IgG1	1:33
CD163-A647	Acris	5C6-FAT	IgG1	1:33
Factor XIII A-A647	Enzyme Research	AP Polyclonal Sheep	NA	1:33
HLA-DR-APC-Cy7	BD	L243	IgG2a	1:50
CD14- PerCP Cy5.5	BD Pharmingen	M5E2	IgG2a	1:25
Lin-FITC	BD	various	IgG1 & IgG2b	1:20
CD1a-FITC	Pharmingen	HI149	IgG1	1:20
CD40-FITC	BD Pharmingen	SC3	IgG1	1:20
CD80-FITC	BD Pharmingen	L307.4	IgG1	1:20
CD86-FITC	BD Pharmingen	23GI (FUN-1)	IgG1	1:20
DEC-205 (CD205)-PE	BioLegend	MG38	IgG2b	1:20
MMR (CD206)-FITC	BD Pharmingen	HI 149	IgG1	1:20
Langerin (CD207)-PE	Immunotech	DCGM4	IgG1	1:20
DC-LAMP (CD208)-PE	Immunotech	104.G4	IgG1	1:20
DC-SIGN (CD209)-FITC	BD Pharmingen	DCN46	IgG2b	1:20
BDCA-2-FITC	Miltenyi	AC144	IgG1	1:50
CD45-FITC	BD Pharmingen	2DI	IgG1	1:20
BDCA-3 – PE or FITC	Miltenyi	AD5-14H12	IgG1	1:50
CD163-FITC	Acris	5C6-FAT	IgG1	1:20
HLA-DR-Alexa 700	BioLegend	L243	IgG2a	1:50
CD16-PE-Cy7	BD Pharmingen	3G8	IgG1	1:33
Lin-PE: CD3, CD19, CD20, CD56	Lab custom	various	IgG1 & IgG2b	1:20
CD3-APC-Alexa 750 or Pacific Blue	eBioscience	OKT3	IgG2a	1:33
CD4-PE-Cy7	eBioscience	RPA-T4	IgG1	1:33
CD8-PerCP-Cy5.5	BD Pharmingen	SK1	IgG1	1:20
IL-17- Alexa 488	eBioscience	eBio64DEC17	rat IgG2a	1:20
IFN γ - Alexa 700	BD Pharmingen	B27	IgG1	1:33
Live-Dead – aqua marina	Invitrogen	NA	NA	1:100

Notes:

All are murine monoclonals unless stated

NA = not available for purchase

CHAPTER 3: Characterization of DC subsets in normal human dermal skin

DCs are a major resident leukocyte population in human skin. Two main types of DCs are found in non-inflamed skin: epidermal Langerhans cells (LC) and dermal DCs (Jenny Valladeau, 2005; Larregina and Falo, 2005). LC express Langerin/CD207, an endocytic receptor that localizes to and forms Birbeck granules, as well as the CD1a class I-like molecule that presents glycolipids (Hunger *et al.*, 2004). Dermal DCs have long been defined on the basis of expression of a clotting factor, the transglutaminase, factor XIIIa (FXIIIa) (Cerio *et al.*, 1989). Studies that define dermal DCs as FXIIIa⁺ have often relied on FACS analysis and functional studies using bulk tissue or enzymatically manipulated émigrés that “crawl out” of the dermis over a variable incubation period, which may increase FXIIIa expression (D. Torocsik, 2005; Per Henriksson, 1985).

Currently, so-called myeloid or conventional DCs in many tissues are often identified on the basis of high expression of HLA-DR antigen presenting molecules and the CD11c integrin. DCs are Lin negative, where Lin is a cocktail of antibodies to other cell lineages, including T cells (CD3), B-cells (CD19 and CD20), monocytes (CD14), granulocytes and NK cells (CD16 and CD56) (Freudenthal and Steinman, 1990; Summers *et al.*, 2001). Circulating myeloid DCs can be further classified as three mutually exclusive subsets: blood dendritic cell antigen (BDCA-1)⁺, CD16⁺, and BDCA-3⁺ (in order of immunostimulatory capacity) (MacDonald *et al.*, 2002), but these markers have seen very little use in studies of the skin.

Since a large number of prevalent dermatologic conditions from atopic dermatitis to psoriasis are characterized by extensive dermal T cell infiltration, and as DCs are pivotal antigen presenting cells for T cells, it is important to pursue these distinct phenotypic definitions of DCs in normal skin and peripheral blood. Here we report that CD11c and FXIIIA marked mutually exclusive populations, the former co-expressing BDCA-1, while FXIIIA⁺ cells were uniformly positively for CD163, a scavenger receptor for hemoglobin/ haptoglobin complexes. CD11c⁺ cells were more typical of DCs with higher HLA-DR expression and T cell stimulating activity. Surprisingly FXIIIA⁺ cells behaved more like macrophages since they were weak initiators of T cell responses in the mixed leukocyte reaction (MLR) and had numerous phagocytosed pigment-containing vacuoles in a tattoo.

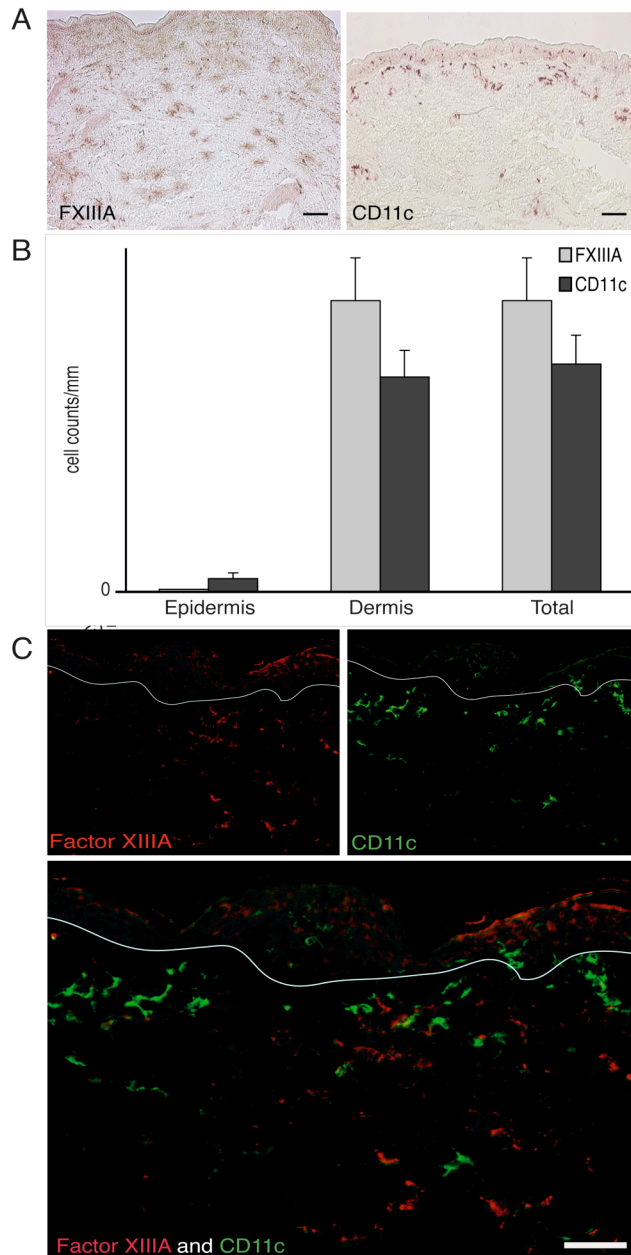
Characterization of dendritic cells in normal human skin and peripheral blood

FXIIIA⁺ and CD11c⁺ cells are discrete dermal populations.

Immunohistochemistry of normal human dermis showed distinct staining patterns for FXIIIA⁺ and CD11c⁺ cells (Figure 1A). The larger FXIIIA⁺ cells were located throughout the dermis. In contrast, CD11c⁺ myeloid cells were located in the papillary and upper reticular dermis. We counted the absolute number of FXIIIA⁺ and CD11c⁺ cells using matched tissue sections from 15 normal volunteers (Figure 1B). In the epidermis there were no FXIIIA⁺ cells and a mean of 4 CD11c⁺ cells (p=0.04) per mm. In the dermis, there was a mean of 83 FXIIIA⁺ cells compared to 61 CD11c⁺ cells per mm (p=0.19). Double-labeled immunofluorescence using FXIIIA (red) and CD11c (green)

confirmed that these two antigens were not expressed on the same cell (Figure 1C). A polyclonal FXIIIA antibody has typically been used for staining skin dermal DCs in the past. We used FXIIIA affinity purified sheep antibody for all of our double label immunofluorescence studies, as it gave the best staining in our hands. There was 100% co-expression of the sheep FXIIIA antibody and a commonly used mouse monoclonal (clone AC-1A1), while other mouse monoclonal antibodies to FXIIIA produced weaker and less consistent staining (data not shown). These data indicate that two commonly used markers for dermal DCs are actually expressed by comparably abundant but different populations.

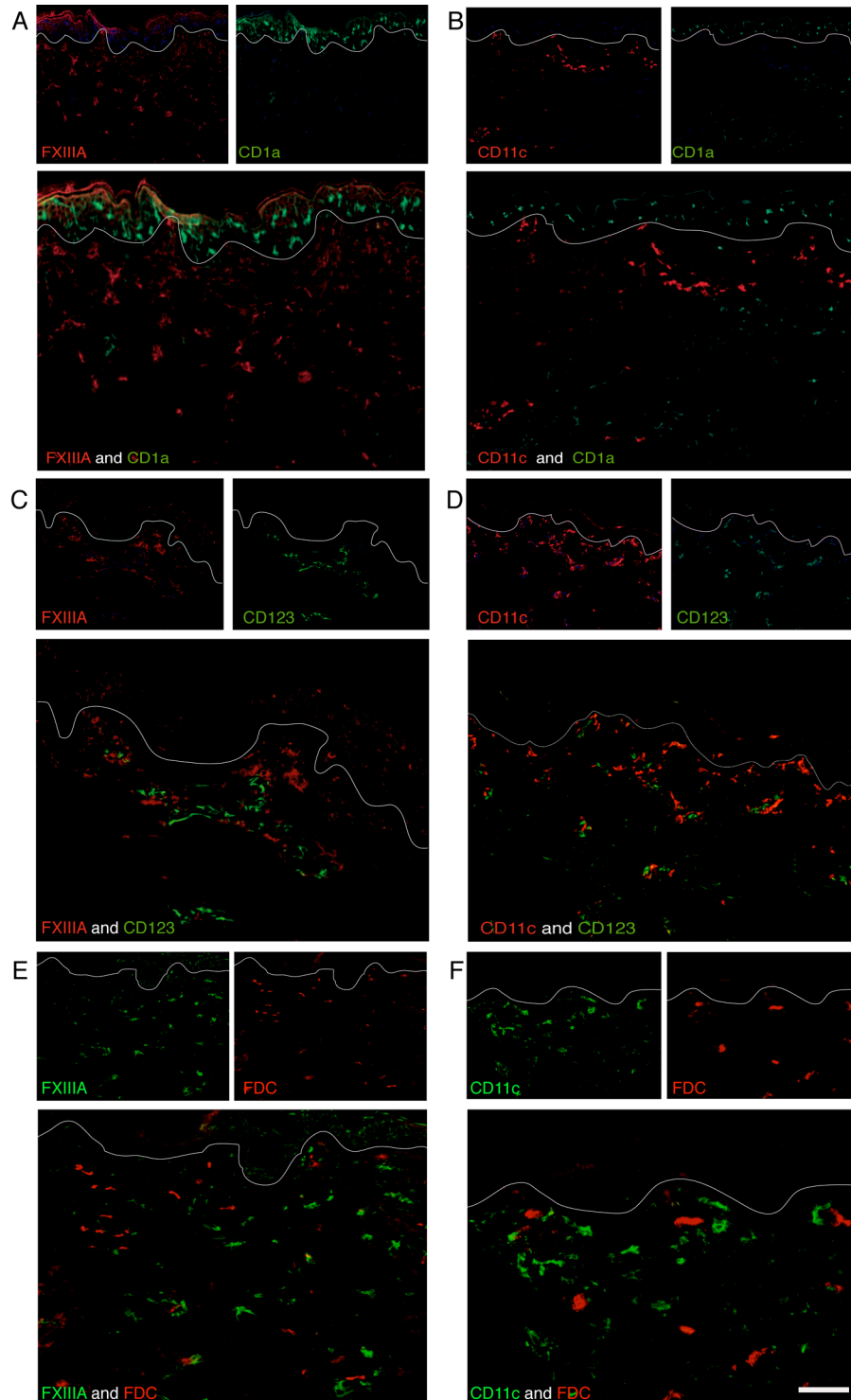
Figure 1. Two antigens used to define normal dermal DCs (FXIIIA and CD11c) are expressed on non-overlapping populations in situ. (A) Immunohistochemistry on normal human skin using FXIIIA (left panel) and CD11c (right panel) antibodies (n=15). FXIIIA⁺ cells were spread throughout the dermis, while CD11c⁺ cells were mainly localized to the superficial dermis. (B) There were similar numbers of CD11c⁺ and FXIIIA⁺ cells per mm in normal dermis. Standard error of the mean indicated. (C) FXIIIA and CD11c identified two discrete populations. Size bar = 100 μ m.



FXIII^A⁺ and CD11c⁺ populations are not Langerhans cells (LC) or plasmacytoid DCs (PDCs).

To further characterize these two dermal cell populations, we first assessed expression of markers used to identify LCs and PDCs, each applied in combination with antibodies to either FXIII^A or CD11c antibody. Neither FXIII^A nor CD11c co-localized with the LC marker CD1a (Figure 2A, B), nor the PDC marker CD123 (Figure 2C, D). Therefore FXIII^A⁺ and CD11c⁺ cells are distinct from additional LC and PDC populations in normal skin.

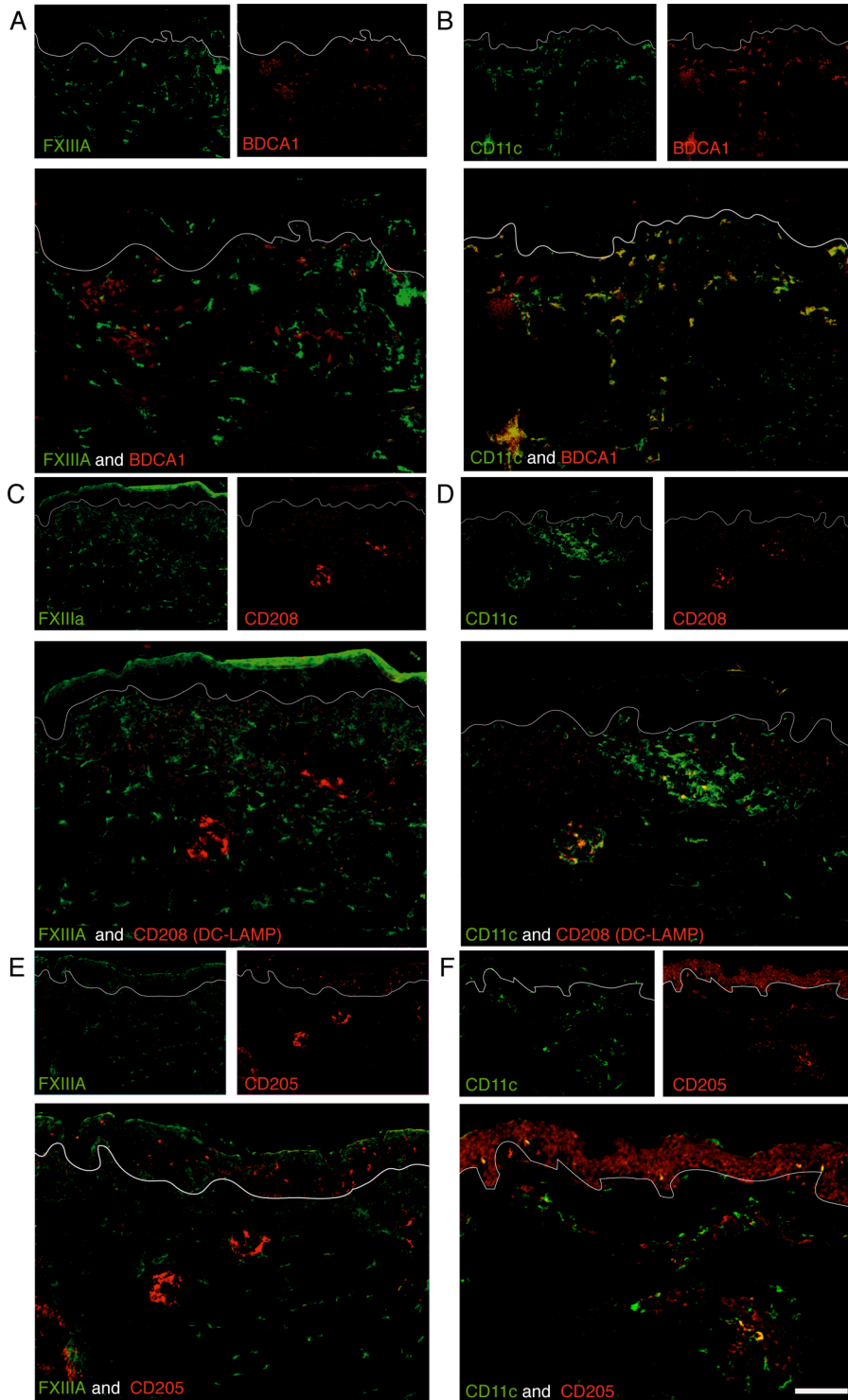
Figure 2. $FXIII^+$ and $CD11c^+$ populations are not Langerhans cells or plasmacytoid DCs. Neither $FXIII^+$ nor $CD11c^+$ showed co-expression with Langerhans antigen $CD1a$ (A, B), plasmacytoid antigen $CD123$ (C, D), or follicular DC antigen (E,F). Size bar = $100\mu m$.



CD11c⁺ cells co-express the blood DC marker BDCA-1/CD1c, and a small fraction also express DC-LAMP/CD208 and DEC-205/CD205.

BDCA-1 also marks a population of CD11c⁺ myeloid DCs in blood, and we noted co-expression of these two markers in the dermis as well (Figure 3A,B). Two other markers that are expressed by tissue DCs are a lysosomal marker, DC-lysosomal-associated membrane protein (DC-LAMP)/CD208, and an endocytic receptor DEC-205/CD205. A small fraction of the CD11c⁺ cells expressed these two markers (Figure 3 C-F). The fraction of CD11c⁺/DC-LAMP⁺ cells is qualitatively much smaller than the fraction of CD11c⁺/DEC-205⁺ cells. DC-LAMP/CD208 is expressed during DC maturation, and we noted that cells positive for this marker were often in dermal aggregates with other DC-LAMP/CD208⁺ CD11c⁺ cells. These results indicate that CD11c⁺ cells in the dermis share features with the myeloid DCs in blood, but some are in a more mature state of differentiation (Granelli-Piperno *et al.*, 2005).

Figure 3. $CD11c^{+}$ cells are defined by *BDCA-1*, *DC-LAMP/CD208*, and *DEC-205/CD205*. *FXIIIa* did not overlap with *BDCA-1* (A), *DC-LAMP/CD208* (C), or *DEC-205/CD205* (E). Most $CD11c^{+}$ cells co-expressed *BDCA-1* (B). Small subsets of $CD11c^{+}$ cells co-expressed *DC-LAMP/CD208* (D), and *DEC-205/CD205* (F). Size bar = 100 μ m.



FXIIIA⁺ cells express the macrophage marker and scavenger receptor, CD163.

CD163, a hemoglobin haptoglobin scavenger receptor identifies tissue resident macrophages, and it was the only marker we studied that was uniformly co-expressed by FXIIIA⁺ cells and not by CD11c⁺ cells (Figure 4A, B). Several other markers were expressed on a fraction of FXIIIA⁺ and CD11c⁺ cells. These included the uptake receptors macrophage mannose receptor (MMR)/CD206 (Figure 4C, D), dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN)/CD209 (Figure 4E, F), CD45, and HLA-DR (Figure 5). In normal skin, both MMR/CD206 and DC-SIGN/CD209 were more abundant on macrophages (FXIIIA⁺ cells) than DCs (CD11c⁺ cells), which is consistent with recent studies in inflamed skin and lymph node (Granelli-Piperno *et al.*, 2005; Krutzyk *et al.*, 2005). Reciprocally, CD45 and HLA-DR were more abundant on CD11c⁺ compared to FXIIIA⁺ cells. All these observations are consistent with the interpretation that CD11c⁺ cells in the dermis are part of the DC pathway of differentiation, while FXIIIA⁺ cells are more macrophage-like.

Figure 4. The macrophage marker CD163 defines $FXIII^+$ cells. $FXIII^+$ cells expressed macrophage marker CD163 (A), and a subset overlapped with MMR/CD206 (MMR) (C) and DC-SIGN/CD209 (E). CD11c did not overlap with CD163 (B), but a subset overlapped with MMR/CD206 (D), and DC-SIGN/CD209 (F). Size bar = 100 μ m.

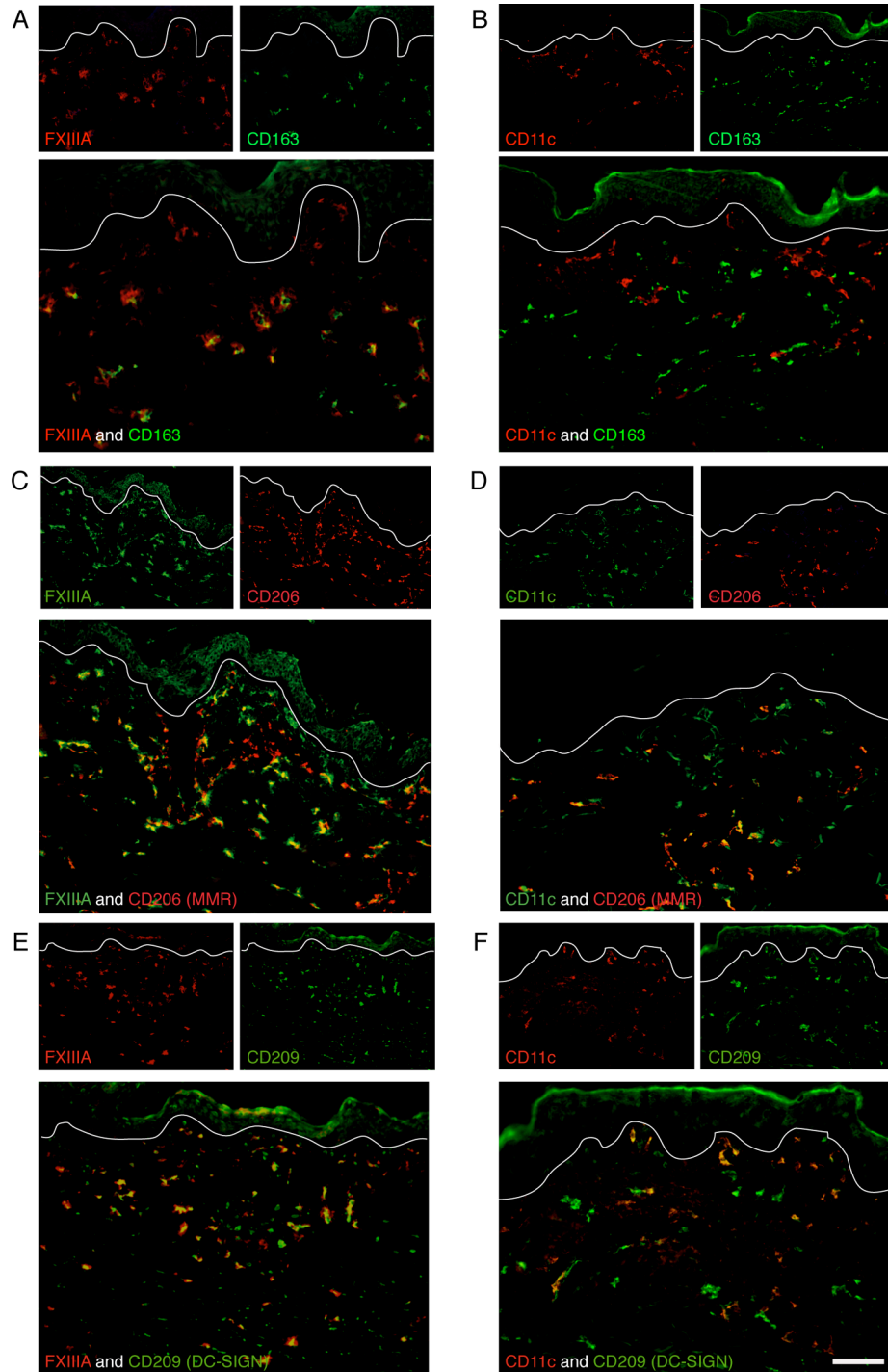
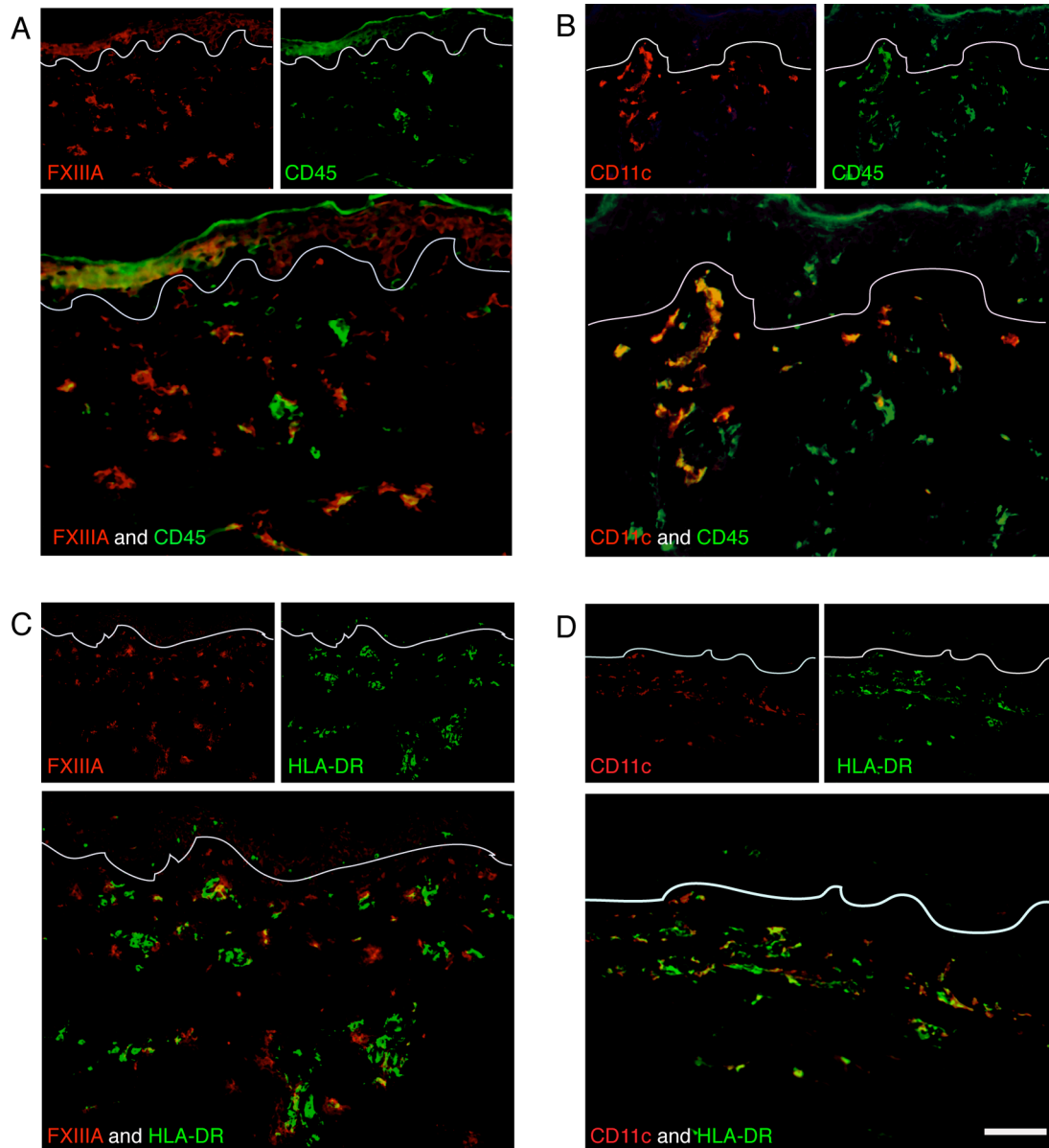


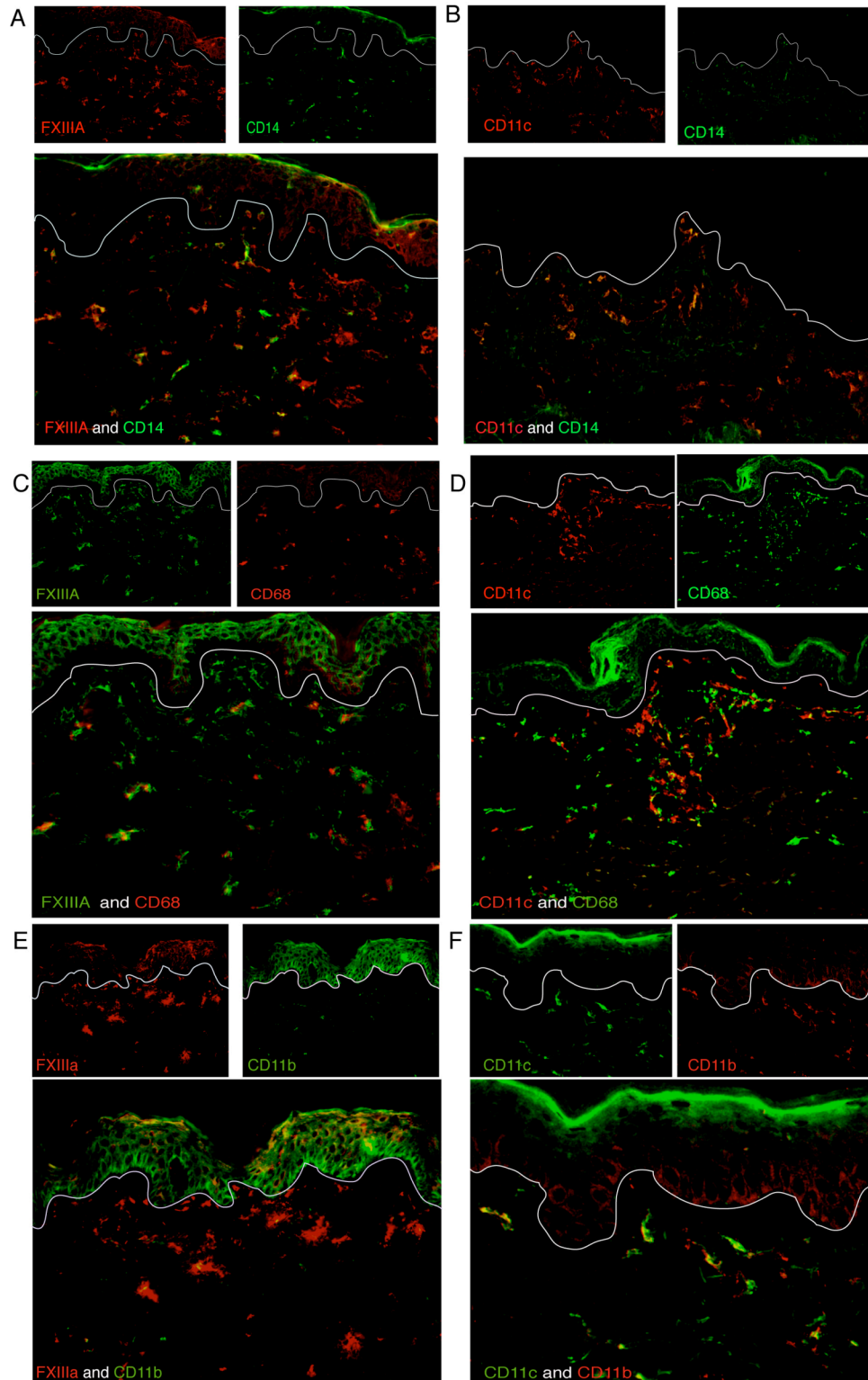
Figure 5. HLA-DR and CD45 mark both $CD11c^{+}$ and $FXIII A^{+}$ cells. $FXIII A^{+}$ cells had a lower expression level of CD45 (A) than $CD11c^{+}$ cells (B). HLA-DR was also expressed to a lower extent on $FXIII A^{+}$ cells (C) than on $CD11c^{+}$ cells (D). Size bar = 100 μ m.

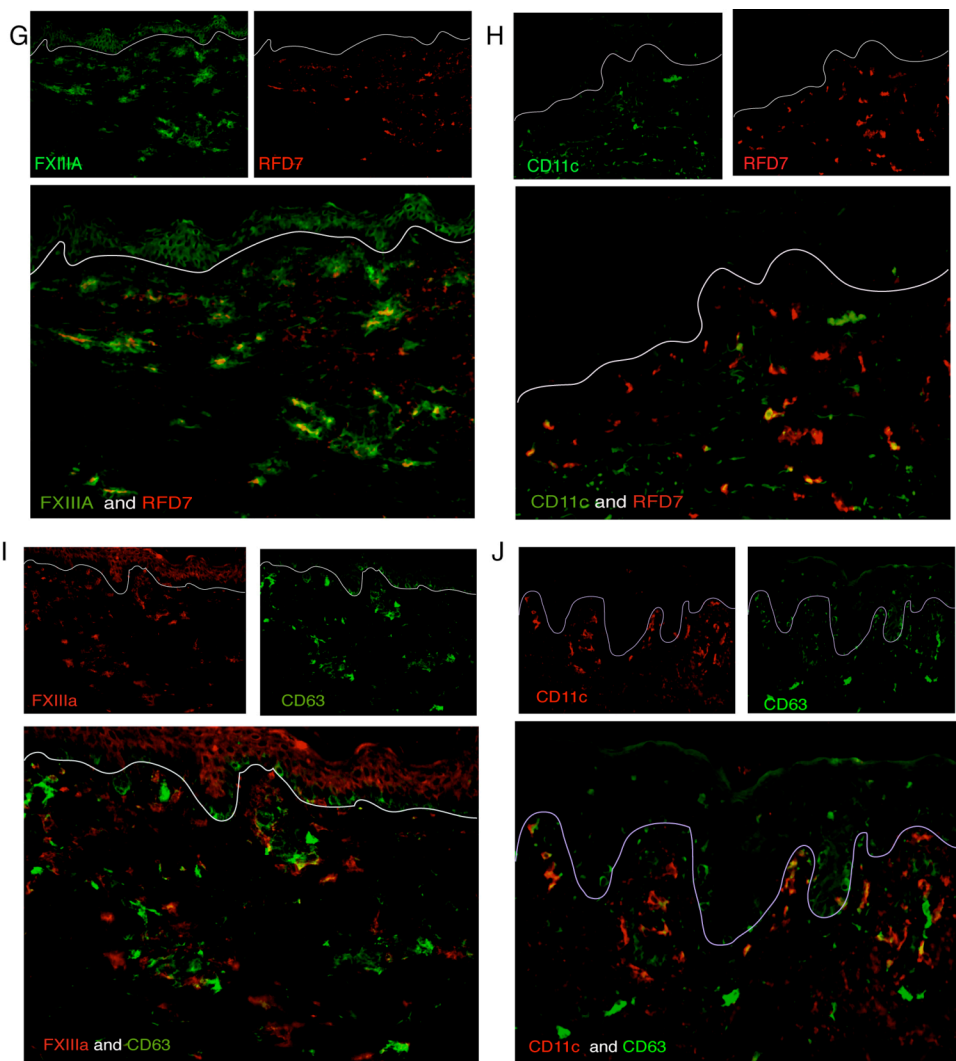


Other markers of potential interest.

Other markers used for characterization of FXIII^A and CD11c cells were CD14 (identifies monocytes), CD68, CD11b, and RFD7 (commonly used macrophage markers) and CD63 (present on macrophages and DCs). These markers were not helpful in discriminating these two populations in normal skin (Figure 6). CD14, a component of the LPS receptor on monocytes (Gordon *et al.*, 2005), was present on a few FXIII^A cells and CD11c⁺ cells. We have previously shown that CD68 identifies a subset of CD11c⁺ cells that produce IL-20 in psoriasis (Wang *et al.*, 2006), so this is not an exclusive macrophage marker. In keeping with this, CD68 also stained both CD11c⁺ and FXIII^A cells. CD11b and RFD7, considered to be common macrophage markers (Gordon *et al.*, 2005; Tormey *et al.*, 1997), were both present on both CD11c and FXIII^A cells. CD63, an MHC Class II internalization antigen, marked occasional CD11c⁺ and FXIII^A cells in normal skin.

Figure 6. Other potential macrophage markers stained both $FXIIIa^+$ and $CD11c^+$ cells. $FXIIIa^+$ and $CD11c^+$ cells showed variable co-expression with CD14 (A, B), CD68 (C, D), CD11b (E, F), RFD7 (G, H), and CD63 (I, J).

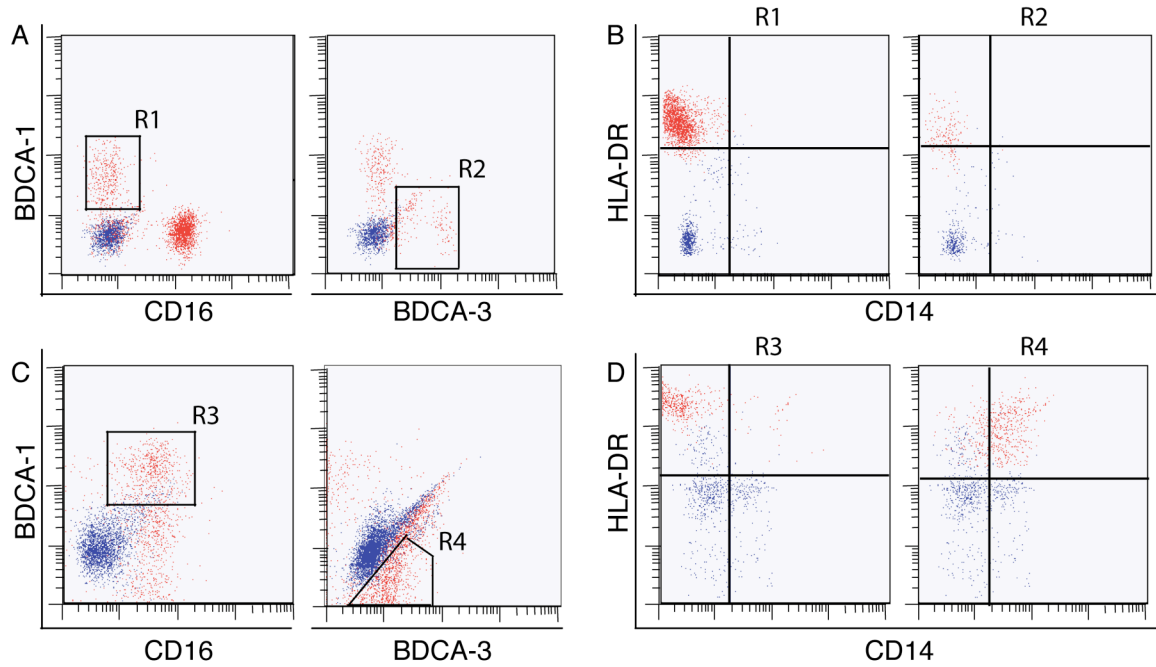




Isolated CD11c⁺ dermal cells contain non-overlapping major and minor populations of BDCA-1⁺ and BDCA-3⁺ cells.

To confirm our in situ data and assess whether or not the three myeloid DC subsets found in blood were also found in tissue, we performed 6-color FACS on single cell suspensions of normal human skin compared to peripheral blood, using a custom made group of mAbs to lineage (Lin) that did not contain mAbs to CD14 or CD16. Peripheral blood FACS dot-plots are shown in Figure 7A and B, and the parallel analysis from dermal cells is shown in Figure 7C and D. There were three discrete Lin⁻ CD11c⁺ HLA-DR⁺ DC populations in peripheral blood: BDCA-1⁺, CD16^{hi}, and BDCA-3⁺ (Fig. 7A), but only two clear populations in skin, BDCA-1⁺ CD16^{lo} and BDCA-3⁺ CD16^{lo} (Figure 7C). In blood, two discrete CD11c⁺ DC populations, BDCA-1⁺ and BDCA-3⁺ cells, were both DR⁺CD14⁻ (Figure 7B). In contrast, BDCA-1⁺ cells from skin increased HLA-DR expression, and BDCA-3⁺ cells acquired low-level CD14 expression (Figure 7D). We also confirmed that BDCA-1 and BDCA-3 identified discrete populations in normal skin in situ (data not shown).

Figure 7. Cutaneous DCs compared to blood DCs. (A) There were three non-overlapping DC populations in peripheral blood, gating on Lin⁻DR⁺CD11c⁺ cells: BDCA-1⁺, CD16^{hi}, and BDCA-3⁺. (B) Blood BDCA-1⁺ (left) and BDCA-3⁺ (right) cells were both HLA-DR⁺ CD14⁻. (C) In dermal single cell suspensions, BDCA-1⁺ cells acquired CD16. (D) BDCA-1⁺ (left) dermal cells increased HLA-DR expression, and BDCA-3⁺ (right) dermal cells acquired low-level CD14 expression.



For FACS studies of isolated dermal cells, BDCA-1 and CD163 are superior markers to CD11c and FXIIIA respectively.

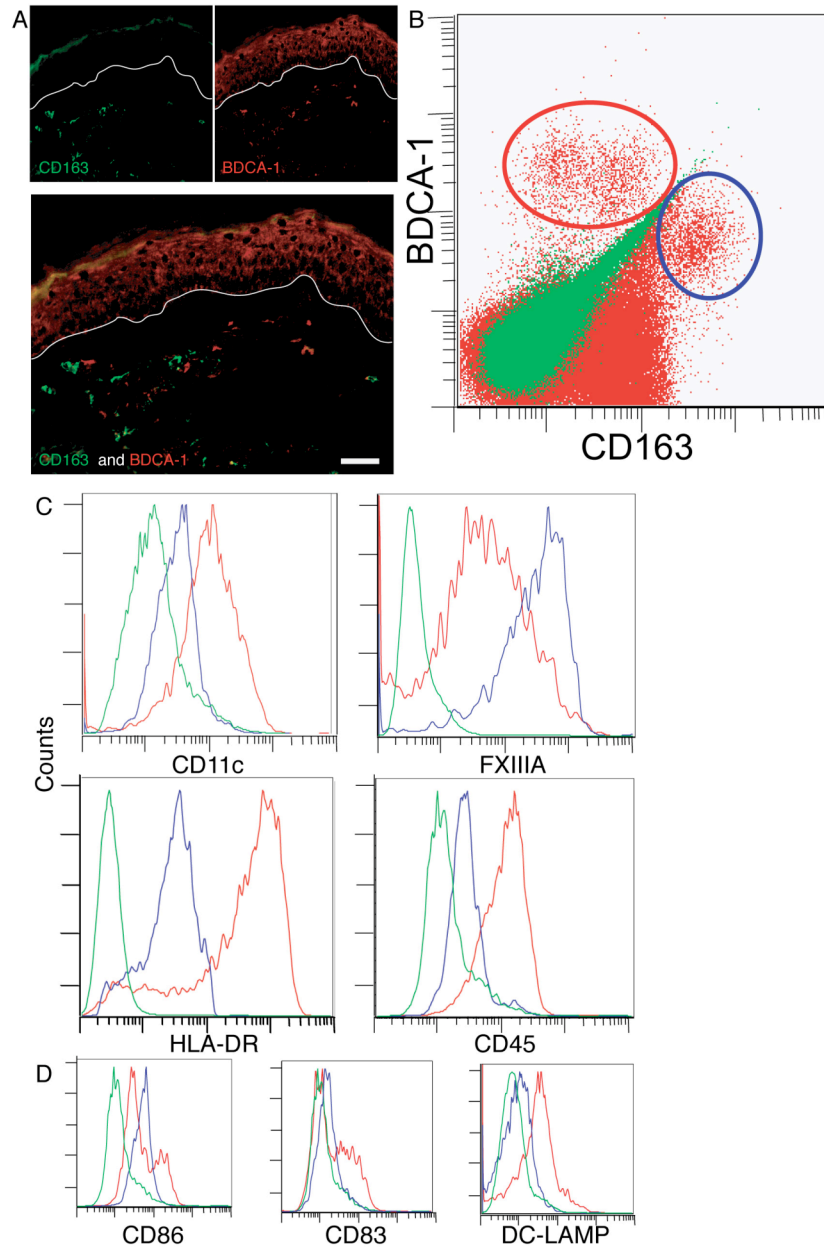
To begin to test the functional properties of cell populations isolated from dermis, we required markers that would be optimal for FACS-sorting. While culture conditions may alter cellular surface phenotype, it was important to compare DC populations using the new BDCA-1 and CD163 markers with previous studies on dermal DCs. It was necessary to balance obtaining sufficient cells to study with as little manipulation as possible. An overnight culture in dispase/collagenase media (Angel *et al.*, 2006) gave insufficient cells to perform our MLR experiments. Enzymatic alteration of surface epitopes is also possible, although experiments on PBMCs did not show loss of FACS staining after culture in dispase collagenase mix (unpublished data). This method was modified to allow 1-2 days of culture so any lost surface markers could be re-expressed, and allow DCs and macrophages to move out of the scored upper and lower dermal surfaces. Comparison of BDCA-1 versus CD163 on the first day of culture (after overnight culture in dispase/collagenase) and 24 and 48 hours culture showed a similar clear distinction of the two populations (data not shown). Culture of bulk dermal cells from normal skin (using this method) with and without DC maturation cytokines (IL-1 β , IL-6, TNF and PGE₂) showed that there was surprisingly little maturation of myeloid DCs (BDCA-1⁺) as measured by HLA-DR and CD83 (data not shown).

We found that BDCA-1, which double labels > 90% of CD11c⁺ cells by in situ immunofluorescence (Figure 3B), had a much higher median fluorescence intensity (MFI) than CD11c (data not shown). Likewise, CD163, which showed 100% co-expression with FXIIIA in situ (Figure 4A), proved to be a much better FACS marker

because FXIIIA antibodies bound non-specifically to dermal cells in suspension perhaps due to adherence of platelet fragments which are rich in FXIIIA (data not shown), and FXIIIA is upregulated during cell culture (D. Torocsik, 2005; Per Henriksson, 1985). Double label immunofluorescence of BDCA-1 and CD163 confirmed that they were distinct populations in situ (Figure 8A), and supported their use as alternative markers for CD11c and FXIIIA, respectively, in FACS studies.

Bulk dermal single cell suspensions showed distinct BDCA-1⁺ (circled in red) and CD163^{hi} (circled in blue) populations by FACS (Figure 8B). BDCA-1⁺ gated cells (red line) had a higher MFI for CD11c than the CD163^{hi} population (blue line) compared to isotype (green line) (Figure 8C upper left), most likely due to increased sensitivity of FACS compared to immunohistochemistry. CD163^{hi} cells had a higher MFI for FXIIIA than BDCA-1⁺ cells (Figure 8C upper right). BDCA-1⁺ gated cells also had a higher MFI for HLA-DR and CD45 than CD163^{hi} gated cells (Figure 8C lower left and right). A subset of BDCA-1⁺ cells was positive for DC maturation markers CD86, CD83, and DC-LAMP/CD208 (Figure 8D). As B cells may also express BDCA-1, we confirmed that none of the BDCA-1⁺ cells were CD19⁺ by FACS (data not shown). Thus the dermal cells have a pattern of expression similar to in situ characterization.

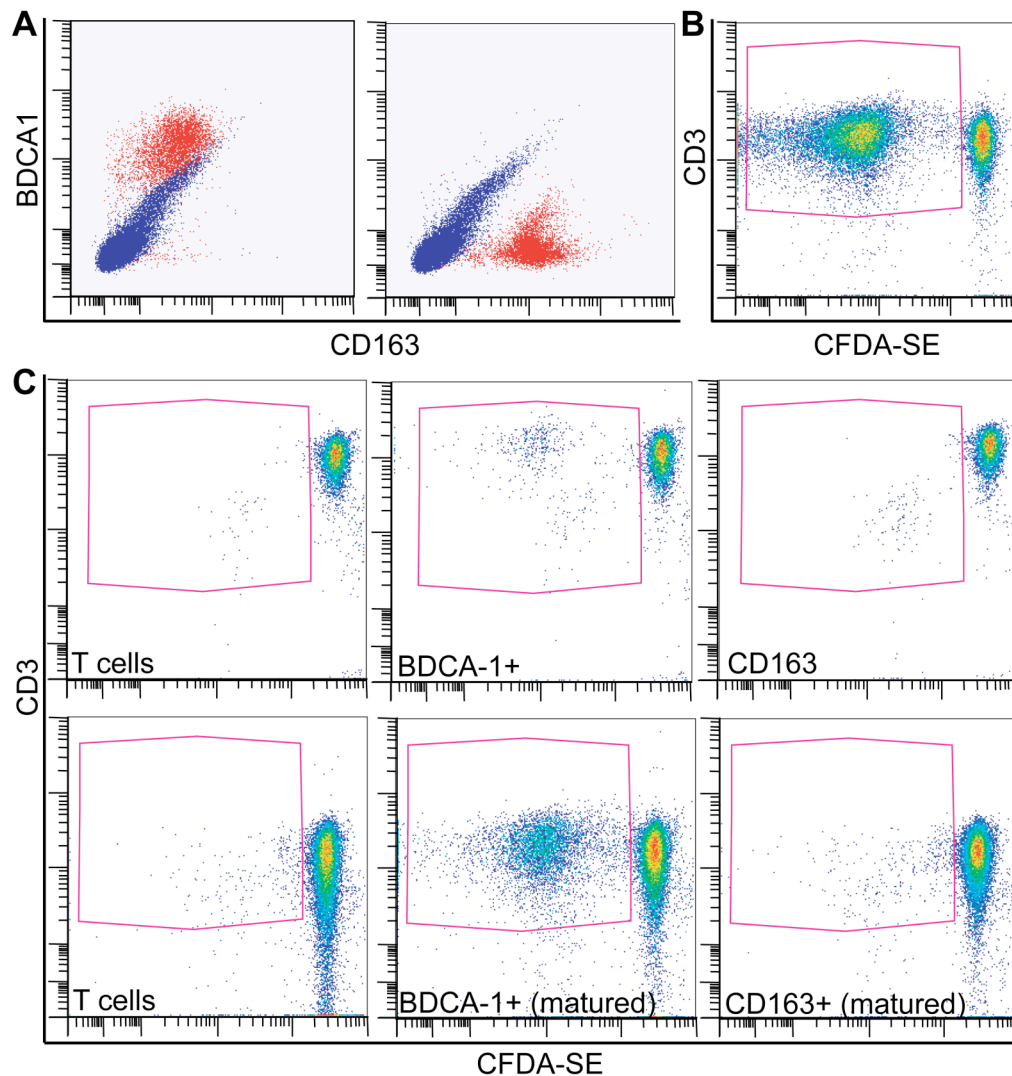
Figure 8. BDCA-1 and CD163 are alternative markers for CD11c and FXIIIa respectively. (A) BDCA-1 and CD163 identified discrete populations of dermal cells. (B) BDCA-1⁺ cells (red circle) and CD163⁺ cells (blue circle) were also discrete populations in dermal single cell suspensions. (C) FACS histograms gated on BDCA-1⁺ cells (red line), CD163⁺ cells (blue line), or isotype (green line). BDCA-1⁺ were CD11c^{hi}, FXIIIa^{lo}, HLA-DR^{hi}, and CD45^{hi}. CD163⁺ cells were CD11c^{mid}, FXIIIa^{hi}, HLA-DR^{mid}, and CD45^{lo}. (D) A subset of BDCA-1⁺ cells was CD86^{hi}, CD83⁺, and DC-LAMP^{hi}. Representative graphs from 3 experiments. Size bar = 100μm.



BDCA-1⁺ cells are a major immunostimulatory population from normal human skin.

To test for the immunostimulatory properties of dermal leukocytes, we focused on FACS-sorted populations of BDCA-1⁺ and CD163^{hi} cells released from the dermis with collagenase. Figure 9 shows a representative FACS plot of the MLR. The post-sorted cells (red) were 99% pure compared to isotype (blue) (Figure 9A). In the MLR induced by in vitro monocyte-derived mature DCs, 63% of the surviving T cells had undergone extensive proliferation at a ratio of stimulators to responders of 1:100 on day 8 post-sort (Figure 9B). In parallel cultures stimulated by BDCA-1⁺ cells, 9.1% of the T cells had proliferated (1:10), compared to CD163⁺ cells (2.1%), and background T cell proliferation (1.0%) (Figure 9C). When these sorted BDCA-1⁺ and CD163⁺ populations were cultured for 2 days in a DC-maturing cytokine cocktail before setting up the MLR, the immunostimulatory capability of BDCA-1⁺ cells was increased to 25.2% (1:100) but the capacity of CD163⁺ cells was unchanged (2.2%; 1:250, low ratio due to low cell survival during 2 day culture period) (Figure 9C). BDCA-1⁺ sorted cells cultured for 2 days without cytokines, and the supernatant from these cells after culture, also increased T cell proliferation (data not shown).

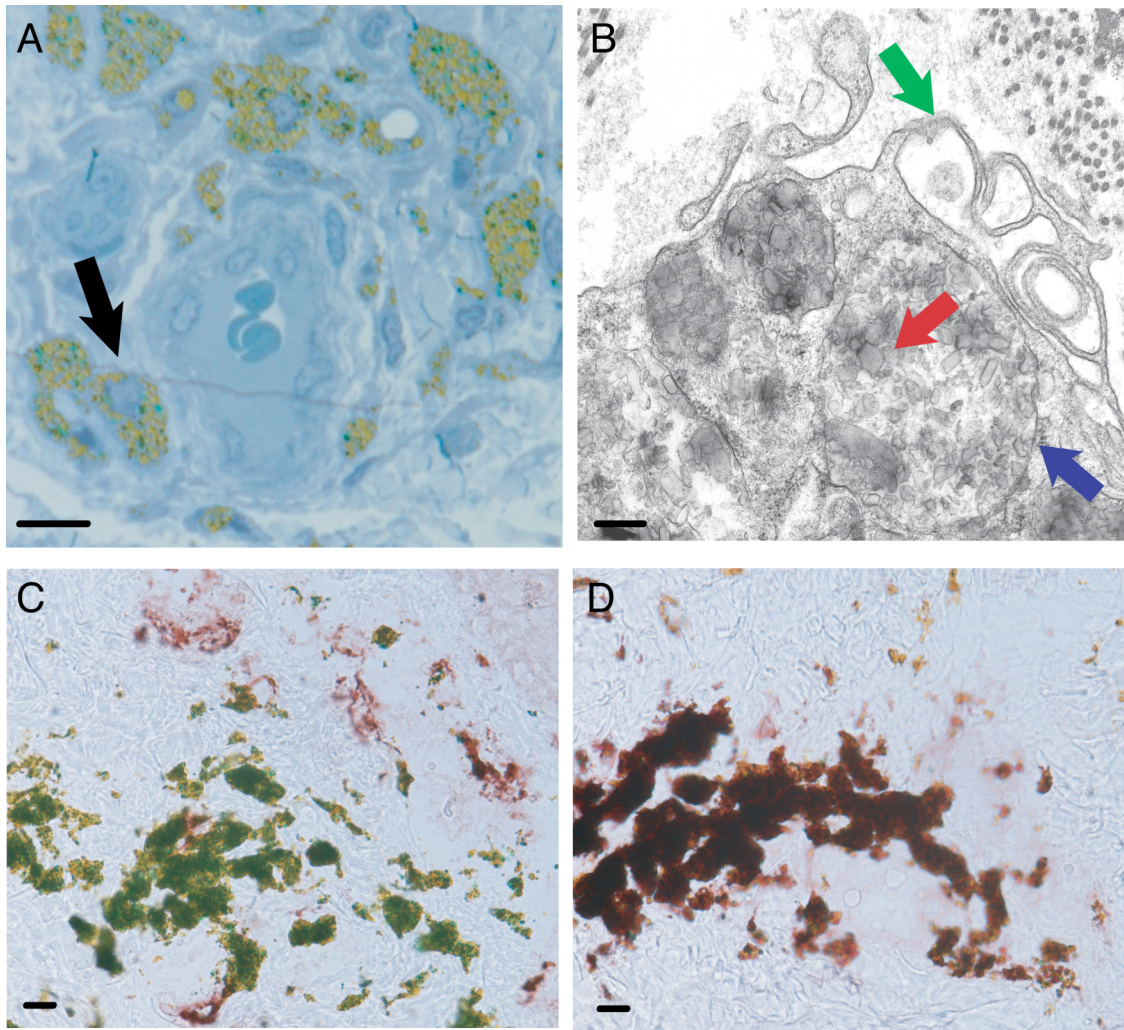
Figure 9. BDCA-1⁺ cells are more immunostimulatory. (A) Post-sort dot-plot of dermal cells from normal skin into BDCA-1⁺ population (red, upper left panel), and CD163⁺ population (red, upper right panel) compared to isotype (blue). (B) Positive control (monocyte-derived mature DC) for MLR on day 8 post sort at 1:100 stimulator to responder ratio. Gate contains CD3⁺ proliferating T cells with left-shifted CFSE. (C) Using BDCA-1⁺ sorted cells as stimulators (1:10), 9.1% of the T cells proliferated; using CD163⁺ cells (1:10), 2.1% of live T cells proliferated. Background proliferation of T cells alone, without stimulation was 1.0%. After cells had been sorted and cultured for 2 days with cytokines to induce maturation, there was a marked increase in the T cell stimulatory capacity of BDCA-1⁺ cells (25.2%, 1:100) versus CD163 (2.2%, 1:250). Representative graphs from 3 experiments.



CD163⁺ cells phagocytose large particles in a tattoo and have the structural features of macrophages.

Ultra-thin sections of a green-dye-tattoo were cut from tattoo-bearing normal skin. These sections confirmed that the dye was intracytoplasmic, and mostly located in cells clustered around blood vessels (Figure 10A). Electron microscopy of the tattoo revealed membrane-bound (Figure 10B, blue arrow) tattoo dye particles (red arrow), and microvillus projections (green arrow) confirming the identity of these cells as macrophages. Immunohistochemistry using BDCA-1 (Figure 10C) or CD163 (Figure 10D) showed that dye laden cells were CD163⁺ and not BDCA-1⁺, confirming that typical macrophages rather than DCs had ingested the pigment.

Figure 10. CD163⁺ cells phagocytose large particles and have the structural features of macrophages. (A) Tattoo skin section (0.5μm) stained with toluidine blue. Cells containing green tattoo dye in their cytoplasm (black arrow) surrounded a blood vessel. (B) Electron microscopy of a tattoo showed that dye particles (red arrow) were membrane bound (blue arrow) within the cytoplasm of a cell with multiple microvillus protrusions (green arrow). (C, D) Cells containing green tattoo dye particles stained for CD163 but not BDCA-1. Size bar = 10μm (A, C, D) and 200nm (B).



Discussion

DCs are important sentinels of the cutaneous immune system, performing central roles in both the innate and acquired immune system. Previous characterization of DC subsets in human dermis has been influenced by results with a rabbit polyclonal antibody to FXIIIA, which identifies dermal cells with a dendritic morphology (Cerio *et al.*, 1989; Deguchi *et al.*, 2002; Frank O. Nestle, 1993; Meunier *et al.*, 1993; Nestle *et al.*, 1994). Here we have characterized populations of cells within the normal dermis and find surprisingly that cells expressing the CD11c integrin and the BDCA-1/CD1c antigen presenting molecule are a distinct population that are functionally differentiated along the DC pathway, whereas FXIIIA⁺ cells are differentiated along the macrophage pathway. Whereas mouse skin contains approximately one CD11c⁺ DC for every five macrophages (Dupasquier *et al.*, 2004), the concentration of CD11c⁺ DCs in human skin is much higher, and is closer to a 1:1 ratio.

CD11c⁺ co-localized with several well-recognized DC markers: BDCA-1, DC-LAMP/CD208, and DEC-205/CD205. BDCA-1, also known as CD1c (Brenner, 2004), is an invariant MHC Class I-like antigen receptor molecule that recognizes lipids in mycobacterial cell walls. BDCA-1 is found on immature and mature DCs, and also on a subset of B Cells. BDCA-1, in the absence of the B cell markers CD19 and CD20, would therefore seem like a valuable marker to compare dermal DCs in both normal skin (Narbutt *et al.*, 2006) and inflammatory skin diseases (David P. Fivenson, 1995). DC-LAMP/CD208 is a lysosomal protein that specifically marks mature DCs (de Saint-Vis *et al.*, 1998). DEC-205/CD205 is a surface receptor that participates in DC antigen

endocytosis; its expression increases during maturation, and it has been previously demonstrated in normal human skin (Ebner *et al.*, 2004). A low frequency of CD11c⁺ DCs expressing these two antigens in normal skin is consistent with their expected immature DC status. CD11c⁺ cells also stain brightly with HLA-DR and CD45, confirming their antigen presenting potential and bone marrow origin, respectively. The phenotype of BDCA-1⁺ cells from skin indicates that they are all CD45^{hi}/HLA-DR^{hi} and a subset are CD86⁺ and CD83⁺.

Our studies show that FXIIIA identifies a population of tissue-resident macrophages in normal skin. The only antibody that overlapped completely with FXIIIA in situ was the scavenger receptor CD163, which is selectively expressed on monocytes and macrophages, reviewed in (Fabriek *et al.*, 2005). The best characterized function of CD163 is to bind hemoglobin/ haptoglobin complexes, which may be important in homeostasis. There was low-level CD45 and HLA-DR expression, consistent with other tissue macrophages, and limited antigen-presenting capacity (Janeway *et al.*, 2001). MMR/CD206 and DC-SIGN/CD209 are C-type lectin receptors that are found on both macrophages and DCs (Granelli-Piperno *et al.*, 2005; McGreal *et al.*, 2005), so it was not surprising that there was expression of both lectin receptors on both FXIIIA⁺ and CD11c⁺ cells in normal human skin.

To evaluate the function of these two dominant dermal populations, we used two approaches. By comparing BDCA-1 and CD163 cells selected from collagenase digests of normal skin as inducers of an allogeneic MLR, we found that BDCA-1⁺ cells were the

main immunostimulatory population. These BDCA-1⁺ cells could induce increased T cell proliferation when cultured before setting up the MLR, but were not as immunostimulatory as in vitro matured DCs, suggesting that there were few mature DCs in normal skin compared to those found in inflammatory conditions such as psoriasis. This result reflects studies by Nestle *et al.* showing that bulk tissue émigrés from normal skin are not as stimulatory as those from psoriasis patients (Nestle *et al.*, 1994). In comparison, CD163^{hi} cells were not immunostimulatory in an allo-MLR, nor could they be induced to be stimulatory, although they may possess some antigen presenting capacity with up-regulation of class II MHC molecules (Steinman, 2006).

Skin tattoos provided a second functional study- the phagocytic activity of tissue macrophages. Pigment granules were found in lysosomal-like cellular structures, and cells containing pigment stained uniformly with CD163. The fate of tattoo pigment injected into dermal tissues has been studied in the past, and fibroblasts were considered to be the primary long-term reservoir of the pigment granules, with pigment in occasional macrophages (Fujita *et al.*, 1988; Lea and Pawlowski, 1987). However, our data suggest that macrophages are indeed a significant store of the dermal pigment. The cells with pigment were CD163⁺ and BDCA-1⁻, round, had numerous microvillous projections, and the pigment is contained within membrane bound structures. These characteristics are consistent with macrophages (Demidem *et al.*, 1991; Fujita *et al.*, 1988).

In summary, we have identified CD11c⁺ cells as immature myeloid DCs in normal human skin, and FXIIIA⁺ cells as tissue-resident macrophages, not DCs as they

were previously classified. In future studies of cutaneous DCs, we would recommend considering the use of CD11c/BDCA-1 and CD163 as alternative markers to identify dermal DCs and macrophages, respectively, as they are more specific and more useful in flow cytometry applications.

CHAPTER 4: Characterization of DC subsets in inflamed human dermal skin

Psoriasis is a common chronic inflammatory skin disease, which results in great morbidity for severely affected patients. In recent years much progress has been made understanding the pathogenesis and treatment of this disease. We now appreciate that psoriasis results from complex interactions between T cells, dendritic cells (DCs), and keratinocytes (Lowes *et al.*, 2007). Until recently, psoriasis has been considered a classical Type 1 autoimmune disease, with a strong IFN γ Th1 signal. However, a new subset of T cells, Th17 cells, have now been described in murine models of autoimmune inflammation (Weaver *et al.*, 2007), and we have reported the presence of these cells in psoriasis (Lowes *et al.*, 2008). Th17 cells produce IL-17, IL-22, and have other important downstream pro-inflammatory effects in skin (Liang *et al.*, 2006; Zheng *et al.*, 2007). DCs may be very central pathogenic players in psoriasis, both by activating T cells and by producing amplifying cytokines and chemokines during inflammation. In the skin the main DC populations include epidermal DCs (Langerhans cells) and dermal DCs (myeloid DCs and plasmacytoid DCs). We were interested in further characterizing dermal myeloid DCs in psoriasis, as these cells may be an important therapeutic target.

Recently, we described that the best marker for identifying dermal myeloid DCs in normal skin is CD11c (Zaba *et al.*, 2007b). Also, we have previously reported that there is a large increase in CD11c⁺ cells in psoriasis (Abrams *et al.*, 1999; Gottlieb *et al.*, 2005; Lowes *et al.*, 2005a). These CD11c⁺ cells include a subset of inflammatory DCs called TNF- and inducible nitric oxide synthase (iNOS) -producing DCs, or Tip-DCs (Lowes *et al.*, 2005a). Tip-DCs were first described in the spleen during a murine model

of *Listeria monocytogenes* infection (Serbina *et al.*, 2003; Tam and Wick, 2004).

Pathogenicity of these Tip-DCs in psoriasis is suggested by the rapid downmodulation of Tip-DC products TNF, iNOS, IL-20, and IL-23 during effective treatment with TNF blocking drugs (Zaba *et al.*, 2007a).

In normal skin, CD11c⁺ cells are nearly all blood dendritic cell antigen (BDCA)-1⁺. BDCA-1 is also known as CD1c, and this molecule is part of the CD1 family of invariant MHC molecules that are important in presentation of lipid antigens to T cells (Barral and Brenner, 2007). In this manuscript, we show that unlike normal skin, most of these CD11c⁺ cells in psoriatic plaques are actually BDCA-1⁻. They are relatively immature DCs, as they do not express DC-LAMP (dendritic cell -lysosomal-membrane associated protein) and DEC-205/CD205. Hence, *in situ* there were two main types of dermal DCs in psoriasis lesions: CD11c⁺BDCA-1⁺ resident DCs, and CD11c⁺BDCA-1⁻ inflammatory DCs. Dermal single cell suspensions for phenotype analysis and functional studies showed that both populations were allo-stimulatory and were able to polarize allogeneic T cells into IL-17 producing Th17 cells.

Characterization of dendritic cells in psoriatic lesional skin and peripheral blood

Psoriatic myeloid dermal DCs are CD11c⁺BDCA-1⁻BDCA-3⁻.

To quantify cells in each dermal DC compartment, we performed immunohistochemistry on normal skin and psoriasis paired lesional/ non-lesional samples (n=20) (Figure 11). Both non-lesional and lesional psoriasis samples had 5-fold fewer

BDCA-1⁺ DCs (Figure 11A) ($p < 0.001$). However, BDCA-1 cell counts did not change significantly in a group of psoriatic patients treated with etanercept (Figure 11C) (Zaba *et al.*, 2007a). There were 2-fold more BDCA-3⁺ DCs compared to normal skin ($p < 0.001$ and $p < 0.05$, respectively) (Figure 11B). CD11c⁺BDCA-1⁻BDCA-3⁻ cell numbers were calculated by subtracting BDCA-1 and BDCA-3 cell counts from CD11c cell counts. While lesional and non-lesional psoriasis sections contained similar numbers of BDCA-1⁺ and BDCA-3⁺ cells, CD11c⁺BDCA-1⁻BDCA-3⁻ cells were increased 10-fold in psoriasis plaques compared to non-lesional skin ($p < 0.001$), and 30-fold compared to normal skin (Figure 11C) ($p < 0.001$). In addition, we performed FACS on whole blood from normal ($n=6$) and psoriasis ($n=6$) subjects, and found that all three previously defined myeloid DC subsets (MacDonald *et al.*, 2002) were decreased in peripheral blood of psoriasis patients compared to normal volunteers (Figure 11D, E).

We next evaluated these populations *in situ* by 2 color immunofluorescence. In previous studies on normal human skin, we have characterized two populations of myeloid CD11c⁺ dermal DCs: BDCA-1⁺ dermal DCs comprise approximately 90% of all CD11c⁺ dermal cells, and the remaining 10% of CD11c⁺ cells are BDCA-1⁻ (Zaba *et al.*, 2007b). We found that in psoriasis, there was a reversal of this ratio of BDCA-1⁺ cells, as the minority of the CD11c⁺ cells co-expressed BDCA-1 (Figure 12A). BDCA-1⁺ cells were aggregated together in dermal clumps (Figure 12A, 2B), compared to CD11c⁺ cells, which were located mostly in the upper reticular dermis and dermal papillae. BDCA-3 identifies an additional population of myeloid DCs in the circulation (MacDonald *et al.*, 2002) and in psoriatic dermis (Figure 12B). This marker was expressed on CD11c⁺ cells scattered throughout the dermis and also on blood vessels (Figure 11C). As

CD11c⁺BDCA-1⁺ cells are the major resident dermal DC population in normal skin, the remainder of our study compares resident CD11c⁺BDCA-1⁺ DCs with CD11c⁺BDCA-1⁻ DCs in the psoriatic inflammatory infiltrate.

Figure 11 . CD11c⁺ dermal DCs are the major DC population accumulating in psoriasis lesional skin and psoriasis peripheral blood contains fewer DCs than normal controls.

(A) Representative immunohistochemistry of BDCA-1⁺ cells, BDCA-3⁺ cells, and CD11c⁺ cells in normal, non-lesional and lesional psoriatic skin. (B) Quantification of myeloid DCs per mm skin stained by immunohistochemistry of normal skin (red boxes; n=20), non-lesional skin (light blue boxes; n=20), and matched psoriatic lesional skin (dark blue boxes; n=20). CD11c⁺BDCA-1⁻BDCA-3⁻ cell numbers were calculated by subtracting BDCA-1 and BDCA-3 cell counts from CD11c cell counts. FACS analysis of 150µL peripheral blood from normal volunteers (n=6) and psoriasis patients (n=6). Cells were gated on CD3⁻CD14⁻CD56⁻CD14⁻DR⁺11c⁺. (A) BDCA-1 cell counts during a clinical trial with etanercept, showing stable cell counts throughout the treatment period. (B) Representative FACS plots comparing circulating BDCA-1⁺ and BDCA-3⁺ cells in normal and psoriatic circulation. (C) Quantification of all patients showing a reduction in the circulation of BDCA-1⁺ and BDCA-3⁺ populations in psoriasis (blue) compared to normal patients (red). Error bars indicate SEM. p<0.05 (*), p<0.01 (**), p<0.001 (***). Size bar = 100µm.

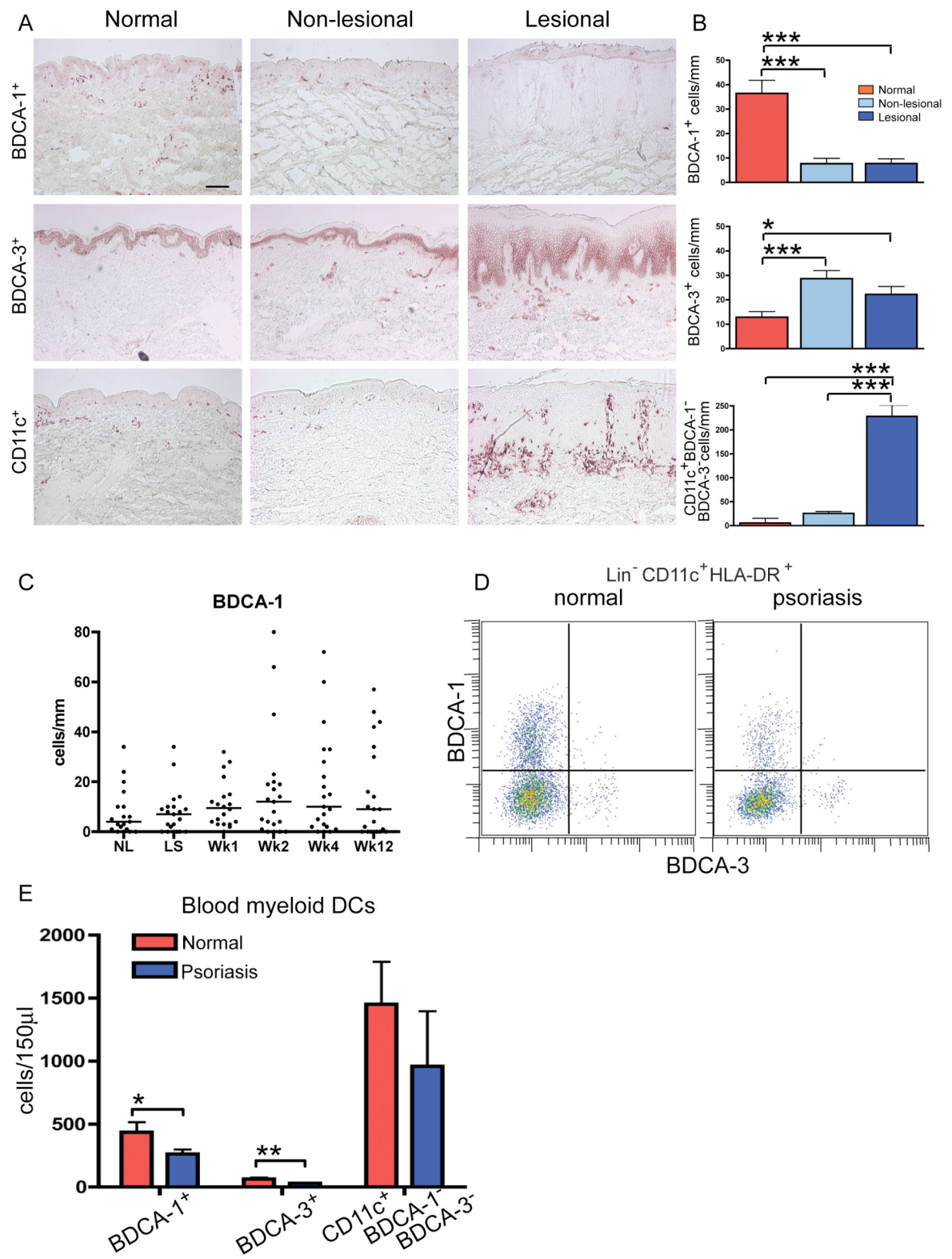
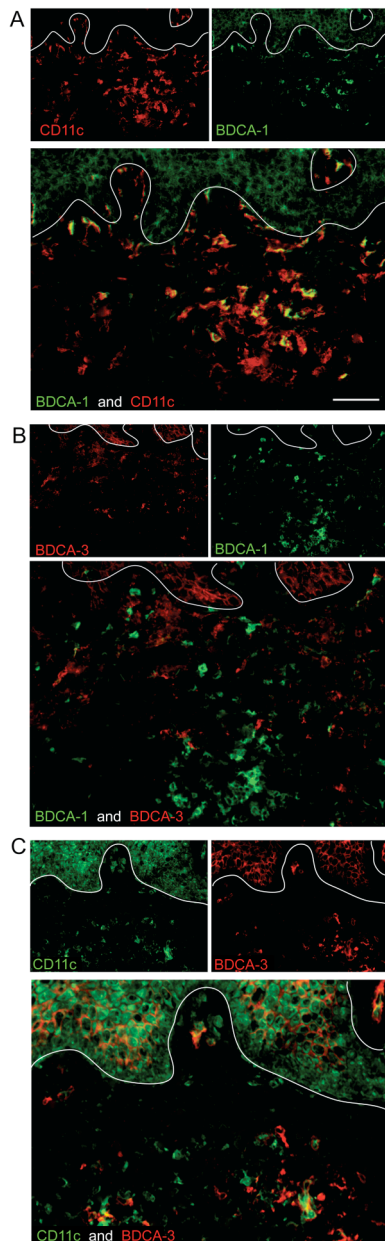


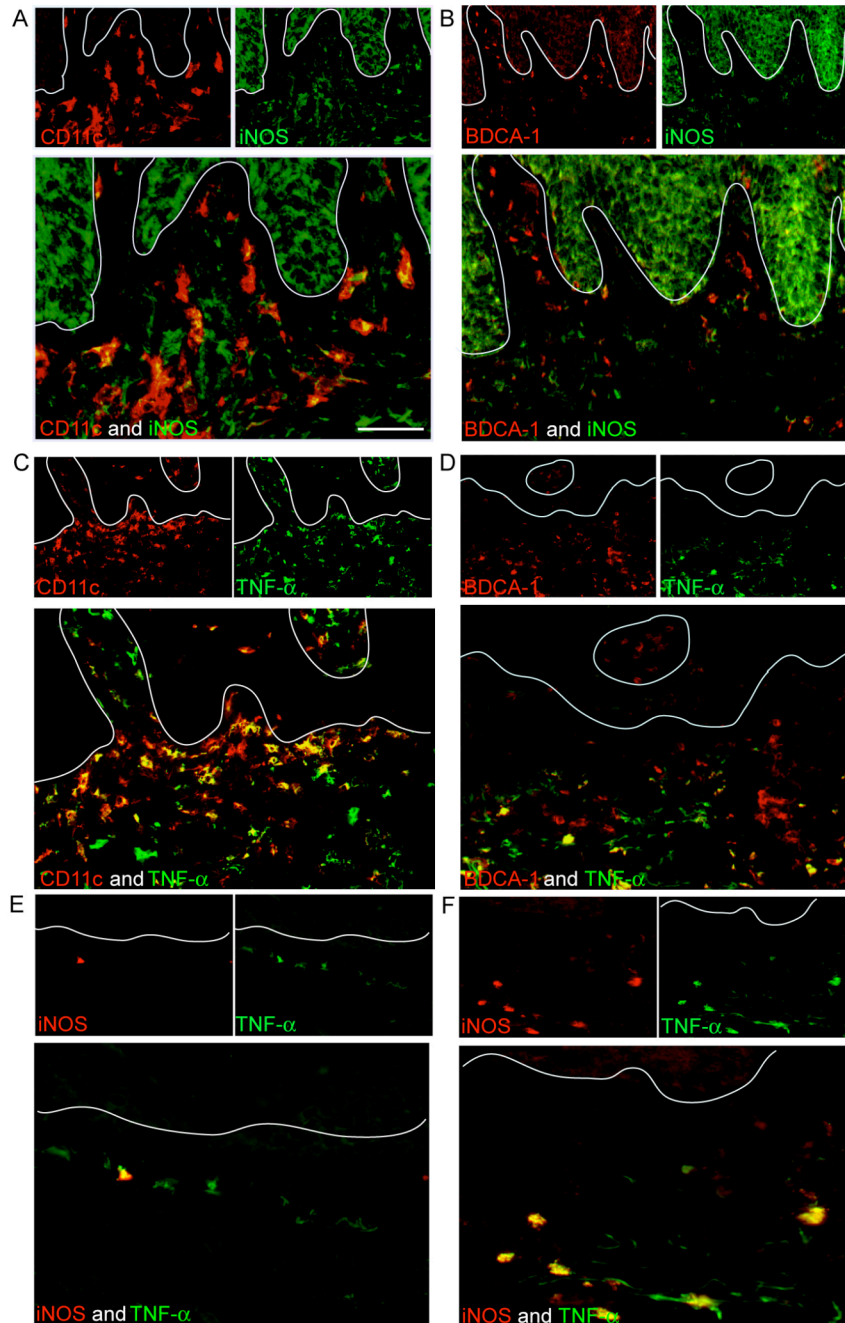
Figure 12 . Most $CD11c^{+}$ myeloid DCs are $BDCA-1^{-}$ in psoriasis lesional skin. (A) The majority of $CD11c^{+}$ cells in psoriatic dermis were $BDCA-1^{-}$, while a small subset of $CD11c^{+}$ cells co-expressed $BDCA-1^{+}$. (B) $BDCA-1$ and $BDCA-3$ identified separate myeloid DC populations in the dermis. (C) Most $BDCA-3^{+}$ cells co-expressed $CD11c$, and some $BDCA-3$ staining was observed on blood vessels. In all IF figures, single stained controls are above the merged image, white line denotes dermo-epidermal junction, dermal collagen fibers gave green autofluorescence, and antibodies conjugated with a fluorochrome often gave background epidermal fluorescence. Size bar = 100 μ m.



CD11c⁺ BDCA-1⁻ myeloid dermal DCs include TNF-and-iNOS producing dendritic cell (Tip-DCs) population.

In psoriasis lesional tissue, the majority of CD11c⁺ cells were iNOS producing (Figure 13A), and the cellular iNOS expression appeared punctate and intracytoplasmic. Resident CD11c⁺ BDCA-1⁺ dermal DCs had <10% iNOS co-expression (Figure 13B). Similarly, TNF was expressed by >90% of CD11c⁺ cells (Figure 13C) and <20% of CD11c⁺ BDCA-1⁺ dermal DCs (Figure 13D). In addition, iNOS and TNF identified occasional non-lesional Tip-DCs (Figure 13E) and many lesional Tip-DCs (Figure 13F), and all cells strongly expressing iNOS also expressed TNF. In conclusion, Tip-DCs were found in the CD11c⁺ BDCA-1⁻ myeloid DC population.

Figure 13 . CD11c⁺BDCA-1⁺ DCs contain the Tip-DC population. (A) Most CD11c⁺ dermal DCs in psoriatic lesional skin co-expressed iNOS compared to (B) BDCA-1⁺ cells. (C) Most CD11c⁺ dermal DCs in lesional psoriatic skin co-expressed TNF compared to (D) BDCA-1⁺ cells. Approximately 25% of BDCA-1⁺ cells co-expressed TNF. (E,F) iNOS and TNF were co-expressed in the same cell, identifying Tip-DCs in situ. Few Tip-DCs were observed in (E) psoriatic non-lesional skin compared to lesional skin (F). Size bar = 100μm.



CD11c⁺ BDCA-1⁻ inflammatory DCs show some expression of macrophage markers compared to BDCA-1⁺ cells.

In order to further characterize the CD11c⁺ myeloid DCs, we evaluated co-expression with a series of myeloid cell markers (Figures 14, 15). Over half of CD11c⁺ DCs expressed low level CD14 (Figure 14A). Most CD11c⁺ cells in the papillary dermis were CD14⁻, while most in the reticular dermis were CD14^{lo}. In contrast, only occasional BDCA-1⁺ cells expressed CD14 (Figure 14B). DC-SIGN/CD209, a marker of both immature DCs and macrophages in normal human skin (Zaba *et al.*, 2007b), partially co-localized with CD11c (Figure 14C) but did not co-localize with BDCA-1 (Figure 14D). Similarly, CD163, a marker of normal dermal macrophages (Zaba *et al.*, 2007b), blood monocytes, and blood DCs (Maniecki *et al.*, 2006), was expressed on some CD11c⁺ cells (Figure 14E), but not on CD11c⁺BDCA-1⁺ DCs. In normal skin, CD11c is not expressed on CD163⁺ macrophages (Zaba *et al.*, 2007b), despite the myeloid origin of macrophages.

Single antigens specific for mature DCs include endocytic receptor DEC-205/CD205 and DC-LAMP/ CD208 (Figure 15). Nearly all DC-LAMP⁺ and DEC-205⁺ cells were in dermal aggregates, and co-expressed CD11c⁺ and BDCA-1⁺. While nearly all BDCA-1⁺ cells co-expressed these two mature DC markers, there were many CD11c⁺ cells that did not, identifying a less mature population of BDCA-1⁻ myeloid dermal DCs.

Figure 14. *CD11c⁺BDCA-1⁺ inflammatory dermal DCs express CD14 and DC-SIGN.*

(A) A subset of *CD11c⁺* cells in the reticular dermis expressed low level *CD14*. (B) Few *BDCA-1⁺* dermal DCs co-expressed *CD14*, and (C) 80% of *CD11c⁺* cells were *DC-SIGN⁺*. This was not an exclusive myeloid DC marker as some *DC-SIGN⁺* cells were not *CD11c⁺*. (D) *BDCA-1⁺* cells did not co-express *DC-SIGN*. (E) *CD163⁺* macrophages showed some *CD11c* co-expression. (F) *BDCA-1⁺* cells did not express *CD163*. Size bar = 100 μ m.

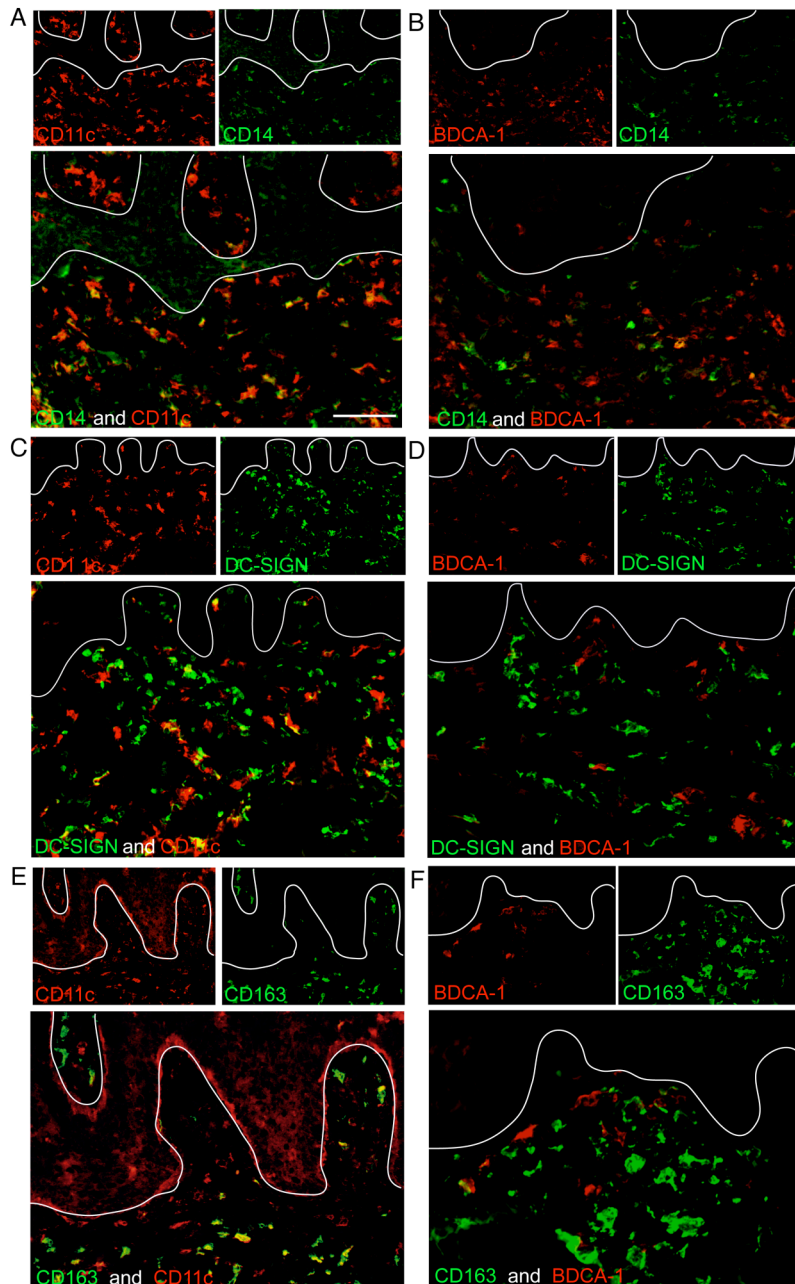
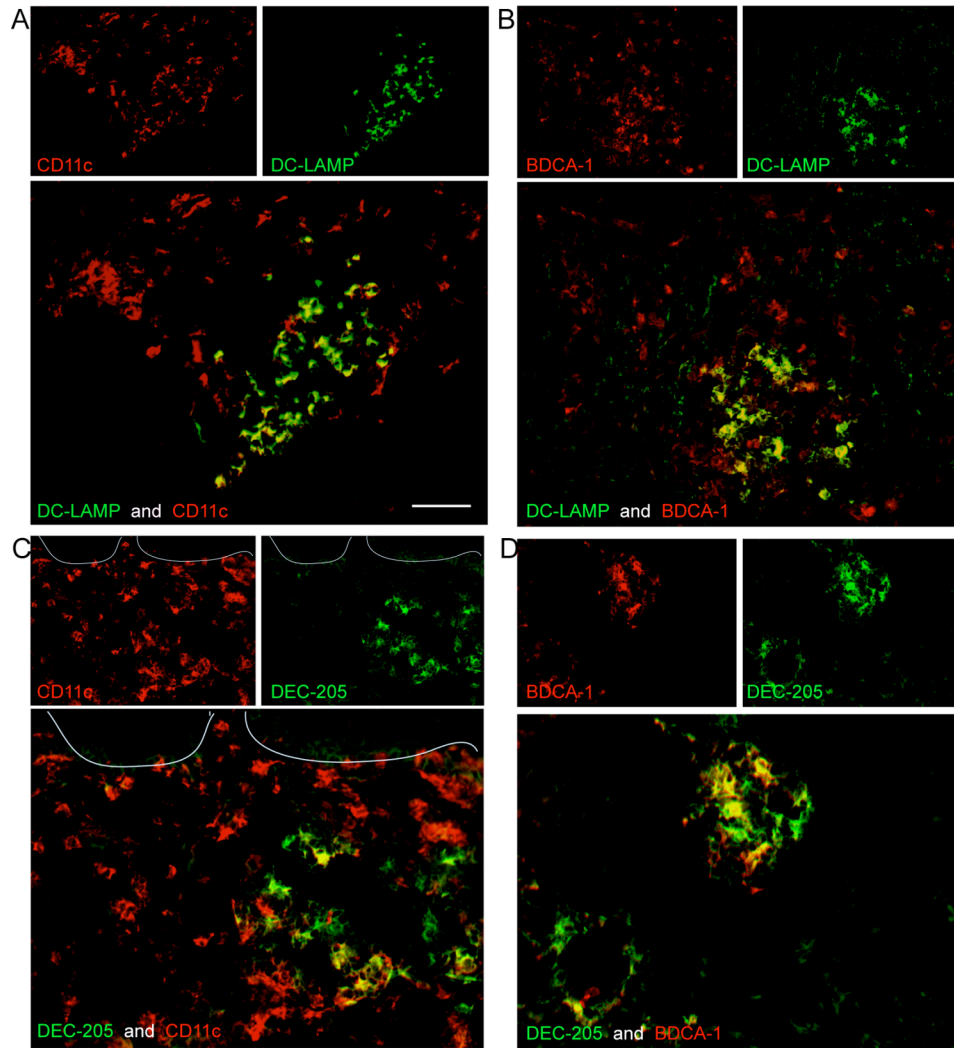


Figure 15. $CD11c^+BDCA-1^+$ cells are phenotypically mature dermal DCs in psoriasis. DC-LAMP and DEC-205 identified mature DCs, often located in dermal aggregates. (A) DC-LAMP⁺ cells were CD11c⁺, and (B) BDCA-1⁺. (C) DEC-205⁺ cells were CD11c⁺, and (D) BDCA-1⁺. There were many CD11c⁺ cells that were not positive for these two markers in the psoriatic dermis. Size bar = 100 μ m.



Myeloid DCs obtained from psoriatic dermis are immunostimulatory and activate Th17 and Th1 cells.

Single cell suspensions of normal skin (n=3) and psoriasis lesions (n=3) were obtained by enzymatic splitting of the epidermis, and then culturing the dermis for 2-3 days to allow the leukocytes to migrate out of the dermal scaffold. For functional experiments, we then FACS-sorted this bulk dermal single cell suspension, obtaining 10,000-50,000 cells in each population.

(a) Surface phenotype. For surface phenotyping of normal and psoriatic dermal myeloid DCs by FACS, large cells were gated on CD11c⁺HLA-DR⁺, a classic definition of a myeloid DC, and further gated according to BDCA-1 expression (Figure 16A). Expression levels of CD11c myeloid lineage marker, HLA-DR MHC-II protein, CD14 LPS receptor, and CD16 FcγRIIIa were measured on BDCA-1⁺ resident DCs from normal dermis (Figure 16A, left, box 1), BDCA-1⁺ resident DCs from psoriatic dermis (Figure 16A, right, box 2), and BDCA-1⁻ inflammatory DCs from psoriatic dermis (Figure 16A, right, box 3). CD11c⁺BDCA-1⁺ resident dermal DCs from both normal (Figure 16B, row 1) and psoriasis skin (Figure 16B, row 2) had similar levels of CD11c, HLA-DR, CD14, and CD16 expression and were similar in size (SSC-A) (isotype light gray fill). In contrast, CD11c⁺BDCA-1⁻ psoriatic dermal DCs (Figure 16B, row 3) had 10-fold decreased HLA-DR expression, 3-6 fold increased CD14 expression, and were 20% smaller than both CD11c⁺ BDCA-1⁺ populations. Blood monocytes expressed similar levels of CD11c and HLA-DR as CD11c⁺ BDCA-1⁻ psoriatic dermal DCs (data not shown), but were 99% CD14^{hi} (Figure 16B, row 3, black line no fill) unlike CD11c⁺

BDCA-1⁻ psoriatic dermal DCs which were CD14^{mid}. Expression of co-stimulatory molecules CD86 was highest on DC in psoriasis CD11c⁺ BDCA-1⁺ in psoriasis, and CD40 was low on all three populations. Thus, psoriatic CD11c⁺BDCA-1⁺ dermal DCs are phenotypically similar to normal resident CD11c⁺BDCA-1⁺ dermal DCs, whereas psoriatic CD11c⁺BDCA-1⁻ cells share phenotypic features of both DCs and monocytes.

(b) *Allo-MLR*. To test the immunostimulatory capacity of psoriatic dermal DC populations, we FACS-sorted DCs (CD11c⁺HLA-DR^{hi}BDCA-1⁺ or CD11c⁺HLA-DR^{hi}BDCA-1⁻) and macrophages (CD163^{hi}) for co-culture as stimulators in an allogeneic mixed leukocyte reactions (allo-MLR) (Figure 17A, right). Mature monocyte derived DCs (MoDCs) and T cells alone served as positive and negative controls, respectively. Figure 7 shows a representative experiment (n=3). 73.2% of living T cells stimulated with MoDCs at a stimulator/responder ratio of 1:50 on day 8 post sort had undergone extensive proliferation (Figure 17B). MLRs without a stimulator population (T cells) alone had 2.5% background proliferation. CD163^{hi} cells were not more immunostimulatory than T cells alone (2.3%), whereas both CD11c⁺BDCA-1⁺ resident DCs and CD11c⁺BDCA-1⁻ inflammatory DCs were similarly immunostimulatory (55.3% and 64.5%, respectively). Bulk psoriatic dermal single cell suspensions stimulate 60% (data not shown). These results suggest that CD163^{hi} cells in psoriasis are non-immunostimulatory macrophages expressing low level CD11c (Figure 14E), and that although CD11c⁺BDCA-1⁻ are phenotypically less mature than the CD11c⁺BDCA-1⁺ cells, they are comparably immunostimulatory.

(c) *Induction of intracellular cytokines in T cells.* We recently demonstrated the presence of Th17 cells from the dermis of psoriatic plaques (Lowes *et al.*, 2008). We now show that CD11c⁺ BDCA-1⁺ and CD11c⁺ BDCA-1⁻ psoriatic dermal DCs induce IL-17 production in allogeneic CD4⁺ T cells. Psoriatic dermal émigrés (n=3) were sorted as previously described into CD11c⁺ BDCA-1⁻ DCs, CD11c⁺ BDCA-1⁺ DCs, or CD163⁺ macrophages and mixed with normal donor T cells at a 1:10 stimulator: responder ratio. Normal skin dermal émigrés (n=2) were sorted into BDCA-1⁺ and CD163⁺ populations. Cells were cultured for 9 days before analysis of T cell intracellular cytokines and IL-17 and IFN γ cytokine production in the supernatant. Figure 18 shows a representative experiment.

There were few T cells positive for IL-17 or IFN γ when the T cells were cultured alone (Figure 18A, left), with marked increases when cells were stimulated with CD3/CD28 beads (Figure 18A, right). Culture of the single cell suspensions alone, without addition of the allogeneic T cells, gave similar results to T cells alone, indicating the capability of the syngeneic T cells in the suspension (Figure 18B). There was marked increase in the intracellular cytokine staining when the dermal DCs were cultured with allogeneic T cells (Figure 18C), much more so for the psoriasis lesions. We particularly noted the increase of the IL-17⁺IFN γ ⁺ cells induced by the psoriatic DCs, from 0.2% to 12.1%. The psoriatic dermal single cell suspensions also induced more IL-17 and IFN γ protein than normal skin (Figure 18D). This was similar to controls of T cells alone, and T cells stimulated with CD3/CD28 beads (data not shown).

The psoriatic BDCA-1⁺ and BDCA-1⁻ populations were able to induce similar percentages of these IL-17⁺IFN γ ⁺ cells, although less than the bulk dermal single cell

suspensions (Figure 18E, 17F, right). This reduction is likely due to the process of FACS-sorting, as sorting reduces the ability of the DCs to polarize T cells by an average of 6-fold (analysis of the ability of bulk single cell suspensions before and after sorting, data not shown). The BDCA-1⁺ DCs from normal skin did not induce IL-17⁺ IFN γ ⁺ cells (Figure 18E, left), nor did the normal or psoriatic skin macrophages (Figure 18G). There was low level of IL-17 and IFN γ cytokines in the supernatants, likely due to the lower cytokine expression in these cells (data not shown).

In summary, normal skin dermal émigrés polarized few Th17 cells, none of which were IL-17- and IFN γ -producing, while psoriatic bulk émigrés and dermal DCs (BDCA-1⁺ and BDCA-1⁻) polarized Th17 cells producing both IL-17 and IFN γ . CD163⁺ macrophages from either normal or psoriatic skin were not able to polarize T cells to produce IL-17.

Figure 16. Psoriatic inflammatory dermal DCs ($CD11c^+ BDCA-1^-$) are less mature than resident $BDCA-1^+$ dermal DCs. Flow cytometric analysis of single cell suspensions of dermal émigrés from normal dermis or psoriatic dermis (n=3 for each). (A) Large cells gated on $CD11c^+ HLA-DR^{hi}$. In normal dermis, most myeloid DCs were $BDCA-1^+$ (box 1). In psoriatic dermis, myeloid DCs were either $BDCA-1^+$ (box 2), or $BDCA-1^-$ (box 3). (B) Histograms in each row were gated on boxes (1-3) as identified above in Figure 2a. Dark grey histogram represents antigen expression, light grey is isotype, and no fill (row 3) is CD14 expression on blood monocytes. MFI indicated in the upper right or upper left corner of each histogram. Resident $BDCA-1^+$ myeloid DCs from normal and psoriatic dermis were phenotypically similar, while the additional population of $BDCA-1^-$ DCs in psoriasis showed lower HLA-DR, wider range of expression of CD14, and were smaller cells.

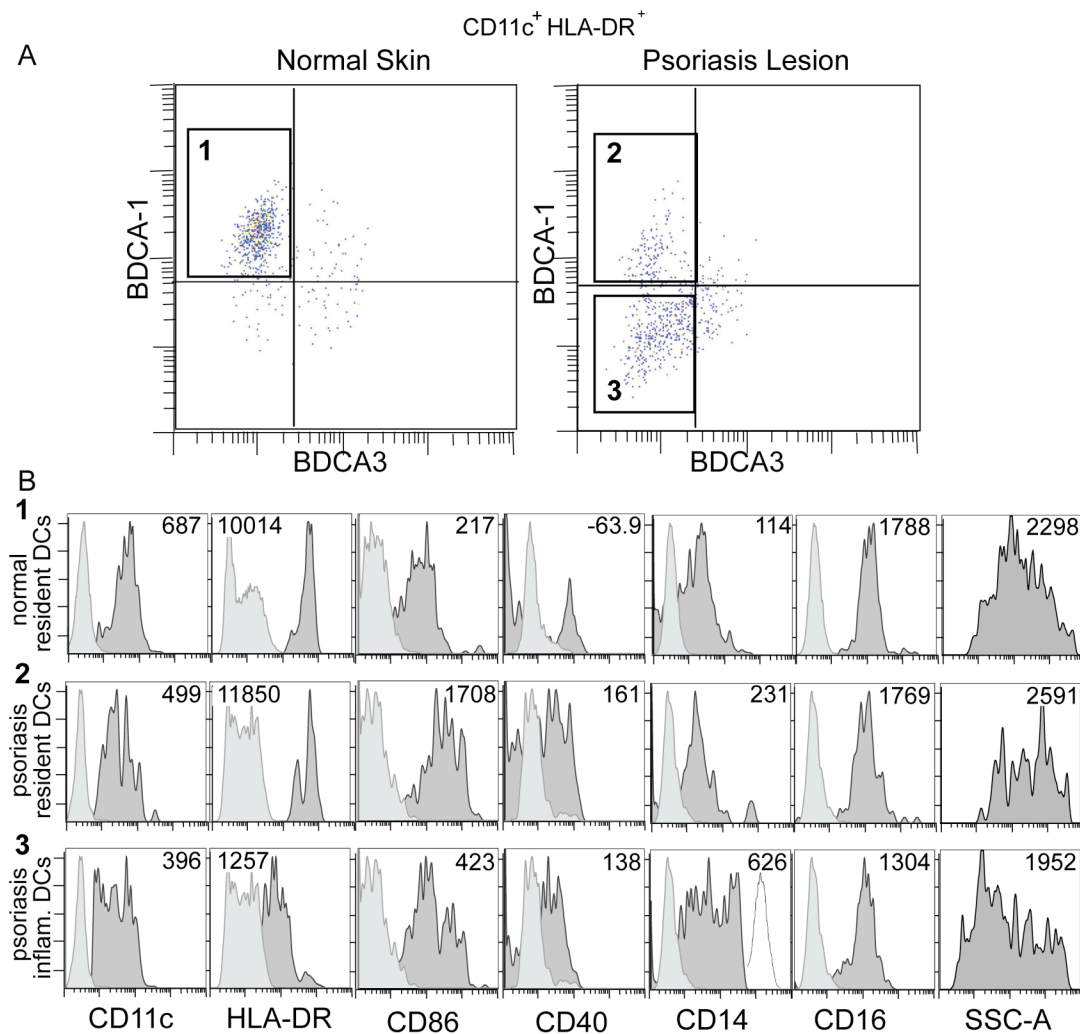


Figure 17. Both psoriatic $CD11c^+BDCA-1^+$ resident DCs and $CD11c^+BDCA-1^-$ inflammatory DCs were immunostimulatory in an *allo-MLR*. Single-cell suspensions of psoriatic dermal émigrés were sorted into (A, left) $CD163^{hi}$ or (A, right), $CD3^-CD45^+HLA-DR^{hi}CD11c^{hi}BDCA-1^+$ or $CD3^-CD45^+HLA-DR^{hi}CD11c^{hi}BDCA-1^-$ compared to isotype (red). (B) Gate contains $CD3^+$ proliferating T cells with left-shifted CFSE as cells proliferated. Positive control (monocyte-derived mature DCs) on day 8-post sort at 1:50 stimulator to responder ratio. Background proliferation of T cells alone (2.5%), $CD163^{hi}$ cells did not stimulate above background. $BDCA-1^+$ and $BDCA-1^-$ cells stimulated T cell proliferation similarly (both >55%). Representative graphs from 3 experiments.

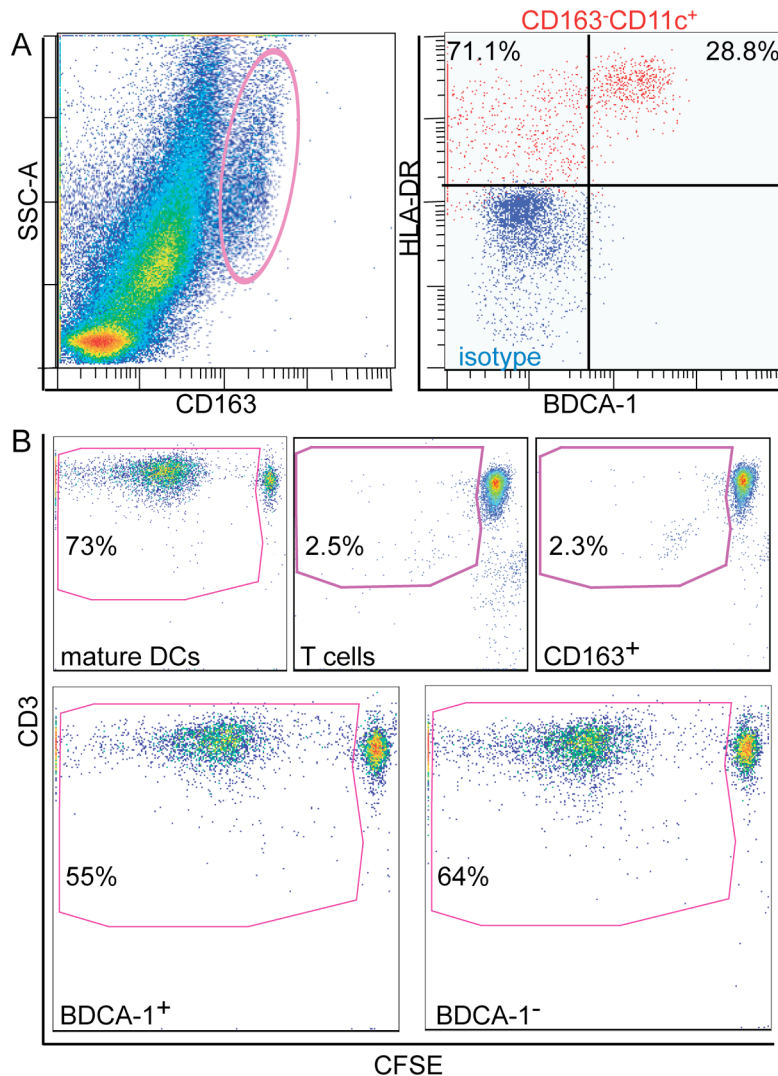
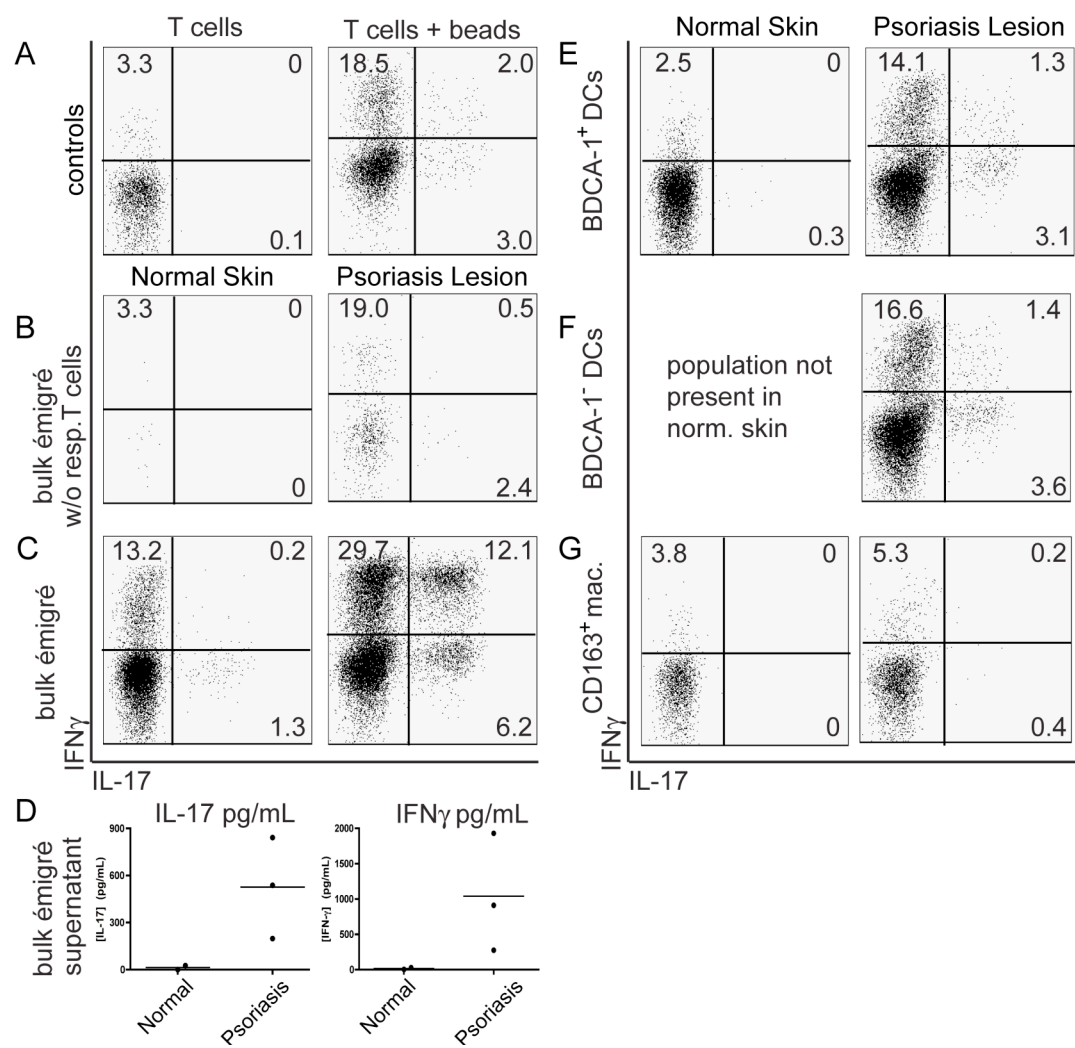


Figure 18. Psoriatic dermal DCs induce IL-17/IFN γ producing T cells. Allogeneic T cell responders were mixed with FACS-sorted stimulator populations described in Figure 6, or with bulk émigrés from either normal or psoriatic dermal skin, at 1:10 ratio for 9 days. Intracellular cytokine expression of T cells (live CD3⁺CD4⁺CD8⁻ cells) measured by flow cytometry (A,B,C,E,F,G) or protein supernatant (D). (B,C,E,F,G) Comparison of T cell polarization of normal dermal DCs (left panels) with psoriatic dermal DCs (right panels). (A) Controls: T cells alone (left panel) demonstrated baseline intracellular cytokine expression and T cells + beads (right panel) measured intracellular cytokine expression following CD3/CD28 bead stimulation. (B) Bulk émigrés w/o responder T cells measured baseline T cell cytokine production in single cell suspensions. Other stimulator populations mixed with responder T cells were (C) unsorted bulk émigrés, (E) sorted HLA-DR⁺CD11c⁺BDCA-1⁺ dermal DCs, (F) sorted HLA-DR⁺CD11c⁺BDCA-1⁻ dermal DCs, or (G) sorted CD163⁺ macrophages. Psoriatic bulk dermal DCs induced a population of IL-17/IFN γ producing T cells, as did BDCA-1⁺ and BDCA-1⁻ DCs (although to a lesser degree, possibly due to sorting). Quadrant percentages are given, representative graphs from 3 experiments. (D) Supernatant of the same cultures was collected for analysis of cytokine protein expression Bulk psoriatic dermal émigrés mixed with allogeneic T cells induced IL-17 and IFN γ production, while normal dermal DCs did not.



Discussion

In psoriasis as well as normal skin, myeloid DCs identified by BDCA-1 were present, and their numbers remained the same during a course of etanercept therapy. These resident BDCA-1⁺ DCs were often in clumps in the dermis, and were positive for mature markers such as DC-LAMP and DEC-205. In psoriasis, there was a marked increase in CD11c⁺ cells currently best identified as BDCA-1⁻, and we have termed these BDCA-1⁻ dermal cells “inflammatory” myeloid DCs. This group of inflammatory cells may be heterogeneous, including the Tip-DCs, IL-20-producing DCs (Wang *et al.*, 2006), and possibly IL-23-producing DCs (Lee *et al.*, 2004; Zaba *et al.*, 2007a). The success of anti-TNF therapies to reduce all these cytokines and mediators and reverse the psoriatic phenotype supports the potential pathogenic role of these DCs (Zaba *et al.*, 2007a). A similar finding was recently described in normal and diseased kidney, with a stable number of BDCA-1⁺ cells during inflammation (Woltman *et al.*, 2007). In this study, DC-SIGN was used as an alternative marker of DCs, and these cells did increase during rejection.

Both BDCA-1⁺ and BDCA-1⁻ DCs were immunostimulatory in the allo-MLR and induced Th1, Th17 and a mixed Th1/Th17 cell type. This lack of difference in their function is perhaps surprising, considering there appears to be two discrete populations *in situ*. We interpret these findings as a confirmation of their DC function, and an indication that the CD11c⁺BDCA-1⁻ cells are not monocytes or macrophages. Their ability to perform in these assays indicates their antigen-presenting potential, rather than being a true characterization of their role *in situ* during inflammation. There may be several

reasons for this lack of difference of function of these two populations, including different migration kinetics or ability, or maturation of the BDCA-1⁻ cells during migration out of the dermis with concomitant up-regulation of defining DC-functions. Thus, although there are several limitations to this system, it is currently the only method available to study the potential functions of these cells.

There are several possible mechanisms to explain an increase in inflammatory myeloid DCs in psoriasis lesional skin, including arising from *in situ* DCs in non-lesional skin, from circulating DC-precursors or monocytes. These cells may arise from BDCA-1⁺ resident myeloid DCs that down-regulate their BDCA-1 surface expression, or even Langerhans cells. This is supported by the xenotransplant psoriasis model, where human transplanted non-lesional skin from psoriasis patients is grafted onto immunocompetent mice and the grafts become psoriatic without further intervention (Nestle and Nickoloff, 2005). In this model, resident cells in the non-lesional skin are sufficient and capable of inducing the psoriatic phenotype, without any contribution from circulating cells. However, the lesional skin may behave differently in this system than *in vivo*, and other cells may take on unconventional roles.

Secondly, these inflammatory myeloid DCs may arise from peripheral blood DC precursors, including hematopoietic stem-cell precursors (HSC) multipotent progenitor (MPP), common myeloid precursor (CMP), circulating pre-DC (CD11c⁺ HLA-DR^{hi}, CD16⁺), or monocytes (Massberg *et al.*, 2007; Piccioli *et al.*, 2007; Randolph *et al.*, 2002; Serbina *et al.*, 2003; Tacke and Randolph, 2006). It is possible that any of these

precursor cells are “pre-inflammatory DCs”, migrating into the skin in response to a chemokine gradient or other stimulus, supported by our observation of a reduction of all circulating DC subsets in psoriasis blood compared to normal. The concept that the inflammatory DCs arise from circulating precursors rather than *in situ* DCs is also consistent with other murine models demonstrating that during steady state, Langerhans cells and dermal DCs are able to locally proliferate, but during active inflammation there is migration of peripheral blood DC precursors into the skin (Bogunovic *et al.*, 2006; Ledgerwood *et al.*, 2007; Liu *et al.*, 2007; Massberg *et al.*, 2007).

CD11c⁺BDCA-1⁻ dermal DCs retain some phenotypic features of their peripheral blood precursors, including smaller size, low-level expression of CD14, and CD163, also supporting the concept of migration of these cells from the blood. CD163^{hi} cells, however, when sorted from psoriasis dermis are not immunostimulatory, thus they can be distinguished functionally from the immunostimulatory CD11c⁺BDCA-1⁻ DC population. Other phenotypic markers that have been attributed to both inflammatory DCs and inflammatory macrophages include CD68 (Wang *et al.*, 2006), CD32 (Dhodapkar *et al.*, 2007), and DC-SIGN/CD209 (Graneli-Piperno *et al.*, 2005). The co-localization of some of these antigens may also be due to plasticity between these immature DC and macrophage populations within the tissue during inflammation.

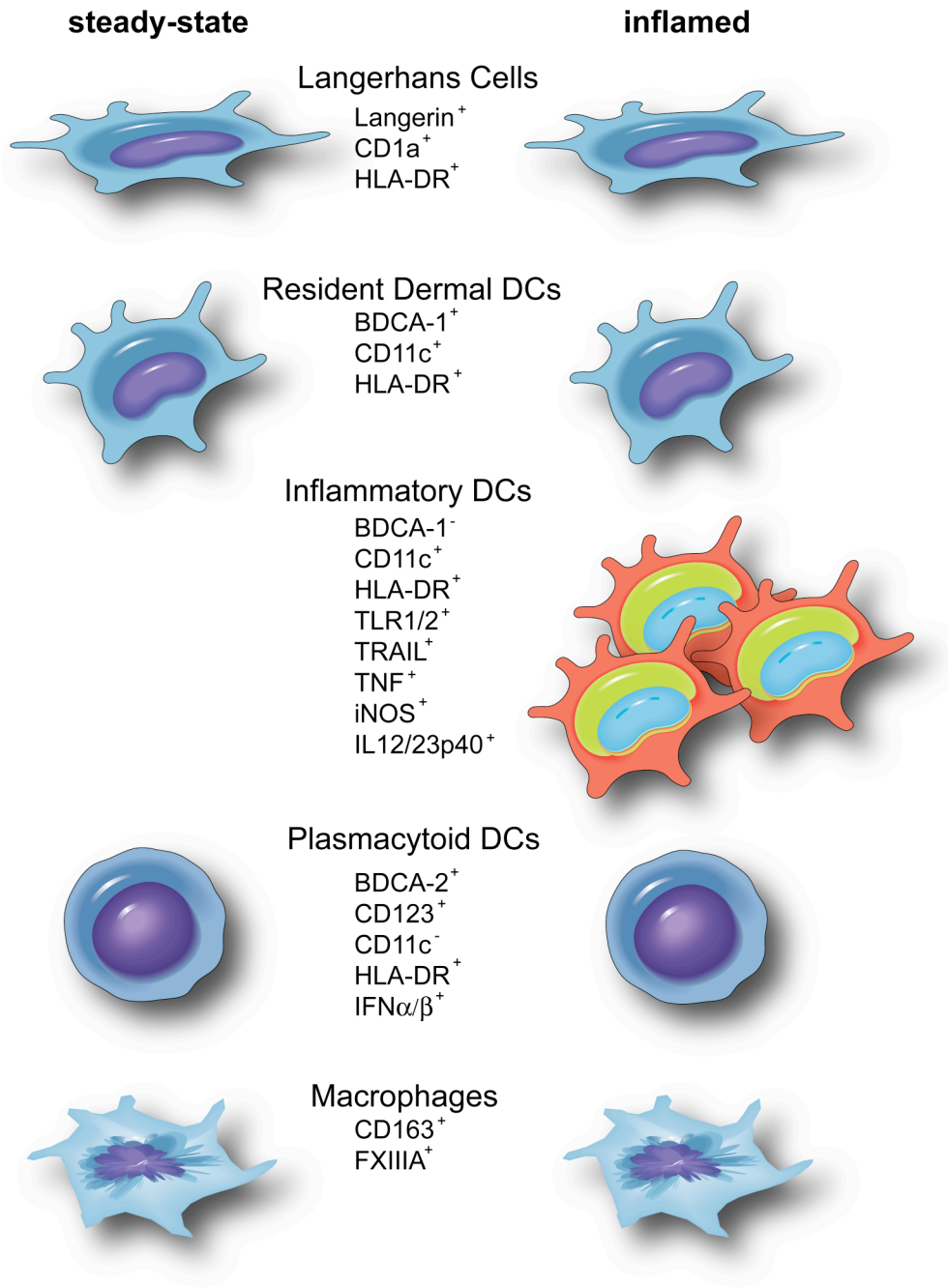
In addition to characterization of psoriatic myeloid dermal DCs, this paper presents evidence for Th17 polarization by psoriatic DCs. Both psoriatic CD11c⁺BDCA-1⁺ resident DCs and CD11c⁺BDCA-1⁻ DCs had the capacity to polarize Th17 cells,

although most polarization was induced by bulk psoriatic dermal cells not damaged by sorting. Moreover, bulk psoriatic émigrés induced Th17 cells producing both IL-17 and IFN γ compared to normal skin. Since psoriasis is now thought of as a mixed Th17/Th1 disease with strong IL-17 and IFN γ signatures (Blauvelt; Ghoreschi *et al.*, 2007; Lowes *et al.*, 2007), it is possible that these IL-17/IFN γ positive T cells induced by psoriatic DCs are pathogenic.

In summary, we have demonstrated a marked increase in CD11c⁺BDCA-1⁻ myeloid DCs in psoriasis, and this group of inflammatory DCs contains Tip-DCs. These cells are immunostimulatory and capable of Th17 polarization, but their most essential contribution may be to secrete inflammatory products such as iNOS, TNF, IL-20, and IL-23. We hypothesize that the resident BDCA-1⁺ are likely the myeloid DCs capable of classic antigen-presentation to cutaneous T cells, although their dermal location and organization into clumps associated with T cells suggests this function may occur within the skin environment (“ectopic lymphoid tissue”) rather than in an extracutaneous lymphoid organ such as a lymph node (Lew *et al.*, 2004a). In contrast, while BDCA-1⁻ DCs are certainly capable of robust antigen presentation in an allo-MLR and have the ability to polarize T cells (DC-defining characteristics), their main role may actually be as an inflammatory mediator production house, amplifying and maintaining psoriatic inflammation. Further studies need to be performed to prove this, dependant on new tools to study DCs *in situ*. We also need new markers to identify these inflammatory DCs in a

positive manner, rather than as a negative population. We need to understand how and where these non-resident CD11c⁺BDCA-1⁻ DCs are generated, in order to be able to specifically shut down their production of pro-inflammatory mediators, and bring about rapid resolution of clinical disease.

Figure 19. Dendritic cell and macrophage populations in human skin during steady-state and inflammation. Non-inflamed skin contains epidermal Langerhans cells, BDCA-1⁺ resident dermal DCs, plasmacytoid DCs, and macrophages. In addition, inflamed skin contains a large population of myeloid “inflammatory” DCs. Common markers used to identify these leukocyte populations are indicated.



CHAPTER 5: Modulating psoriasis immune pathogenesis with TNF inhibition

In previous chapters we characterized DC populations in normal and psoriasis skin, and showed that the dominant population of inflammatory DCs in psoriatic lesions are immunostimulatory. While these data suggest that DCs play a role in psoriasis immunopathogenesis, a DC “knock-out” model is necessary to determine the role of these DCs in psoriasis inflammation. Using the immune modulating drug etanercept, a TNF blocking agent, we are able to create an effective knock-out of TNF, a cytokine produced by DCs and other cells. In this chapter we monitored changes in the immune milieu of psoriatic plaques during the course of etanercept treatment to further explore the role of DCs in psoriasis inflammation.

Until recently, IFN γ -producing Th1 T cells were implicated as the main pathogenic cells (Blauvelt, 2007) as certain T cell-targeted therapies were successful in clearing psoriasis (Lowes *et al.*, 2007), and clonal T cells have been found in psoriatic skin (Prinz *et al.*, 1994). However, we are beginning to appreciate that there may be an important pathogenic contribution from a recently-recognized subset of T cells – Th17 cells producing IL-17 and IL-22 (Bettelli *et al.*, 2007; Blauvelt, 2007). In model systems, IL-17 stimulates keratinocyte production of innate inflammatory “danger signals” such as defensins and S100 proteins as well as IL-8 neutrophil chemokine (Liang *et al.*, 2006), while IL-22 modulates defensins (Wolk *et al.*, 2006) and keratinocyte hyperproliferation (Sa *et al.*, 2007; Zheng *et al.*, 2007). Upstream inducers of Th17 cells are still being understood, as most experiments have been performed in murine model systems. Mediators may include IL-1, IL-6, and TGF β , which stimulate differentiation of naïve

CD4⁺ T cells into activated memory Th17 cells (Annunziato *et al.*, 2007; Mangan *et al.*, 2006; Sutton *et al.*, 2006), and IL-23 which drives Th17 proliferation (Vanden Eijnden *et al.*, 2005).

Th17 T cells producing IL-17 and IL-22 have been implicated as pathogenic in murine models of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and inflammatory bowel disease (IBD) (Komiyama *et al.*, 2006; Nakae *et al.*, 2002; Nakae *et al.*, 2003; Park *et al.*, 2005). IL-17 knockout mice are resistant to both EAE and CIA. Also, mice with EAE have increased numbers of Th17 cells, but are resistant to disease if immunized against IL-17 (Uyttenhove and Van Snick, 2006). The dendritic cell (DC) product IL-23, a survival factor for Th17 cells, also appears to be necessary for IBD pathogenesis in mice (Yen *et al.*, 2006). Thus a model is emerging of autoimmune inflammation that begins with activated antigen presenting cells (APCs) producing IL-23, subsequent Th17 cell proliferation and IL-17/IL-22 release, and downstream inflammatory tissue damage.

Most studies on Th17 cells have been performed in murine models or *in vitro*. However, there is some human data also supporting a similar model of Th17-mediated autoimmune inflammation. Patients with IBD have elevated IL-17 and IL-22 in affected colonic tissue and serum, dependent on disease activity and severity (Fujino *et al.*, 2003; Nielsen *et al.*, 2003; Te Velde *et al.*, 2006), and patients with rheumatoid arthritis (RA) have elevated IL-17 and IL-22 protein in synovial fluid (Ikeuchi *et al.*, 2005; Kotake *et al.*, 1999). In psoriasis patients, IL-17 mRNA has been demonstrated within lesions (Li *et al.*, 2004), but protein levels are not increased in the serum (Arican *et al.*, 2005). IL-22 protein is increased in psoriatic serum compared to normal, and mRNA is increased in

lesional tissue (Wolk *et al.*, 2006). High levels of IL-23 have also been detected in psoriasis lesions (Piskin *et al.*, 2006), and are strongly diminished by effective therapies for psoriasis (Lowes *et al.*, 2005b).

Biological treatments provide researchers with tools to directly target components of the immune system, and begin to dissect molecular circuitry and pathogenic pathways. Treatment of psoriasis patients with etanercept, a TNFR-Ig fusion protein (etanercept, Amgen), presents an opportunity to further understand effects of blocking TNF at a molecular and cellular level. Comparative modulation of Th17 vs. Th1 T cell activation in psoriasis within the context of a therapeutic trial has not been previously reported. We found that psoriasis disease improvement correlated with rapid down-modulation of DC and Th17 cell products and downstream effector molecules, and final disease resolution correlated with late down-modulation of Th1 cells.

***In vivo* anti-inflammatory and immune modulating effects of TNF inhibition at multiple time points during progressively resolving psoriasis with drug treatment**

Clinical and histological responses

In this study, 20 patients were given 50mg of etanercept biweekly for 12 weeks. PASI was decreased by a mean of 36% (range 9-67%) after 4 weeks of treatment, and 69% (range 33-96%) after 12 weeks of treatment (data not shown). The time course and extent of improvement with biweekly etanercept treatment in this trial were similar to outcomes seen in larger, double-blind clinical trials (Papp *et al.*, 2005; Tying *et al.*, 2006).

The effects of etanercept on disease histopathology, epidermal thickness, expression of K16 (immunohistochemistry and quantitative mRNA measures), and Ki67 cell counts are illustrated in Figure 20A,B. After 12 weeks of treatment, epidermal thinning and normalization of keratinocyte differentiation occurred in 16 of 20 patients, which we considered to be histological responders (Gottlieb *et al.*, 2005). The data presented is from the 16 histological responders in order to study immunologic response within the target lesion.

Average PASI score for histological responders was 7.1 (range 0.6-22, SEM 1.4) with an average % clearance of 74.5 (range 38.9-97.5, SEM 4.9) (Figure 20A). Mean epidermal thickness was significantly reduced by week 1 compared to baseline lesional skin ($p < 0.05$). K16 mRNA levels (a measure of epidermal regenerative activation) and Ki67 cell number/mm (a measure of keratinocyte proliferation) were also significantly

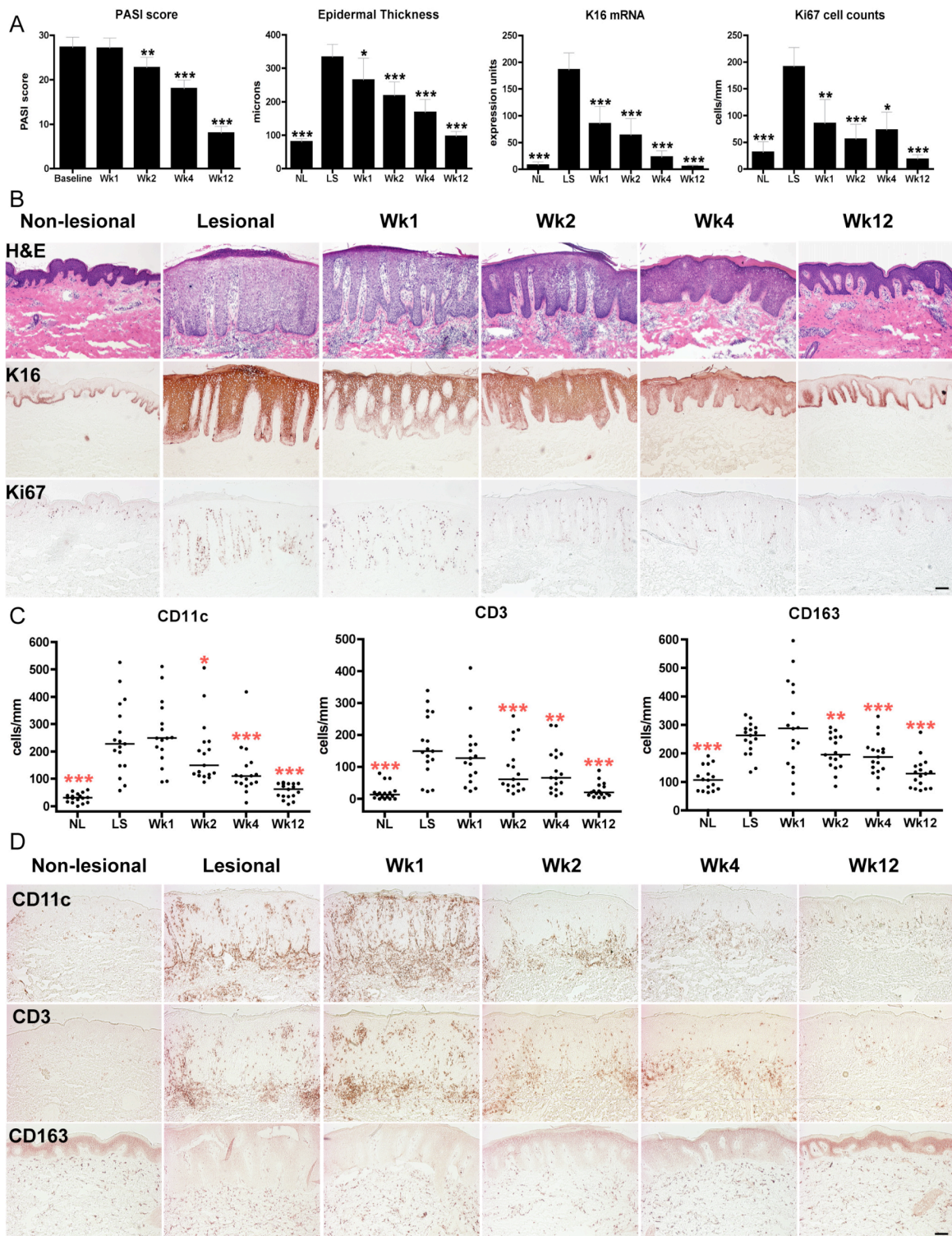
reduced by week 1 ($p<0.001$ and $p<0.01$, respectively) . Representative haematoxylin and eosin (H&E), K16, and Ki67 immunostaining for a responding patient is shown (Figure 20B). Thus, keratinocyte acanthosis, differentiation, and proliferation were all rapidly down modulated at week 1 of treatment.

Inflammatory infiltrate in psoriasis skin was reduced with etanercept treatment

Non-lesional skin contained relatively low numbers of CD11c⁺ myeloid DCs, CD3⁺ T cells, and CD163⁺ macrophages (Figure 20C). In psoriasis plaques, inflammatory cell numbers were increased 2-4 times above normal. Little or no change in inflammatory cell infiltrate was seen by week 1 of etanercept treatment. By week 2 cell numbers began to decrease, but did not approximate baseline values until week 12. At week 12, CD11c, CD3, and CD163 cell counts were not significantly different from non-lesional values. Representative immunohistochemistry for CD11c, CD3, and CD163 antigens at each biopsy time point is shown in Figure 20D. Therefore, decreased dermal inflammatory infiltrate with etanercept treatment lagged behind decreased keratinocyte acanthosis.

Figure 20. Clinical and histological resolution of psoriasis with etanercept treatment.

(A) Average PASI scores, epidermal thickness, keratin 16 (K16) mRNA expression, and Ki67 cell counts in histological responders (n=16) during treatment with etanercept. Clinical response measured at baseline, wks 1, 2, 4 and 12; biopsies evaluated in non-lesional skin (NL), lesional skin (LS), and in the lesional index plaque at weeks 1, 2, 4, and 12. Baseline lesional values compared with other time points, $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). (B) Histology and immunohistochemistry showing H&E, K16, and Ki67 expression during treatment. Size bar = 100 μ m. (C) CD11c⁺ myeloid DCs, CD3⁺ T cells, and CD163⁺ macrophages per mm in non-lesional skin (NL) lesional skin (LS), and in the lesional index plaque at weeks 1, 2, 4, and 12. Baseline lesional values compared with other time points, $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). (D) Immunohistochemistry showing CD11c, CD3 and CD163 expression during treatment. Size bar = 100 μ m.



Etanercept rapidly down modulated Th17 T cell products and had a delayed effect on Th1 and Th2 T cell products

IL-17 and IL-22, the hallmark cytokines of Th17 cells, were rapidly down modulated in histologic responders by week 1 ($p=0.05$) and week 2 ($p=0.05$) of etanercept treatment respectively (Figure 21A). Variability in IL-17 expression at weeks 2 and 4 resulted in p-values that approached significance ($p=0.056$ and $p=0.057$, respectively). In contrast, IFN γ , the hallmark cytokine of Th1 response was not down modulated until week 12 ($p<0.01$) (Figure 21B). LTA-1, another Th1 response cytokine, was also down modulated at week 12 ($p<0.05$) (Figure 21C).

To assess the biological significance of early Th17 cytokine down modulation and late Th1 cytokine down modulation with etanercept treatment, we used multivariate U-statistics to correlate “Th17 score” (a composite of IL-17 and IL-22 mRNA expression values) or “Th1 score” (IFN γ and LTA-1) and correlated them with histological disease improvement “response score” (epidermal thickness, K16 expression, and Ki67 counts) (Figure 21C). There was a strong correlation between Th17 cytokines and epidermal response score ($R=0.89$, $p=3.7 \times 10^{-6}$) and less so between Th1 cytokines and epidermal response score ($R=0.49$, $p=0.055$). We further confirmed biological significance of early Th17 down modulation by measuring genes regulated by IL-17, CCL20 and DEFB4 (Figure 21D). CCL20 and DEFB4 were both down modulated by week 1 of etanercept treatment ($p=0.01$, $p=0.05$) and were consistently suppressed at all weeks of treatment. In contrast, an IFN γ regulated gene, MX-1, was not significantly reduced until week 4 ($p=0.05$) and even more strongly suppressed by week 12 ($p<0.001$) (Figure 21E). Also of

interest was IL-4, the defining cytokine of the Th2 T cell, was up regulated at week 12 (p=0.09) (Figure 21F).

Other inflammatory cytokines rapidly down modulated with etanercept were IL-1 β (week 1, p<0.01), IL-6 (week 2, p<0.05), and IL-8 (week 1, p<0.01), findings previously reported by our group at 1 month, the earliest time point of that study (Gottlieb *et al.*, 2005). In contrast, TGF β was not significantly altered with treatment (Figure 22). In summary, while Th17 T cell products and downstream effector molecules regulating keratinocyte hyperplasia are modulated rapidly during the course of etanercept treatment, Th1 and Th2 products are modulated late, months after disease has significantly improved.

Figure 21. *Th17 cell products and downstream mediators are rapidly down modulated with etanercept treatment compared to Th1 and Th2 cell products.* mRNA expression normalized to HARP for (A) Th17 cell products IL-17 and IL-22 and (B) Th1 cell products IFN γ and LTA-1. (C) Multivariate U-statistics correlating the change in Th17 or Th1 cell products with histological response (epidermal thickness, K16, Ki67) over time. (D) Downstream effectors of Th17 cells, CCL20 and DEFB4 (b-defensin) (E) Downstream effector of Th1 cells MX-1. (F) Th2 cell product IL-4. All mRNA evaluated in non-lesional skin (NL), lesional skin (LS), and in the lesional index plaque at weeks 1, 2, 4, and 12. Baseline lesional values compared with other time points, $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

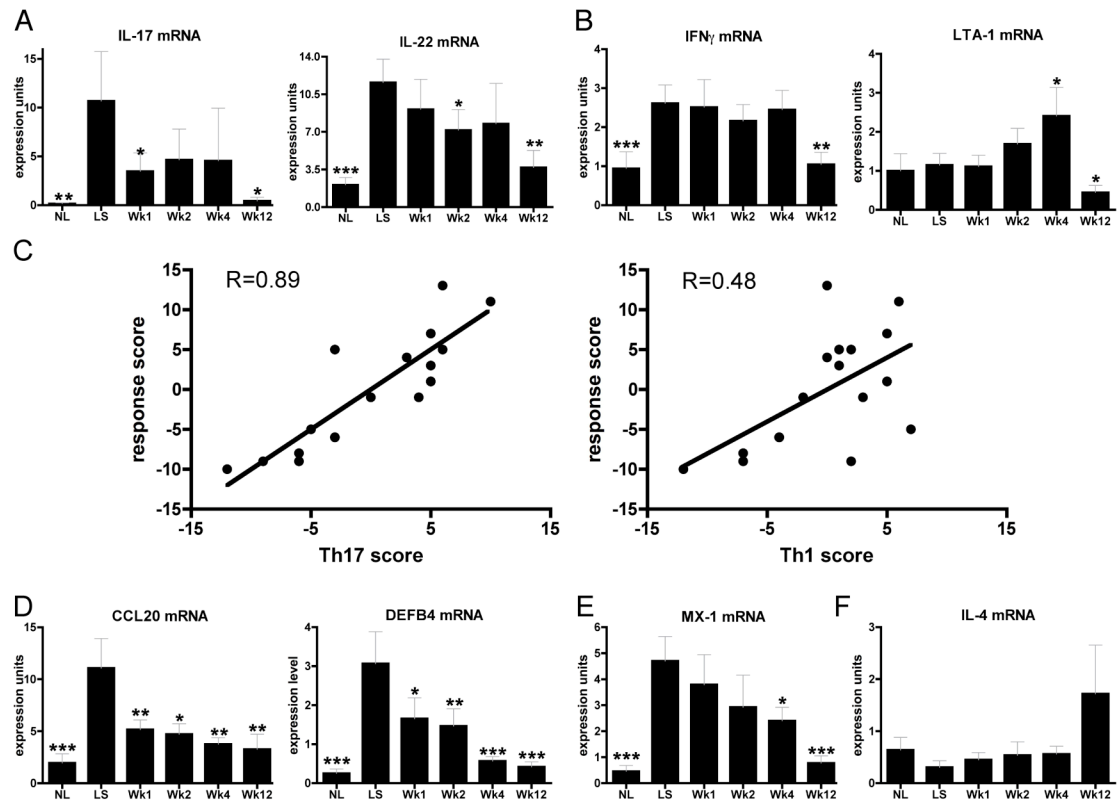
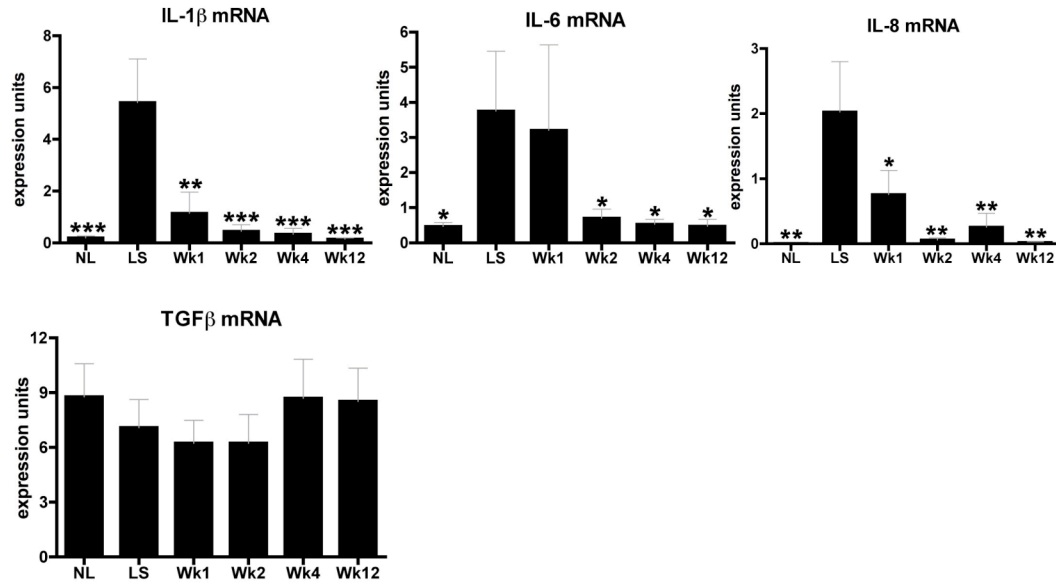


Figure 22. *IL-1 β , IL-6, and IL-8 are rapidly down modulated with etanercept treatment.* mRNA expression normalized to HARP for IL-1 β , IL-6, IL-8, and TGF β in non lesional skin (NL) lesional skin (LS), weeks 1, 2, 4 and 12. Baseline lesional values compared with other time points, $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)).



Products of TipDCs were rapidly down modulated with etanercept treatment

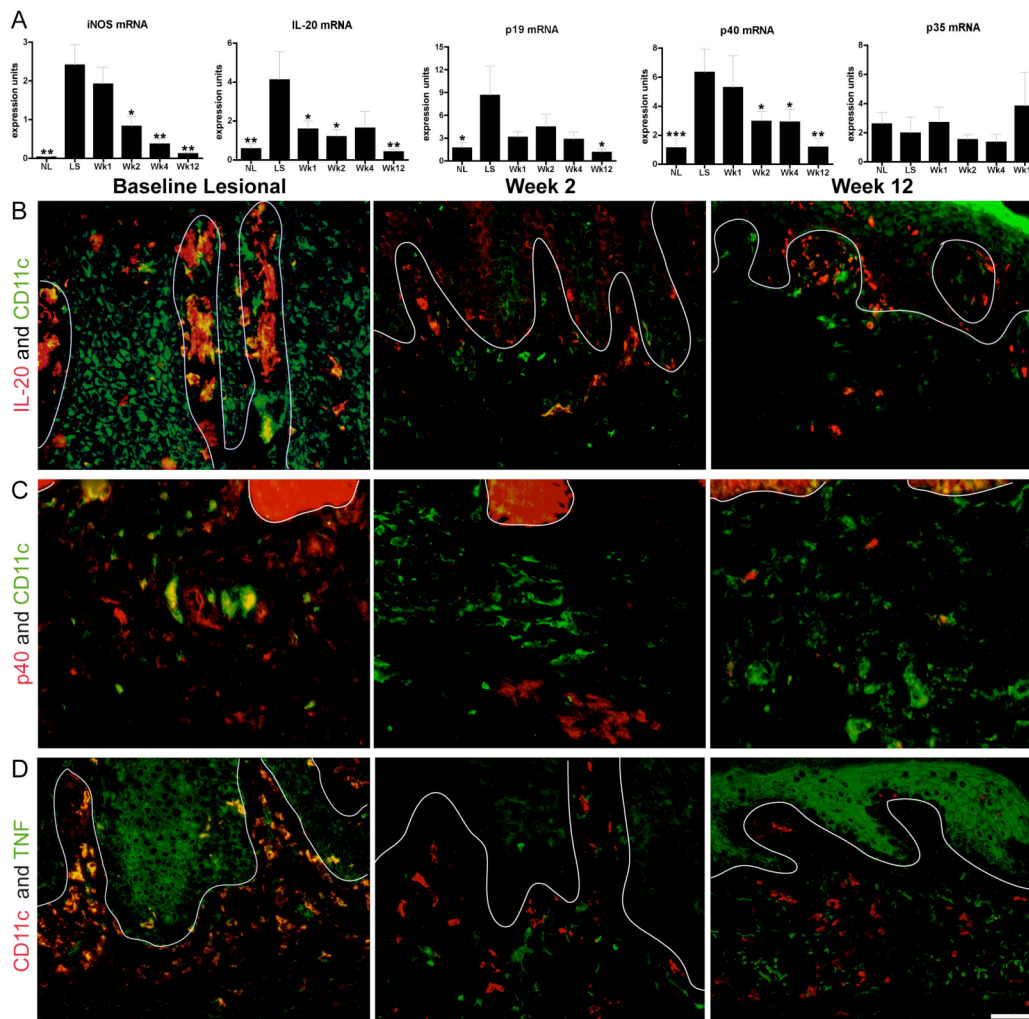
We have previously described the TNF and inducible nitric oxide synthase (iNOS) producing DC (Tip-DC) as a major pathogenic cell in psoriasis (Lowes *et al.*, 2005a). Using RT-PCR and double-label immunofluorescence we show that Tip-DC products were rapidly down modulated with etanercept treatment (Figure 23A). iNOS mRNA was significantly decreased by week 2 ($p<0.05$), IL-20 mRNA decreased by week 1 ($p<0.05$), and both IL-23 subunits (p19 and p40) were reduced by weeks 1 and 2 ($p=0.06$, $p<0.05$ respectively). In contrast, transcription of the IL-12 p35 subunit was not modulated by etanercept.

We confirmed that IL-20 was primarily a product of CD11c⁺ myeloid DCs in untreated psoriasis using double-label immunofluorescence showing >90% co-localization (yellow color) of IL-20 antigen with CD11c antigen in baseline lesional sections (Figure 23B). IL-20⁺CD11c⁺ cells were clustered in elongated dermal papillae where there is an extensive vascular supply, and a few cells invaded the epidermis. At week 2 of etanercept treatment <10% of CD11c⁺ cells produced IL-20, and by week 12 no visible overlap was apparent. Similarly, IL23p40 subunit was produced by 100% of CD11c⁺ cells in psoriasis lesional skin, but was not detected at weeks 2 and 12 of etanercept treatment (Figure 23C).

TNF was produced in >95% of CD11c⁺ DCs within untreated psoriasis plaques as indicated by the yellow cells clustering near the dermal-epidermal junction and infiltrating the epidermis (Figure 23D). At weeks 2 and 12 of etanercept treatment, no

visible overlap was apparent. iNOS protein in CD11c⁺ DCs is also down modulated by etanercept treatment, as previously published by our group (Gottlieb *et al.*, 2005). Hence, iNOS, TNF, IL-20, and IL-23 are Tip-DC products down modulated within the first 2 weeks of etanercept treatment.

Figure 23. *Inflammatory DC cell products are rapidly down modulated with etanercept treatment.* (A) mRNA expression normalized to HARP for inflammatory DC cell products iNOS, IL-20, IL-23 p19, IL-23/IL-12 p40, and IL-12 p35 in non-lesional skin (NL) lesional skin (LS), weeks 1, 2, 4 and 12. Baseline lesional values compared with other time points, $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). (B-D) Double-label immunofluorescence of myeloid DCs (CD11c) and various mediators (IL-20, IL-12/23 p40, and TNF) demonstrating co-expression (yellow color) in baseline lesional skin, compared to weeks 2 and 12, showing a reduction in myeloid DCs and their products with etanercept treatment. (B) CD11c (green) and IL-20 (red); (C) CD11c (green) and IL-23/IL-12 p40 (red); (D) CD11c (red) and TNF (green). The white line identifies the dermal epidermal junction. Autofluorescent keratinocytes appear in all panels. Size bar = 100 μ m.



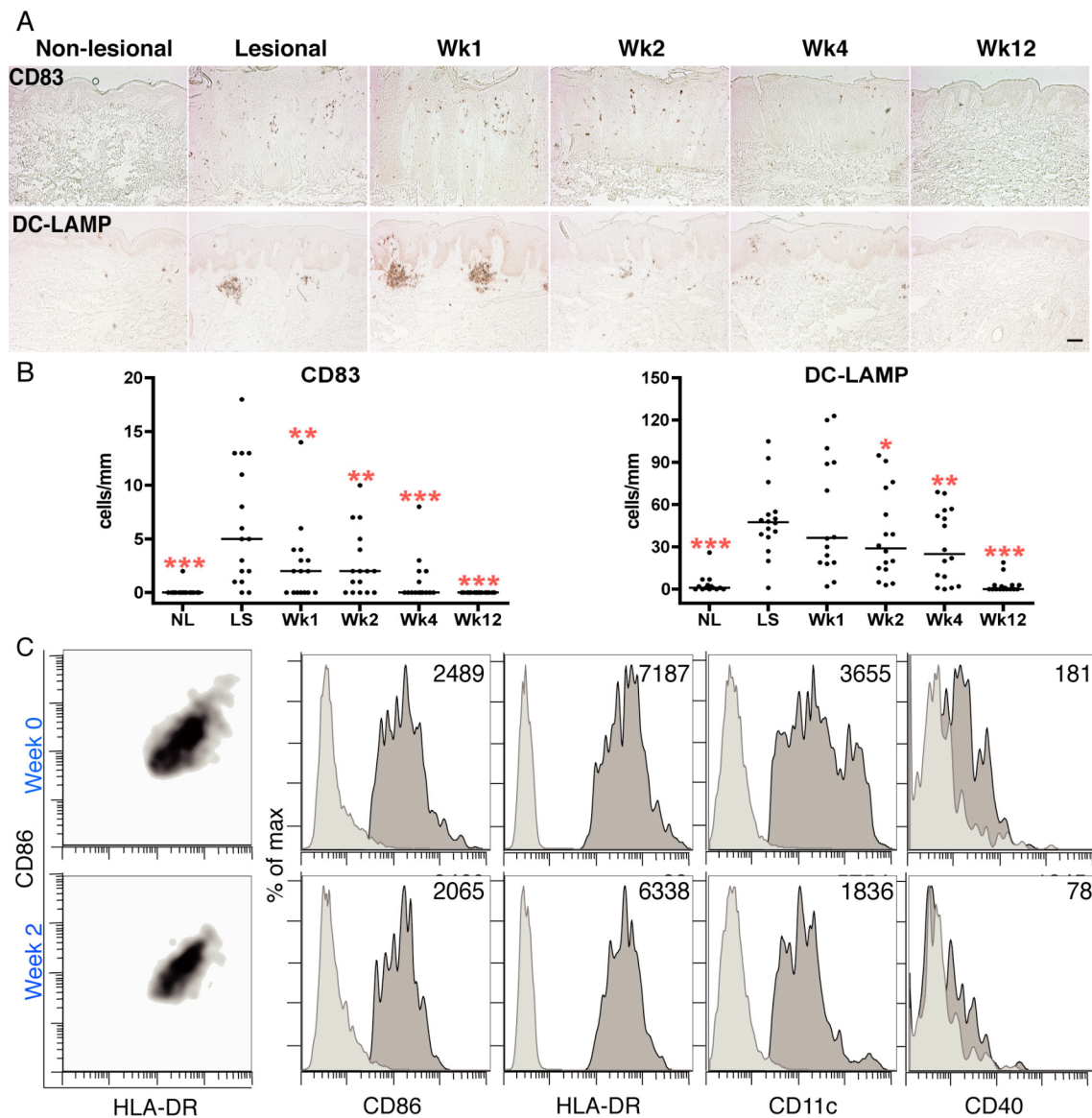
Myeloid DCs in the skin down regulated maturation markers by week 2 of etanercept treatment

Single antigens specific for mature DC identification include CD83 and/or DC-LAMP. In responding patients, CD83⁺ DCs were scattered throughout psoriatic epidermis and upper-dermis, while DC-LAMP⁺ DCs aggregated together in clusters in the upper reticular dermis (Figure 24A). CD83 and DC-LAMP were significantly decreased by weeks 1 and 2 of etanercept treatment ($p < 0.01$ and $p < 0.05$, respectively) (Figure 24B). The larger average number of CD11c⁺ myeloid cells in lesional skin (247 cells/mm, standard error of the mean (SEM) = 31.9, Figure 20C) compared to CD83⁺ (9 cells/mm, SEM=3.0, Figure 24B) and DC-LAMP⁺ DCs (49 cells/mm, SEM=6.8, Figure 24B) suggests that mature DCs were a subset of lesional DC infiltrate.

Maturation of migrant dermal DCs as measured by levels of surface co-stimulatory molecules, was also decreased by week 2 of etanercept treatment (Figure 24C). Using FACS analysis, we gated on cells that met the classic definition of DCs (Lin⁻CD11c⁺HLA-DR⁺) and determined the expression levels of CD86, HLA-DR, CD40, and CD11c on cells emigrating from the dermis at baseline and week 2 (n=5 patients). At baseline (week 0) there was a subset of CD86^{hi}HLA-DR^{hi} cells that was not present after 2 weeks of etanercept treatment. Mean fluorescence intensity (MFI) of DC activation markers CD86, HLA-DR, and CD40 decreased in all week 2 samples (representative patient shown in Figure 24C). Myeloid lineage marker CD11c was decreased in 3

samples and increased in 2 samples. Cell size (FSC-H) and cell complexity (SSC-A) decreased in all samples (data not shown). Thus, myeloid dermal DC activation (CD86, HLA-DR, CD40) and cell size/complexity are reduced by week 2 of etanercept treatment compared to baseline.

Figure 24. DCs down regulate maturation and co-stimulatory molecules with etanercept treatment. (A) Immunohistochemistry for mature DC markers CD83 and DC-LAMP in non-lesional skin (NL), lesional skin (LS), and in the lesional index plaque at weeks 1, 2, 4, and 12. Size bar = 100 μ m. (B) Quantification of CD83⁺ and DC-LAMP⁺ cells per mm (n=16) during etanercept treatment. Baseline lesional values compared with other time points, p<0.05 (*), p<0.01 (**), and p<0.001 (***). (C) FACS analysis at baseline (week 0) and matched week 2 etanercept-treated lesional dermal single cell suspensions. Acquired cells gated on myeloid DCs (Lin⁻ HLA-DR⁺ CD11c⁺). MFI is indicated in upper right corner of each histogram, isotypes shown in light grey.



Etanercept blocked in vitro derived DC maturation and IL-23 production, immunostimulatory capacity, and shifted differentiation towards a macrophage-like phenotype

Monocyte derived DCs cultured with etanercept decreased CD86 expression 3-fold and HLA-DR expression 5-fold (Figure 25A). CD11c expression decreased slightly, as did cell granularity (SSC-A). RT-PCR on three paired biological replicates showed a significant decrease in IL-23 subunits p19 and p40 ($p=0.02$ and $p=0.05$, respectively) but there was no significant decrease in IL-12 subunit p35 ($p=0.25$) (Figure 26). Likewise, IL-6 was down regulated ($p=0.04$) while TGF β 1 was up regulated ($p=0.05$). MoDCs cultured with etanercept were also an average of 2-3 fold less stimulatory than control DCs in a mixed leukocyte reaction (MLR) ($n=2$). Etanercept did not affect stimulation of T cells alone or T cells stimulated with CD3/CD28 beads (Figure 25B).

Gene array on control monocyte-derived DCs compared to those cultured with etanercept revealed that CD163, a macrophage scavenger receptor, was strongly up regulated (6.5-fold increase, $p<0.05$) (Figure 25C). We confirmed these results using FACS analysis, and identified upregulation of CD163 protein on CD11c⁺ cells matured with etanercept compared with control DCs (Figure 25D). Etanercept had no significant effect on expression of Th1 (IFN- γ) or Th17 (IL-17 and IL-22) cytokine mRNAs in activated T cells with or without etanercept ($n=3$) (data not shown).

The small number of non-responders in this trial ($n=4$) limits statistical comparison with responders ($n=16$). However, for interest, we have included data from

non-responders in Figure 27. Of note, IL-17 response genes CCL20 and DEFB4 are not downmodulated as rapidly or consistently in non-responders (Figure 27C) as they are in responders (Figure 21D). Reactive epidermal hyperplasia is also not suppressed to the same extent as in responders.

Figure 25. In vitro monocyte derived DCs generated in the presence of etanercept are less mature, less immunostimulatory, and express macrophage antigen CD163. (A) FACS analysis of monocyte derived DCs generated without or with etanercept. Acquired cells gated on myeloid DCs ($\text{Lin}^- \text{HLA-DR}^+ \text{CD11c}^+$). MFI is indicated in upper right corner of each histogram. Isotypes shown in light grey. (B) Mixed leukocyte reaction comparing monocyte derived DCs matured with and without etanercept (T cells + iDC). “T cells alone” and “T cells +CD3/28” serve as negative and positive controls respectively. Percent proliferation is indicated in lower left corner of each FACS plot. (C) Comparison of CD163 mRNA expression (gene array) in monocyte derived DCs generated without (blue) or with (red) etanercept, $p < 0.05$ (). (D) Increased surface expression of CD163 on monocyte derived DCs generated with etanercept confirmed by flow cytometry.*

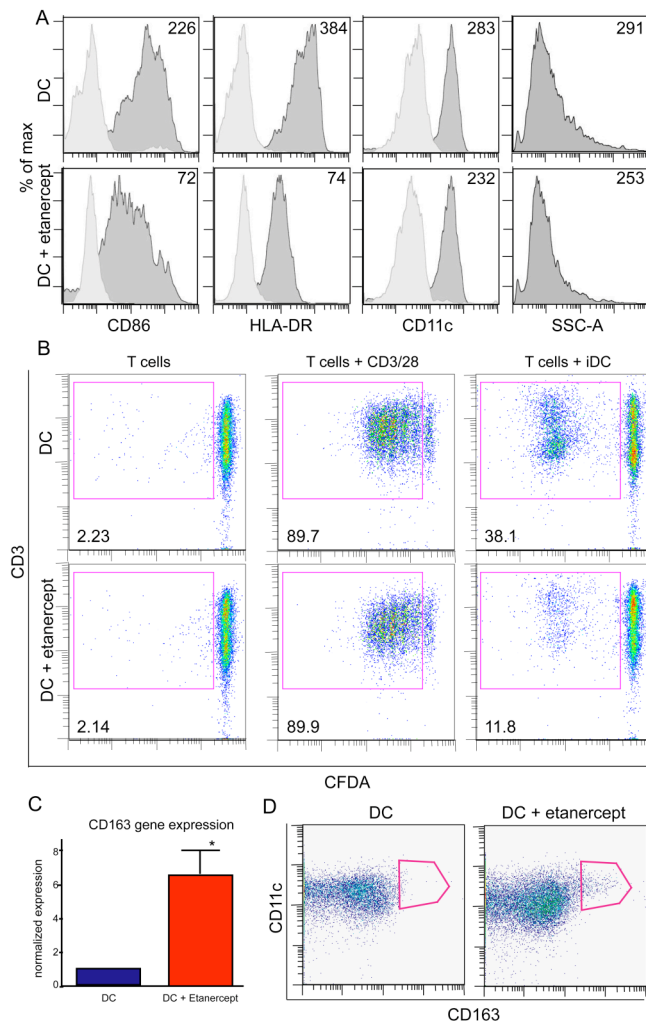


Figure 26. *In vitro* derived DCs matured with etanercept produce less IL-23 and are less immunostimulatory than control DCs. mRNA expression normalized to HARP for p19, p40, p35, IL-6, and TGF β *in vitro* derived DCs matured without (control) and with etanercept (+ etanercept).

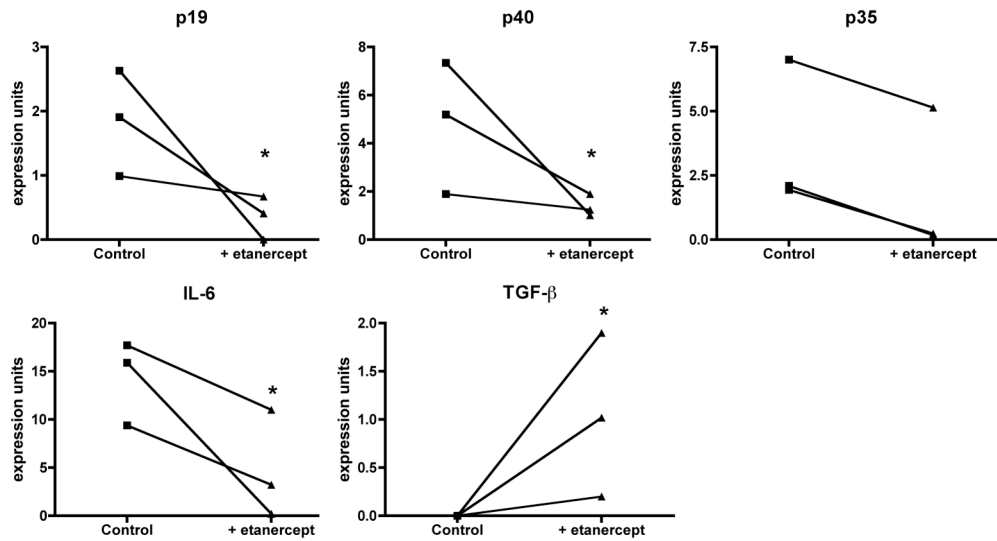
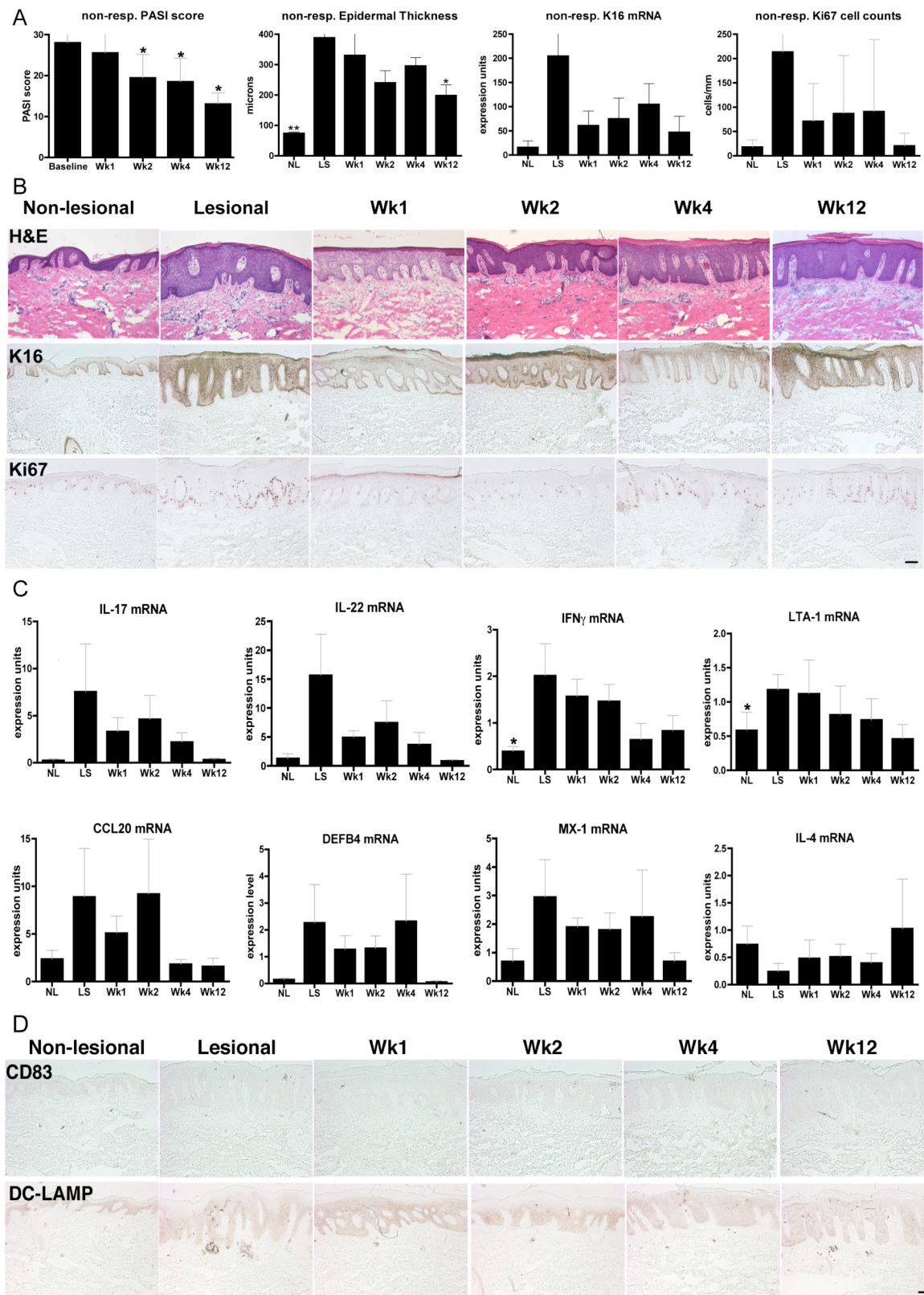


Figure 27. Non-responder data. (A) Average PASI scores, epidermal thickness, keratin 16 (K16) mRNA expression, and Ki67 cell counts in non-responders (n=4) during treatment with etanercept. Clinical response measured at baseline, wks 1, 2, 4 and 12; biopsies evaluated in non-lesional skin (NL), lesional skin (LS), and in the lesional index plaque at weeks 1, 2, 4, and 12. Baseline lesional values compared with other time points, $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***). (B) Histology and immunohistochemistry showing H&E, K16, and Ki67 expression during treatment. Size bar = 100 μ m. (C) mRNA expression normalized to HARP for Th17 cell products IL-17 and IL-22, Th1 cell products IFN γ and LTA-1, downstream effectors of Th17 cells, CCL20 and DEFB4 (b-defensin), downstream effector of Th1 cells MX-1, and Th2 cell product IL-4. All mRNA evaluated in non-lesional skin (NL), lesional skin (LS), and in the lesional index plaque at weeks 1, 2, 4, and 12. Baseline lesional values compared with other time points, $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***). (D) Immunohistochemistry for mature DC markers CD83 and DC-LAMP in non-lesional skin (NL), lesional skin (LS), and in the lesional index plaque at weeks 1, 2, 4, and 12. Size bar = 100 μ m.



Discussion

This study contains new information that informs on two separate but related topics: therapeutic mechanisms of the TNF inhibitor etanercept, and the network of inflammatory cytokines and leukocytes that drive psoriasis pathogenesis. Presently, there are three TNF inhibitors in widespread use for treatment of psoriasis, psoriatic arthritis, rheumatoid arthritis, inflammatory bowel disease and ankylosing spondylitis – Infliximab and adalimumab are monoclonal TNF antibodies, and etanercept is a dimeric TNFRII Fc fusion protein (Atzeni *et al.*, 2005; Scott and Kingsley, 2006). While often considered as a therapeutic class, these agents are structurally different, have different affinities for TNF, and are not uniformly effective for all inflammatory diseases (Furst *et al.*, 2006). Although over one million patients have been treated with these drugs, there is surprisingly little data on therapeutic mechanisms in human inflammatory diseases. In this paper, we show that psoriasis disease improvement correlated with early reduction in DC and Th17 cell products and downstream effector molecules, and final disease resolution correlated with late down modulation of Th1 cells.

When considering previous research on TNF inhibitor mechanism, it is useful to divide response into early (hours to days) versus late (weeks to months) effects. In the case of infliximab and adalimumab, there are reports suggesting that broad apoptosis of inflammatory leukocytes is induced within hours of drug delivery (Lugering *et al.*, 2001; Shen *et al.*, 2005). With these agents, reduction of cytokine-driven inflammation is likely a combination of inhibition of TNF-dependent cytokine production, as well as reducing cytokine-producing cells via apoptosis. Early apoptosis, however, is not a feature of

etanercept treatment. Studies on psoriasis lesions show some leukocyte apoptosis after 1 month of treatment (Malaviya *et al.*, 2006), suggesting that apoptosis is a secondary mechanism following growth factor/TNF withdrawal.

In this paper we propose that an early mechanism of etanercept is to inhibit inflammatory DC cytokine production and maturation, leading to reduction in activity of Th17 T cells. Recently, a new type of inflammatory myeloid CD11c⁺ DC was described in psoriasis, the Tip-DC (Lowe *et al.*, 2005a). This cell type was first identified in a murine model of innate immune response to listeria infection (Serbina *et al.*, 2003). In a previous clinical trial using etanercept, iNOS mRNA and protein, along with various other DC and T cell inflammatory cytokines and chemokines, were decreased by 1 month treatment (the earliest time point in that study) (Gottlieb *et al.*, 2005). Our current study uses even earlier time points to recreate the hierarchy of TNF dependent mediators, and separate primary (early) versus secondary (late) response. We now show that multiple inflammatory products of TipDCs, including iNOS, TNF, IL-20, and IL23 p40 subunit, are reduced within 1-2 weeks after beginning etanercept, while the number of CD11c⁺ DCs in the tissue is minimally affected during this time, suggesting initial blockade of cytokine production by these cells rather than cell reduction. This suggests that TNF is an autocrine or paracrine inducer of TipDC inflammatory products, which is blocked by etanercept. This direct effect on DCs is supported by our in vitro studies with monocyte-derived DCs showing that etanercept blocked up regulation of co-stimulatory and MHC-II molecules, IL-23 production, and immunostimulatory capacity.

The early modulation of Tip-DCs by etanercept may rapidly impact Th17 cells, beginning the process of molecular resolution before reduction in cellular infiltrates and long before clinical resolution. Our proposed psoriatic inflammatory pathway involves the production of IL-23 from these inflammatory Tip-DCs causing proliferation of Th17 cells, with subsequent induction of IL-17, IL-22, and other products (Figure 36). IL-17 appears to serve as an inducer of keratinocytes to produce antimicrobial peptides like β -defensin 4, S100 acute phase proteins, and chemokines such as IL-8 (Kao *et al.*, 2004). Models of psoriasis suggest that IL-22 strongly induces keratinocyte hyperplasia, and mediates IL-23 induced dermal inflammation and acanthosis (Zheng *et al.*, 2007). All of these products were down modulated within 1-2 weeks of etanercept treatment. The involvement of Th17 cells in psoriasis may now help explain the following: hyperplasia of psoriatic keratinocytes (IL-22); why psoriatics are relatively protected from bacterial infection (defensins); and why neutrophils that are normally reserved for acute inflammatory processes appear in a chronic inflammatory disease (IL-8). Moreover, histological resolution of the disease, as defined by decreased epidermal thickness and normalization of keratinocyte proliferation (Ki67) and differentiation (K16), correlates with rapidly decreased Tip-DC and Th17 products. Thus, these results suggest that Th17 cells are important for disease pathogenesis, and may be modified by etanercept at an early time point.

Finally, while there is an emerging role for Th17 driving inflammation in psoriasis, Th1 cells may still be important for final disease resolution. While Tip-DC and Th17 products are down modulated within 2 weeks of etanercept treatment, IFN γ is not

decreased until week 12, and STAT-1 (an IFN γ -dependent transcription factor) is not significantly decreased until several months of treatment (Gottlieb *et al.*, 2005). So although histological disease resolution begins within weeks, complete remission does not occur until several months of treatment when both Th17 and Th1 products have been down modulated. IFN γ is a major inducer of MHC-II, and acts synergistically with IL-17 to upregulate keratinocyte ICAM-1 adhesion molecules and IL-8 production (Teunissen *et al.*, 1998), suggesting that Th1 cells may be important for leukocyte activation. Activated T cells are required in the epidermis for psoriasis to develop (Conrad *et al.*, 2007; Conrad *et al.*, 2005), and most epidermal T cells are Type 1 CD8⁺ cells (Gudjonsson *et al.*, 2004); it follows that they must be ablated for disease resolution. Thus, while Th17 cells may be the major drivers of keratinocyte hyperplasia and inflammatory cytokine production, Th1 cells may be important for leukocyte activation and for sustaining a network of over 100 genes linked to IFN γ signaling (Lew *et al.*, 2004b). In addition, there may be important functional interactions between Th17 and Th1 T cells, as cross-regulation has been recently demonstrated in model systems (Nakae *et al.*, 2007; Park *et al.*, 2005). More work needs to be done to delineate the specific roles of Th17 and Th1 cells in psoriasis, and in other examples of autoimmune inflammation.

CHAPTER 6: Global characterization of genes modulated by etanercept

In the previous chapter we used RT-PCR as a genomic tool for probing specific gene products we hypothesized were modulated by etanercept. In this chapter we use gene array as a tool for probing the genomic profile of etanercept modulated genes in an unbiased way. Using gene array we confirm our initial hypothesis that IL-17 and TNF pathways are rapidly downregulated with etanercept treatment and IFN γ is downregulated later during final disease resolution. We also show that myeloid genes are the first genes modulated by blocking TNF, and that T cell genes are affected secondarily. We propose that the close association of downregulated TNF and IL-17 pathways is through inhibition of DC-dependent IL-23 production.

Gene array analysis on skin samples from the etanercept clinical trial

Comparison of “psoriasis genes” identified using the newer Affymetrix U133A2.0 platform with previously published gene lists using the Affymetrix U95 platform

Previous work that used gene array technology to identify transcripts that were differentially expressed among psoriasis lesional and non-lesional paired samples was done on the 63,100-element Affymetrix U95A, B, C, D and E platform (Zhou *et al.*, 2003). The samples in this trial were interrogated using the 22,215-element Affymetrix U133A2.0 platform. On the newer U133A2.0 platform, we found that 599 genes (728 probesets) were upregulated 2-fold or more in lesional skin compared to non-lesional skin, and 732 genes (890 probesets) were downregulated to this extent. Overall, the U133A2.0 platform detected more genes differentially expressed than the previous

studies using U95 array sets, despite our using stricter statistical criteria to detect differences.

Cluster analysis on genes modulated by etanercept

We first identified genes that were significantly different from lesional baseline at any time during etanercept treatment (FDR<0.05 and 2-fold change), and found that etanercept downregulated 978 probesets and upregulated 999 probesets.

For cluster analysis on genes modulated by etanercept, we clustered time profiles for responders using Consensus Clustering method to determine the optimal number of clusters in the hierarchical clustering algorithm. Consensus Clustering produces the Consensus Matrix and the delta area curve for each scenario of user defined number of clusters (k) in each gene set (Swift *et al.*, 2004).

In the case of genes downmodulated by etanercept, Consensus Clustering identified a scenario with a high delta area for either 3 or 4 clusters (k=3 or k=4) (Figure 28A). Visualization of the Consensus Matrix showed more defined clustering for k=4 (Figure 28B). The average gene expression profile in each cluster at each time point is visualized in Figure 28C (left) with corresponding heatmap (right). Cluster #1 containing 31 probesets was defined as “down early,” Cluster #2 with 168 probesets was “down mid,” Cluster #3 with 616 probesets was “down late,” and cluster #4 with 163 probesets was “down latest”. The relative velocity of gene downmodulation is visualized in each cluster graph by the dotted red line, marking the time taken to achieve 1/2 maximal gene expression ($t_{1/2}$). “Down early” genes achieved $t_{1/2}$ between weeks 0 and 1 of etanercept treatment, “down mid” $t_{1/2}$ fell between weeks 1 and 2, “down late” was between weeks 2

and 4, and “down latest” was between weeks 4 and 12. Downregulated genes from clusters 2-4 are listed in Supplementary Table 2, and “down early” cluster #1 genes with corresponding heatmap is shown in Figure 2a.

Etanercept upregulated genes clustered into three groups (Figure 29A, B). Cluster #1 genes (23 probesets) were upregulated “early”, cluster #2 genes (43 probesets) were upregulated “mid,” and cluster #3 genes (933 probesets) were upregulated “late” (Figure 29C, D).

Figure 28. Gene array analysis groups genes down-modulated by etanercept into 4 clusters: down early, down mid, down late, and down latest. Consensus Clustering on genes downmodulated with etanercept produced a delta curve (A) and Consensus Matrix (B) that identify 4 clusters (C). Red dashed line denotes time taken to achieve 1/2 maximal gene expression. Corresponding heat map for each cluster is to the right.

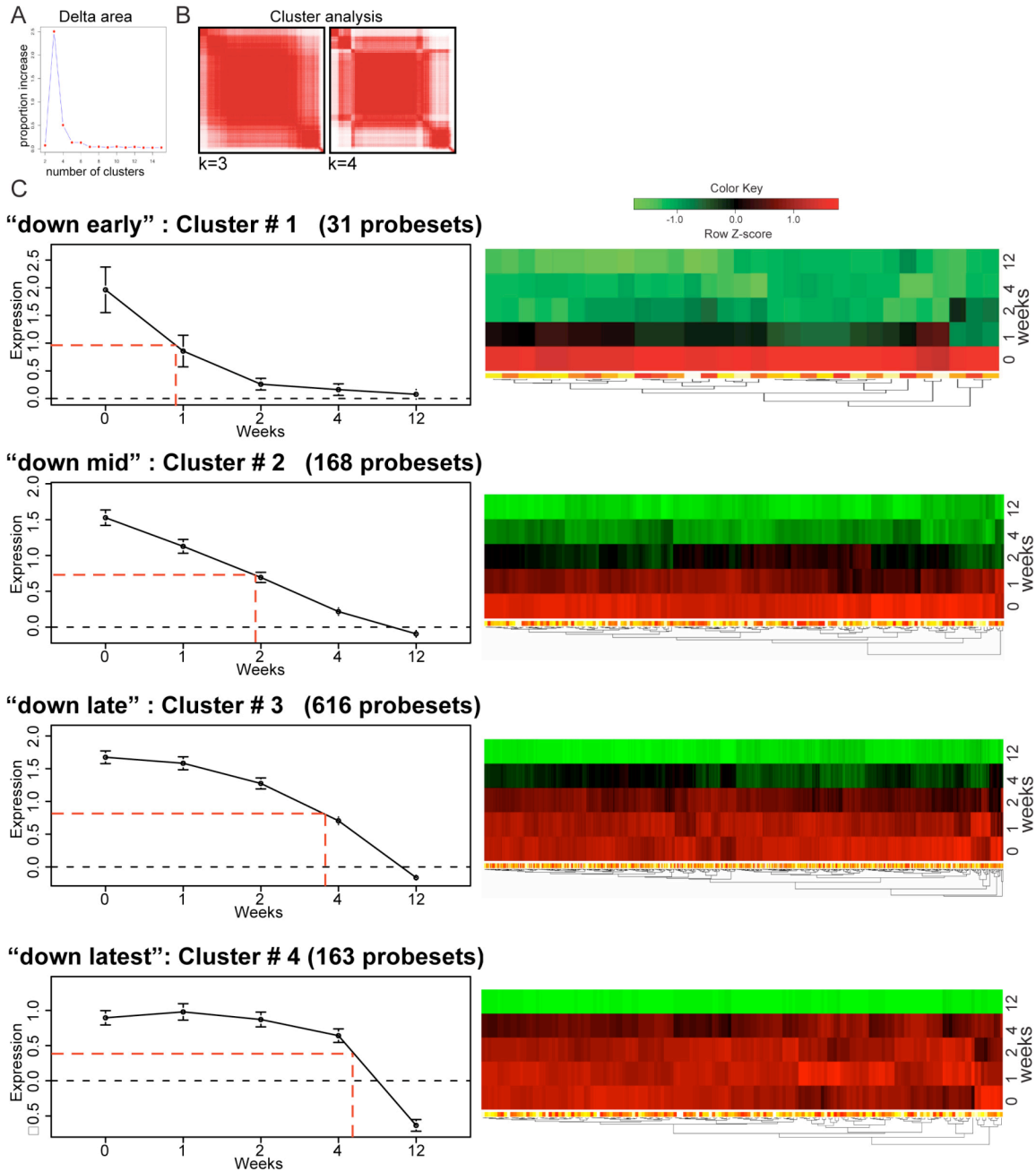
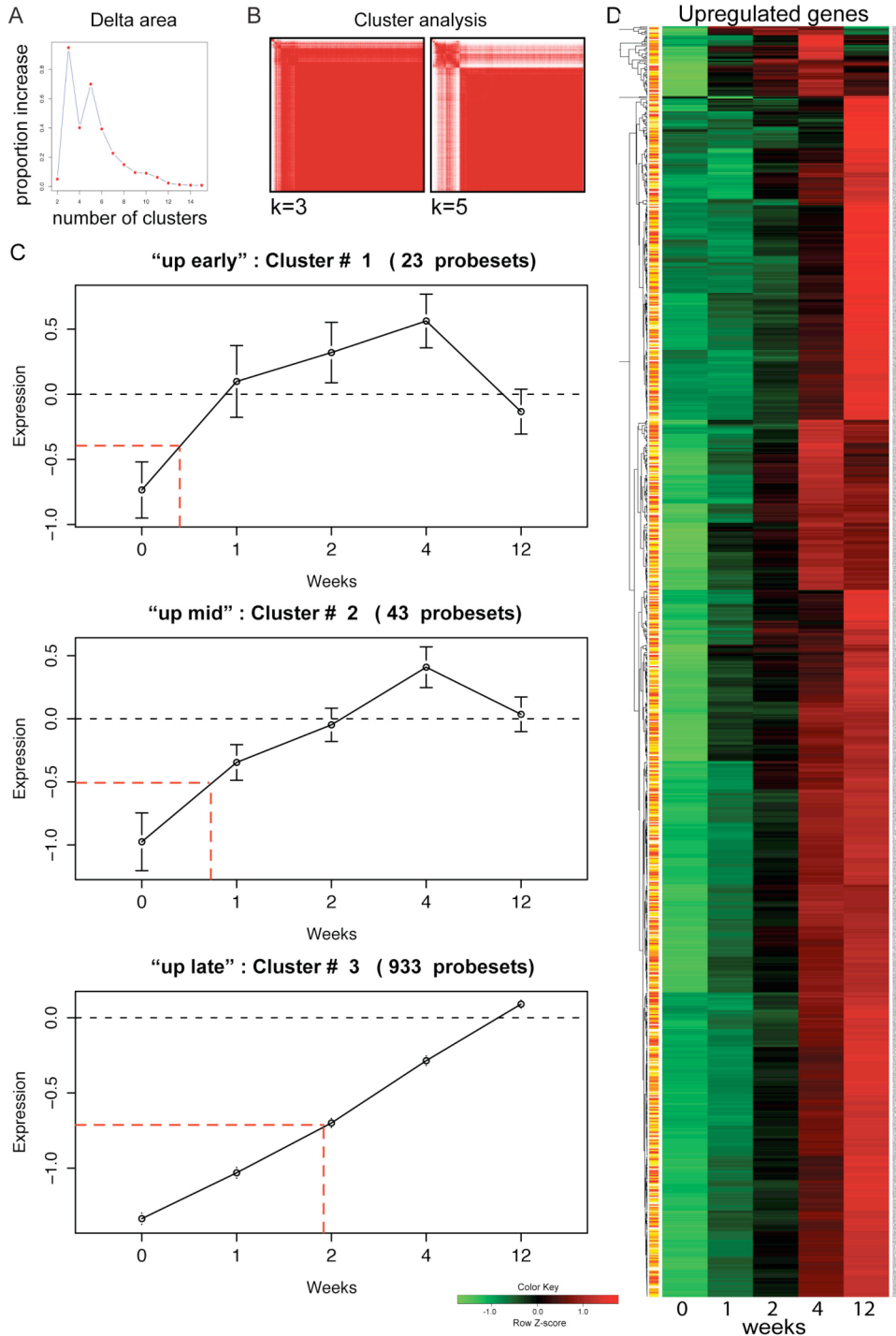


Figure 29. Gene array analysis groups genes upregulated by etanercept into three clusters: up early, up mid, and up late. Consensus Clustering on genes upregulated with etanercept produced a delta curve (A) and Consensus Matrix (B) that identify 3 clusters (k=3) as the best clustering model (C). Red dashed line denotes time taken to achieve 1/2 maximal gene expression. Corresponding heat map for all upregulated genes (D).



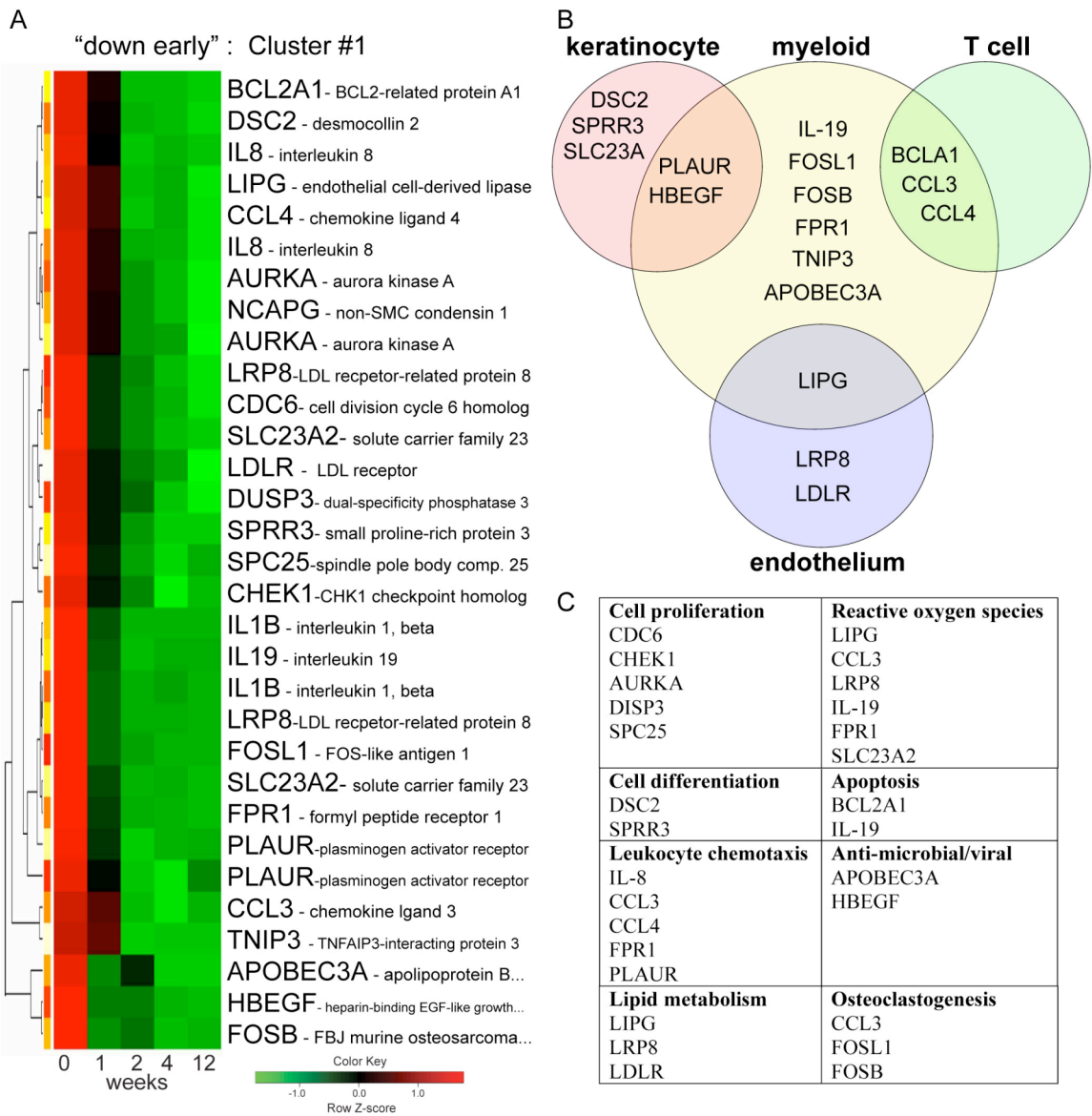
Genes rapidly downmodulated during etanercept treatment

To identify which cell types are rapidly modulated by TNF inhibition, we grouped “down early” cluster #1 (Figure 30A) genes according to the cell lineage(s) in which the genes are expressed (Figure 30B). These “down early” genes were mostly products of activated myeloid cells (monocytes, DCs, macrophages, or osteoclasts), but were also expressed by T cells, endothelium, keratinocytes, or were ubiquitously expressed. As previously reported, cutaneous CD11c⁺ cell counts remain constant during the first week of etanercept treatment (Zaba *et al.*, 2007a), suggesting that rapidly decreased myeloid gene expression was secondary to cell inhibition, not decreased cell number. In summary, these data suggest that myeloid cell inhibition is the earliest target for TNF inhibitors.

Mechanisms of TNF inhibition were further elucidated by classifying “down early” genes according to gene function (Figure 30C). Genes regulating cell proliferation/ mitosis rapidly downmodulated with etanercept were CDC6, CHEK1, AURKA, DUSP3, and SPC25. Keratinocyte differentiation genes were DSC2 and SPRR3; Leukocyte chemotaxis genes were IL8, CCL4, CCL3, FPR1, PLAUR, and LIPG; Lipid metabolism genes were LIPG, LRP8, and LDLR; Reactive oxygen species modulating genes were LIPG, CCL3, LRP8, IL-19, and FPR1 and SLC23A2; Pro and anti-apoptotic genes were IL-19 and BCL2A1 respectively; Anti-microbial / anti-viral genes were HBEGF and APOBEC3A; and osteoclastogenesis promoting genes were CCL3, FOSL1, and FOSB. Known NFκB-dependent genes include BCL2A1, IL-8, IL-

1b, FPR1, TNIP3, LIPG, SPRR3, and HBEGF, although it can be argued that all genes rapidly downmodulated by TNF inhibition are likely downstream of NF κ B. Implications of these rapidly downmodulated genes for psoriasis disease pathogenesis are further elucidated in the discussion of this thesis.

Figure 30. Genes downmodulated rapidly by etanercept. 27 genes (31 probesets) were rapidly downmodulated with etanercept treatment (A). Most of these genes were produced by myeloid lineage cells (B). Primary function of rapidly downmodulated genes are listed (C).



Gene set enrichment analysis on genes downmodulated by etanercept

After establishing myeloid cell inactivation as a likely primary target of TNF inhibition, we used Gene Set Enrichment Analysis (GSEA) to identify secondary and tertiary effects of etanercept (Table 3). GSEA confirmed that “down early” cluster #1 was enriched with TLR/ NF κ B signaling pathway activation genes and cytokine-cytokine receptor interactions. Clusters #2 and #3 were enriched with cell cycle genes, p53 signaling pathway genes, pyrimidine metabolism, folate biosynthesis, amino acid metabolism, epithelial cell signaling, and starch/sucrose metabolism genes – all indicators of decreased rate of keratinocyte proliferation. “Down latest” cluster #4 was again enriched with TLR/ NF κ B signaling pathway genes during final disease resolution. Therefore, etanercept directly decreased TNF-dependent myeloid cytokine/chemokine production, secondarily decreased keratinocyte proliferation/differentiation, and lastly decreased leukocyte activation even more extensively during final disease resolution.

Table 3. Gene set enrichment analysis on genes downmodulated by etanercept. Gene Set Enrichment Analysis on downregulated genes to reveal over-represented pathways within each cluster.

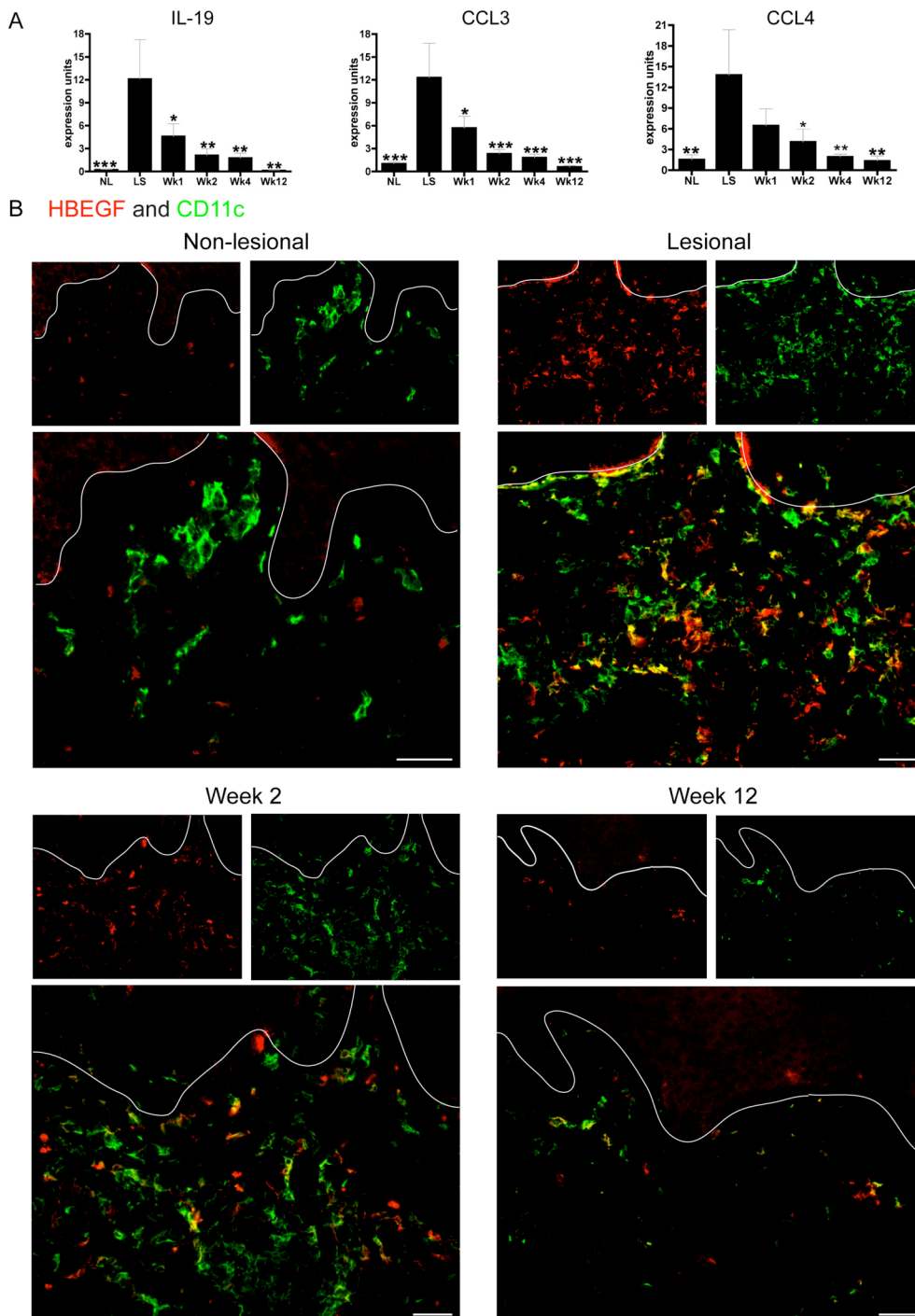
KEGGID	Pathway	p-value	odds ratio	cluster
4620	TLR signaling pathway	6×10^{-4}	13.2	1
4060	Cytokine-cytokine receptor interaction	2×10^{-3}	7.2	1
4110	Cell cycle	6×10^{-6}	7.3	2
4115	p53 signaling pathway	5×10^{-4}	6.8	2
240	Pyrimidine metabolism	6×10^{-3}	4.8	2
860	Porphyrin and chlorophyll metabolism	2×10^{-3}	5.1	3
790	Folate biosynthesis	6×10^{-3}	4.1	3
220	Urea cycle and metabolism of amino groups	7×10^{-3}	4.8	3
5120	Epithelial cell signaling in Helicobacter pylori infection	8×10^{-3}	3.1	3
500	Starch and sucrose metabolism	0.01	2.9	3
4620	TLR signaling pathway	0.01	4.1	4

Confirmation of gene array results with RT-PCR and immunofluorescence

Rapidly downmodulated genes were confirmed using RT-PCR and immunofluorescence (Figure 31). “Down early” cluster #1 chemokines IL-19, CCL3 (MIP1 α), and CCL4 (MIP1 β) expression was decreased by 62%, 54%, and 50% ($p < 0.05$, $p < 0.05$, $p = 0.055$, respectively) after 1 week of etanercept treatment (Figure 31A).

Another “down early” cluster #1 gene, heparin-binding epidermal growth factor-like protein (HBEGF), was a rapidly downmodulated myeloid cell product (Figure 31B). Using image-J we measured red (HBEGF), green (CD11c), and red+green (yellow) pixel area normalized to dermal area in non-lesional, lesional, week 2, and week 12 double-labeled immunofluorescence images. Using this digital analysis technique we compared relative HBEGF and CD11c expression as well as overlap (CD11c⁺ cells expressing HBEGF). After 2 weeks of etanercept treatment, there is a 70.0% reduction in CD11c⁺ cells that express HBEGF and a 58.3% reduction in total HBEGF production compared to lesional baseline. HBEGF is implicated in promotion of antimicrobial expression in keratinocytes by binding to the EGF receptor, upregulating defensin β -103A (formerly DEFB3) and lipocalin. Thus, both RT-PCR and immunofluorescence confirmed rapid downregulation of gene array-defined cluster #1 genes.

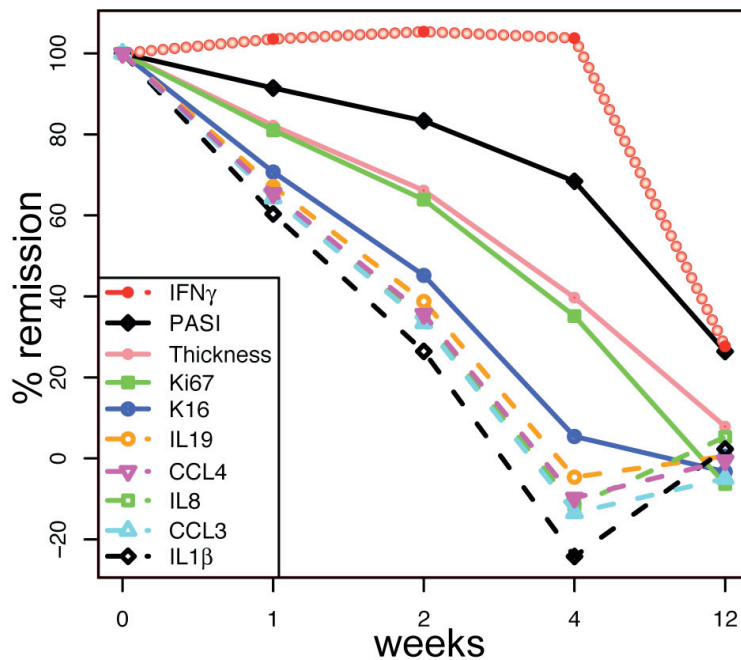
Figure 31. Confirmation of gene array results with RT-PCR and immunofluorescence. RT-PCR on IL-19, CCL3, and CCL4 cytokines show downregulation by week 1 of etanercept treatment (A) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (p-value of CCL4 at week 1 = 0.055). Double label immunofluorescence shows rapid downregulation of HBEGF (red) and CD11c (green) after 2 weeks etanercept treatment (B). Size bar = 100 μ m



Comparison of early and late response genes with clinical markers of disease resolution

Cluster #1 etanercept early response cytokines confirmed by RT-PCR include IL-19, CCL3, and CCL4 (Figure 31A) as well as IL-8 and IL-1 β which were also rapidly downregulated by week 1 as previously reported ($p < 0.01$ and $p < 0.05$, respectively) (Zaba *et al.*, 2007a). To understand how early response gene downregulation may impact clinical phenotype and histological disease resolution, we graphed early response genes (Figure 32, dashed lines) and clinical disease markers (Figure 32, solid lines) by percent remission. As a point of comparison, we included RT-PCR data from known etanercept late response gene IFN γ (Figure 32, dotted red line). The four clinical/histological disease markers we examined were PASI, epidermal thickness, Ki67 cell counts, and K16 gene expression: PASI is a global measurement of % body mass covered with psoriasis plaque, epidermal thickness is a linear measurement (in mm) on H&E sections, Ki67 is a marker of cell proliferation, and K16 gene expression indicates aberrant keratinocyte maturation. K16 expression was rapidly downregulated along with etanercept early response genes, suggesting that aberrant epidermal differentiation was caused by TNF directly or by TNF dependent early response genes. In contrast, both markers of keratinocyte proliferation (Ki67 and epidermal thickness) were downregulated later, a result that was supported by Gene Set Enrichment Analysis (Table 3) in which keratinocyte proliferation genes were enriched in clusters 2 and 3. PASI was located in between keratinocyte proliferation markers and IFN γ etanercept late response gene, suggesting that global disease resolution depended on both early and late TNF response gene downregulation.

Figure 32. Comparison of early and late response genes with clinical markers of disease resolution. % remission is calculated as $\log_2(t / NL)$ where $t = 0, 1, 2, 4$, or 12 . Data for $IFN\gamma$, K16, IL-19, CCL4, IL-8, CCL3, and IL-1 β was obtained using RT-PCR. PASI is a clinical score of psoriasis disease severity, thickness is a measure (in mm) of histologic epidermal thickness, and Ki67 cell count/ mm^2 was obtained from immunohistochemistry on skin sections.



IL-17 and TNF response genes are downregulated more rapidly than IFN γ response genes during etanercept treatment

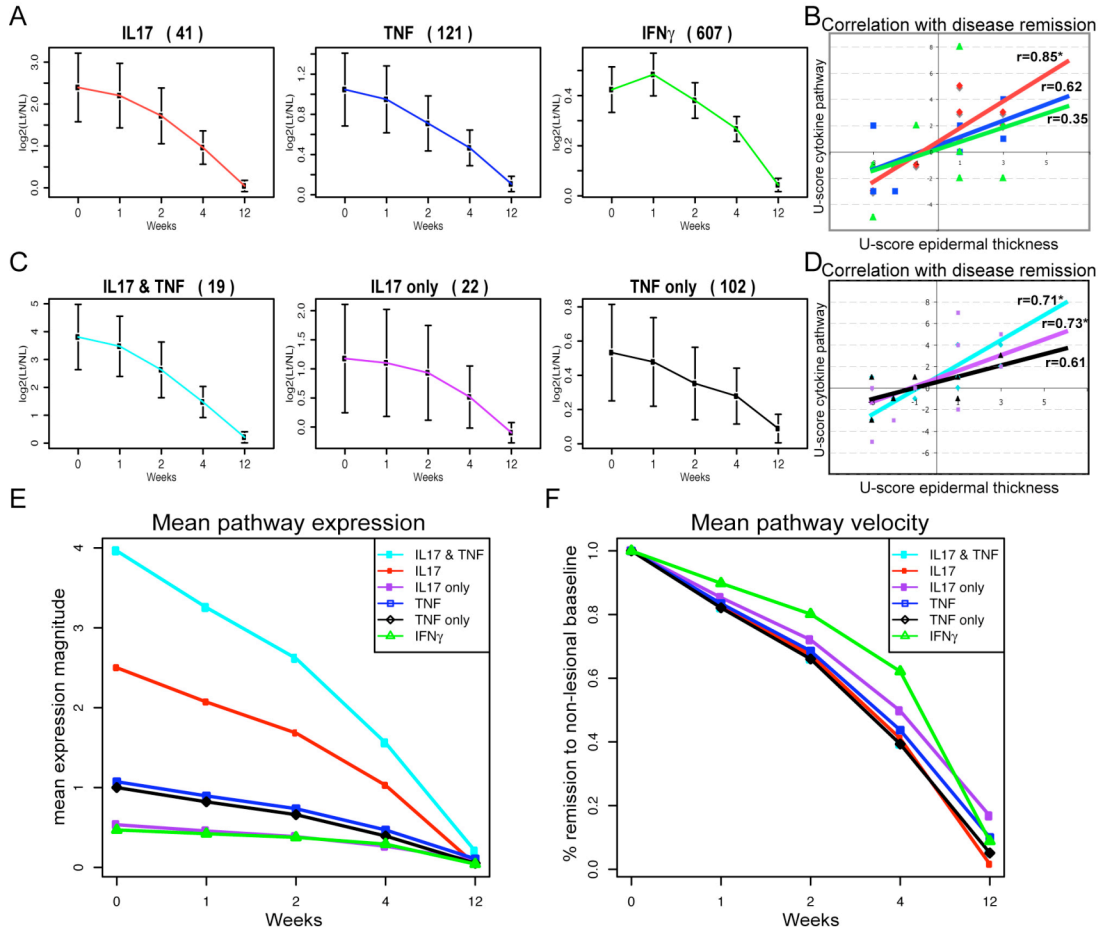
To assess the relative contribution of IL-17, TNF, and IFN γ response genes to etanercept-mediated disease resolution, we first defined a set of keratinocyte genes induced when cultured with each cytokine for 24 hours (n=3 for each condition) (Nogales et. al., submitted). IL-17 modulated 41 keratinocyte genes, TNF modulated 121 genes, and IFN γ modulated 607 genes. Each cytokine pathway gene set was then culled from the etanercept trial data set and normalized to baseline non-lesional expression over time (Figure 33A). IL-17 pathway genes were overexpressed in lesional biopsies and decreased rapidly with etanercept treatment. TNF pathway genes were not as highly expressed as IL-17 genes, but also decreased rapidly with etanercept treatment. In contrast, IFN γ modulated gene expression increased during the first of etanercept treatment, and was not significantly downmodulated until week 12. Downregulation of IL-17 pathway genes correlated significantly with decreased epidermal thickness, a histologic indicator of disease resolution ($r=0.85$, $p<0.05$) (Figure 33B). Neither TNF pathway nor IFN γ pathway gene modulation correlated significantly with decreased epidermal thickness ($r=0.62$ and $r=0.35$, respectively).

Intersection of IL-17 and TNF pathway gene lists revealed 19 commonly modulated genes, 22 unique “IL-17 only” genes, and 102 unique “TNF only” genes (Figure 33C). IL-17 and TNF common pathway genes were highly expressed in baseline lesional skin, more so than unique IL-17 or unique TNF genes (Figure 33C) suggesting cooperativity between IL-17 and TNF pathways. Correlation of each gene set with epidermal thickness (Figure 33D) showed significant correlation for cooperatively

regulated IL-17/TNF genes ($r=0.71$, $p<0.05$) and for uniquely IL-17 regulated genes ($r=0.73$, $p<0.05$), but not for uniquely TNF regulated genes ($r=0.61$, $p<0.05$).

All six pathway mean expression values are plotted in Figure 33E, demonstrating the relatively large downregulation of cooperative IL-17/TNF and IL-17 genes during etanercept treatment compared to genes in other pathways. To visualize relative velocity of pathway recovery to non-lesional expression levels, we plotted mean gene expression % remission over time (Figure 33F). As suggested by the mean expression curves in Figure 32, we found that all pathways had a similar velocity of recovery except for the IFN γ pathway, which was downregulated later. These results solidify the importance of IL-17 pathway downmodulation for etanercept-mediated psoriatic phenotype resolution.

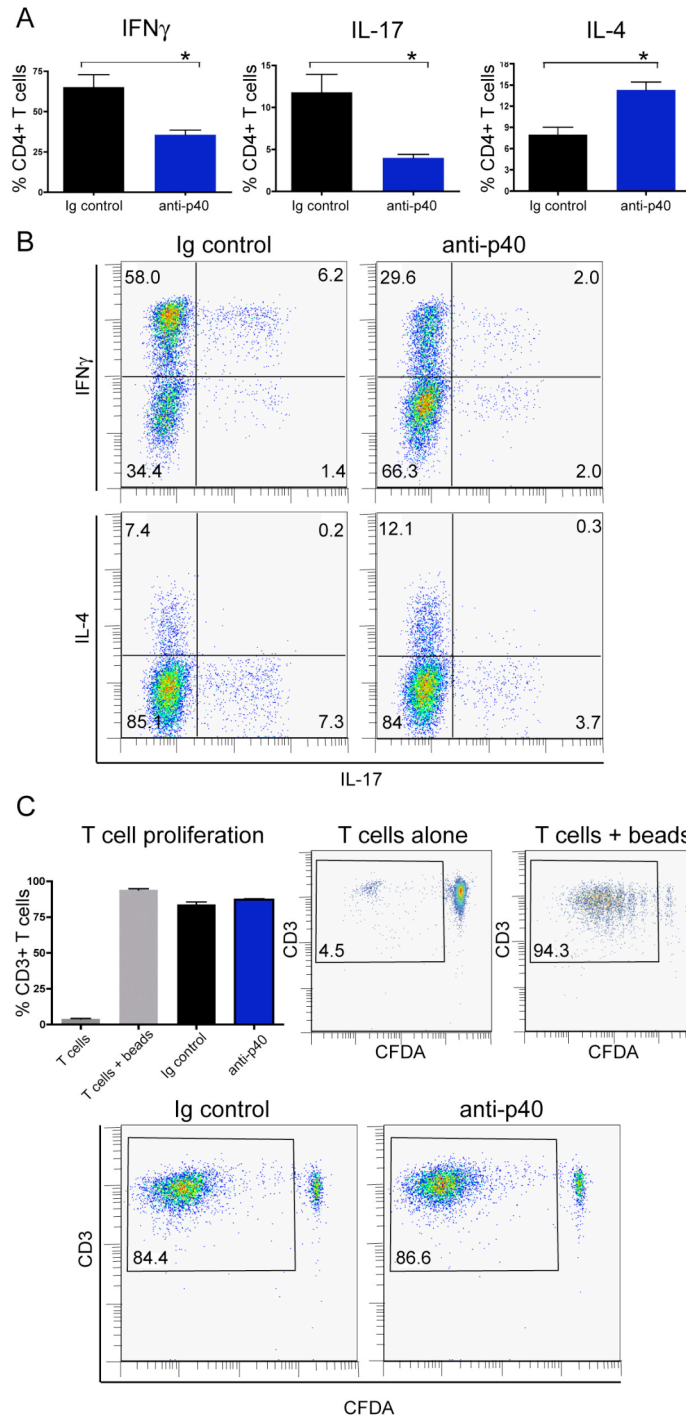
Figure 33. *IL-17 and TNF response genes are downregulated more rapidly than IFN γ response genes during etanercept treatment.* Mean expression of IL-17, TNF, IFN γ , IL-17 and TNF, IL-17 only, and TNF only pathway genes in patients treated with etanercept over time (A,C,E). U-score correlation of pathway data and epidermal thickness (a proxy for disease severity) (B,D). Velocity of pathway modulation over time (F).



Functional link between TNF inhibition and Th17 downmodulation

Previous work established that p40 IL-23/IL-12 subunit, but not p35 IL-12 subunit, is a TNF early response gene produced by activated dendritic cells (Zaba *et al.*, 2007a). As IL-23 supports the differentiation and survival of IL-17 producing Th17 cells, it is likely that TNF modulates Th17 cells indirectly through p40 expression – a hypothesis supported by rapid downmodulation of IL-17 pathway genes with TNF inhibition (Figure 33A). To further explore the link between TNF inhibition and rapid downmodulation of Th17 pathway genes, we performed an allo-MLR mixing psoriatic dermal émigrés (n=3) with donor T cells \pm p40 neutralizing antibody (Figure 34). p40 neutralization significantly decreased CD4⁺ T cell IFN γ production by 46% (p<0.05), and IL-17 production by 66% (p<0.05), with a reciprocal 44% upregulation of Th2 cytokine IL-4 (p<0.05) (Figure 34A). A representative patient's FACS plots are shown in Figure 34B. Neutralization of p40 did not; however, inhibit T cell proliferation (Figure 34C), confirming that downregulation of cytokine production was not due to decreased cell viability. These results provide functional validation for the link between TNF inhibition and rapid downregulation of Th17 adaptive immunity.

Figure 34. Functional link between TNF inhibition and Th17 downmodulation. MLR using psoriatic dermal émigrés and donor T cells with or without p40 neutralizing antibody. Intracellular cytokine staining determines effects of p40 blockade on CD4⁺ T cell IFN γ , IL-17, and IL-4 production (A,B). CFDA labeling measures T cell proliferation with and without p40 neutralization (C).



Differences between patients who responded to etanercept treatment and those who did not

Applying consensus clustering to non-responder vs. responder data normalized to baseline, the delta area statistics suggested there were either 6 or 7 gene clusters (Figure 35A). By inspection of the consensus matrix, a 7 cluster structure clearly emerged (Figure 35B). Gene expression values for genes within the four largest clusters are presented in Figure 35C. Cluster #2 is likely the most biologically meaningful to explain differential response, as non-responders had elevated expression of these genes throughout treatment, while responder gene expression steadily declined. Cluster #2 genes and the corresponding heatmaps for non-responders and responders are shown in Figure 35D.

In contrast to leukocyte-dominated genes downregulated rapidly in patients who responded to etanercept treatment (Figure 30), only 8 out of 35 genes in cluster #2 are reportedly expressed by or act on bone marrow derived cells (HPSE, CD24, HSPA4, JUNB, ZFP36, STAT3, VDAC1, and CCL20). The other genes in cluster #2 regulate keratinocyte proliferation, differentiation, and maturation or oxygen radical formation and quenching. This is an expected result since non-response to drug therapy is defined as continued keratinocyte hyperplasia and parakeratosis. Of the 8 “non-keratinocyte” genes differentially expressed by non-responders and responders, 4 promote leukocyte chemotaxis Heparinase (HPSE), CD24, JUNB, and CCL20), heat shock 70kDa protein 4 (HSPA4) increases TLR4 expression on monocytes promoting an innate immune response, STAT3 promotes Th17 polarization, voltage-dependent anion channel 1 (VDAC1) is also involved in T cell polarization, and zinc finger protein 36 (ZFP36)

targets TNF for proteasome degradation. In summary, responders have decreased keratinocyte proliferation, leukocyte migration, Th17 polarization, and TNF negative feedback gene expression compared to non-responders.

One possible explanation for non-response to etanercept treatment is that TNF was not completely inhibited by the drug. To ascertain the level of TNF inhibition in non-responders we compared non-responder (n=4) and responder (n=16) expression of TNF early response genes IL-1 β , IL-8, and iNOS (Figure 35E). Although early gene downregulation is not statistically significant due to lack of sufficient power, these genes are downmodulated by week 1. In contrast, other TNF modulated genes including p40 and CCL3 are not downmodulated in non-responders, and IL-20 is not downregulated as rapidly (Figure 35F). A summary of our proposed DC / T cell / keratinocyte interaction is presented in Figure 36.

Figure 35. Differences between patients who responded to etanercept treatment and those who did not. Consensus Clustering on genes differentially modulated in etanercept non-responders and responders produced a delta curve (A) and Consensus Matrix (B) that identify 7 clusters, 4 of which had more than 4 genes (C). Corresponding heat map for cluster #2 (D). RT-PCR on TNF early response genes in non-responders vs. responders (E), and downstream TNF-mediated genes (F).

Figure 7

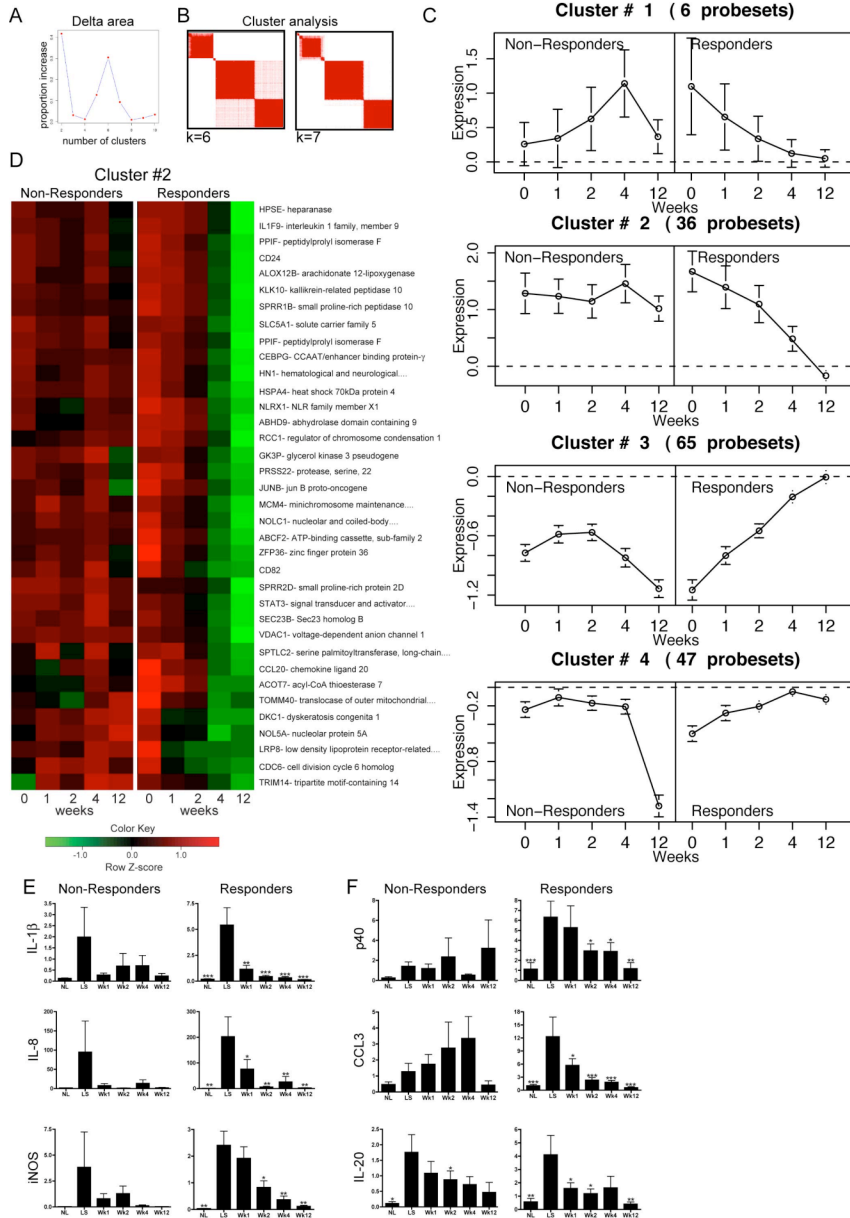
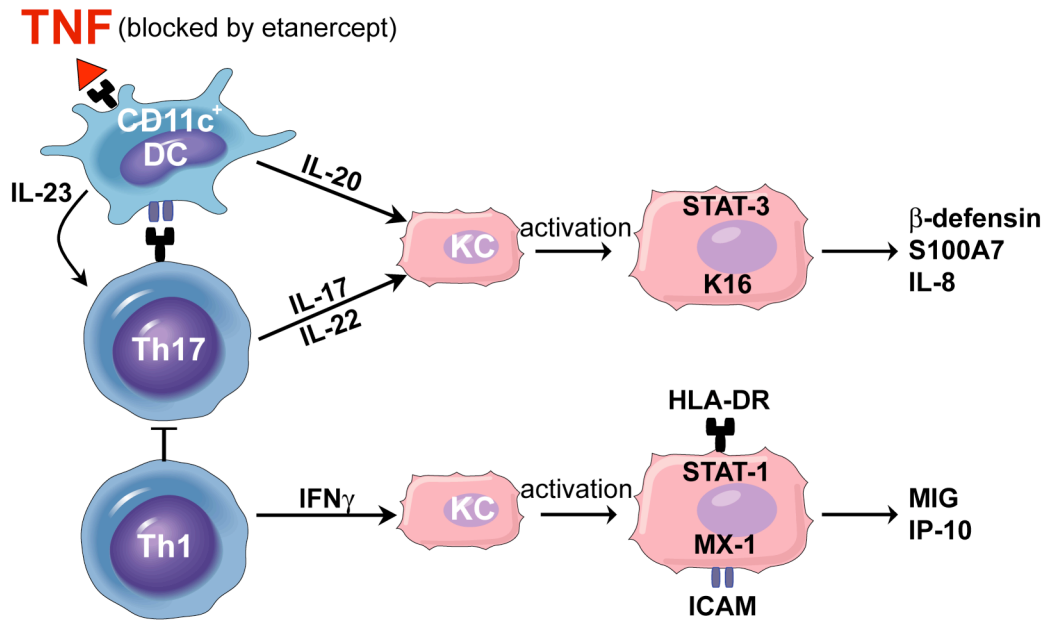


Figure 36. Proposed role of Th17 and Th1 T cells in psoriasis pathogenesis. TNF stimulates CD11c⁺ inflammatory DCs to produce IL-23 and IL-20. DC activation and production of IL-23 supports Th17 survival and proliferation, and induces production of IL-17 and IL-22. DC and Th17 products activate keratinocytes, promoting release of innate inflammatory molecules such as β -defensin, S100A7, and IL-8. Concurrently, Th1 cells producing IFN γ activate keratinocytes to upregulate MHC-II molecules (HLA-DR) and integrins (ICAM), and release cytokines including MIG and IP-10. Th1 and Th17 cells may suppress each other's development, but IFN γ can also act synergistically with IL-17 to increase ICAM expression and IL-8 release from keratinocytes. In psoriasis, etanercept may proximally inhibit this IL-23/IL-17 pathway to normalize keratinocyte proliferation and leukocyte infiltration.



Discussion

Tumor Necrosis Factor (TNF) was initially identified in 1976 as the soluble, LPS-dependent serum factor that killed murine fibrosarcoma cells but not normal fibroblasts (Green *et al.*, 1976). TNF was later identified as a macrophage-derived product that rapidly induced expression of IL-1, IL-6, and IL-8, leading to recruitment of innate inflammatory leukocytes within hours of the infectious insult (Clark *et al.*, 1981; Hoffman, 1986). This cytokine sequence, which might be termed the “sepsis cascade model,” has largely dominated thinking about the function of TNF in chronic inflammatory diseases. Indeed, the initial rationale for testing TNF antagonists in rheumatoid arthritis was based on demonstrated increases in all sepsis cascade cytokines in synovial fluid of affected joints, as well as abundant neutrophils producing collagen-destroying matrix metalloproteases (van Meurs *et al.*, 1999). Extension of these findings led to clinical trials of TNF antagonists in Inflammatory Bowel Disease and Psoriasis with subsequent therapeutic success and FDA approval. Consequently, there has been a strong temptation to link pathogenesis of human inflammatory diseases, and especially those successfully treated with TNF antagonists such as psoriasis and rheumatoid arthritis, to direct effects of sepsis cascade cytokines on innate immunity. However, despite the success of TNF blocking agents, psoriasis vulgaris clearly has some elements of pathogenesis that are not well explained by sepsis cascade cytokines and neutrophil activation. First, there is clearly a strong T cell component in psoriasis, including multiple reports of T cell clonality, and there is considerable clinical benefit provided by T cell targeted therapeutics (Chang *et al.*, 1997; Diluvio *et al.*, 2006; Gottlieb *et al.*, 1995; Prinz *et al.*, 1994; Weinshenker *et al.*, 1989). Second, psoriasis has been induced in

several transplanted skin models without apparent involvement of neutrophils in converted grafts (Nestle and Nickoloff, 2005). Third, recent success in treating psoriasis with antibodies to the p40 cytokine subunit shared between IL-12 and IL-23 implies a central role for Th1 and/or Th17 T cells in the pathogenesis of this disease (Gottlieb *et al.*, 2007; Krueger *et al.*, 2007; McKenzie *et al.*, 2006; Toichi *et al.*, 2006; Yen *et al.*, 2006). How then is TNF linked to pathogenic actions of T cells in psoriasis?

We believe that a critical link between TNF and the adaptive immune system occurs through a group of myeloid dendritic cells (DCs) termed TIP-DCs (TNF- and iNOS-producing DCs) (Lowes *et al.*, 2005a; Serbina *et al.*, 2003; Tam and Wick, 2004). In many cases, TIP-DCs are the most abundant type of leukocyte in psoriatic skin lesions and these cells have the ability to make key “downstream” cytokines such as IL-20 and IL-23 that affect other cell types in psoriasis lesions (Lowes *et al.*, 2005a; Wang *et al.*, 2006). For example, IL-20 has direct trophic effects on epidermal keratinocytes, where it serves as an inducer of NFkB and directly modulates several cellular/molecular features of psoriasis in model systems (Sa *et al.*, 2007). IL-23 is now viewed as an essential survival cytokine for Th17 T cells which are major pathogenic effector cells in psoriasis (Annunziato *et al.*, 2007; McKenzie *et al.*, 2006; Vanden Eijnden *et al.*, 2005). TIP-DCs are not only characterized by expressing large amounts of TNF cytokine, but also have high levels of TNF receptors, reflecting TNF’s ability to positively autoregulate TIP-DCs (Gottlieb *et al.*, 2005). TNF effects on DCs include DC maturation, stimulation of IL-23 or IL-12 synthesis, increased antigen presenting capacity, and effects on secondary lymphoid tissue organization (Ware, 2005; Zaba *et al.*, 2007b). TNF induction of DC-

dependent IL-23 production and downstream Th17 T cell activation provides a plausible TNF mediated link between the innate and adaptive immune system.

In prior work, we have established that TNF blockade with etanercept rapidly represses synthesis of several key TIP-DC cytokines, including TNF, IL-20, and IL-23, rapidly attenuates Th17 T cell products IL-17 and IL-22, and more slowly attenuates Th1 IFN γ synthesis (Zaba *et al.*, 2007a). Targeted gene analysis using RT-PCR did not, however, provide a “global” view of qualitative/quantitative and temporal relationships of genes modulated by etanercept. We believe the present results use gene array analysis to help clarify several elements in the inflammatory cascade that links TNF to adaptive immune responses in psoriasis. First, while the sepsis cascade is self-amplifying and reaches completion within hours of the initial stimulus, the resolution of psoriasis might be viewed as the opposite process in which removal of a key stimulus “de-amplifies” progressive waves of gene transcription over a period of several weeks. By studying the response at multiple time points after etanercept is administered, we have identified a cluster of genes downmodulated “early” and 3 other sets of progressive reductions in gene transcripts. Second, the majority of genes downmodulated early were myeloid derived, suggesting that these cells are the primary target of TNF inhibitors. Third, we demonstrate a functional link between IL-23p40 inhibition and decreased Th17 T cell polarization in a mixed leukocyte reaction, completing the journey from innate to adaptive immunity.

This data has also given us the first view of global gene transcripts modulated by TNF, IL-17, or interferon-gamma in epidermal keratinocytes, and how these cytokine pathways are modulated by TNF neutralization. Genes most significantly and rapidly

downmodulated following TNF inhibition were genes regulated by both TNF and IL-17, suggesting cytokine synergy or redundancy, a theory supported by known common regulation of NFkB (Awane *et al.*, 1999; Qian *et al.*, 2007).

Lastly, this study may inform on possible mechanisms for TNF-mediated extracutaneous manifestations in psoriasis vulgaris, such as arthritis and cardiovascular disease (CVD). TNF inhibitors are frequently used as first-line drugs for psoriatic arthritis patients because of rapid joint disease resolution. TNF is a known inducer of osteoclastogenesis and pathologic bone reabsorption in TNF-mediated autoimmune diseases through induction of AP-1, a dimeric complex containing FOS proteins (c-Fos, FOSB, FOSL1/Fra-1, Fra-2), Jun and ATF proteins (Asagiri and Takayanagi, 2007; Karin *et al.*, 1997; Lam *et al.*, 2000; Romas *et al.*, 2002). AP-1 potentiates RANKL on myeloid lineage cells, initiating differentiation into osteoclasts (Lam *et al.*, 2000). Although murine models have been used to establish these mechanisms, our *in vivo* human model of TNF inhibition shows rapid downregulation of FOSB and FOSL1/Fra-1 which may translate into decreased osteoclast mediated joint destruction. Another rapidly downregulated gene, endothelial lipase (EL/LIPG), may explain known dyslipidemia associated with psoriasis. TNF is an inducer of LIPG which cleaves triglycerides off of HDL and decreases plasma HDL concentrations (Kempe *et al.*, 2005), and increases monocyte adhesion (Kojima *et al.*, 2004). At physiological levels, HDL protects against cardiovascular disease by reverse cholesterol transport (RCT), acting as a sink for cholesterol that could otherwise be deposited in macrophages in atherosclerotic plaques (Popa *et al.*, 2007). Our data suggest a potential mechanism for

TNF causing low HDL in psoriasis patients, and that etanercept may reverse this pathway.

CHAPTER 7

General Discussion

Our work has re-classified dermal dendritic cells in normal skin and inflamed psoriatic skin. Until recently, normal and psoriatic dermal DCs were defined by FXIIIa expression, which we have shown to be a macrophage marker (Zaba *et al.*, 2007b). The immunohistochemical distinction between dermal DC and dermal macrophages is medically important as evidenced by recent discovery of FXIIIa⁺ cells mediating nephrogenic systemic fibrosis (NSF) in dialysis patients who have received gadolinium contrast (Kucher *et al.*, 2005; Parsons *et al.*, 2007). NSF is characterized by fibrosis of the dermis and subcutaneous septae as well as fibrosis of organs, including the heart, liver, lungs and muscle. Biopsy of lesional skin reveals increased CD68⁺FXIIIa⁺ cells containing gadolinium particles. In the literature, these cells have been described as either dendritic cells, dermal dendrocytes, or fibroblasts, but as in our tattoo model, we suggest that the gadolinium particles are collected inside macrophages. Accurately defining these FXIIIa⁺ cells as inflammatory macrophages and not antigen-presenting dendritic cells may have consequences on therapeutic targets for NSF treatment.

In normal skin, we suggest that mature, immunostimulatory BDCA-1⁺ cells are the major resident dermal dendritic cell population. Other groups have suggested a different classification system in normal skin with two non-overlapping subsets of immunostimulatory CD1a^{hi} and non-immunostimulatory CD14⁺ cells (Angel *et al.*, 2006; Angel *et al.*, 2007). The CD1a⁺ subset described by Angel *et al.* is also BDCA-1⁺, and

therefore corresponds to our definition of BDCA-1⁺ resident dermal DCs. In our hands using the “gold standard” immunohistochemistry and immunofluorescence for *in situ* characterization of dermal cells, we did not find many CD14⁺ cells. Therefore, we believe that this subset of CD14⁺ cells are blood monocytes contaminating the preparation.

Prior work on psoriatic dermal DCs defined them as FXIIIa⁺ immunostimulatory cells (Nestle *et al.*, 1994). FXIIIa is upregulated on all dermal dendritic cell populations during culture and emigration (unpublished data), and therefore the Nestle et al. model does not contrast with our own. Functional studies showed that both BDCA-1⁺ and BDCA-1⁻ myeloid DC psoriatic dermal emigres are equally immunostimulatory and TH17 polarizing. This result is somewhat surprising considering that *in situ* double label immunofluorescence clearly shows that all CD205⁺DC-LAMP⁺ cells are BDCA-1⁺, suggesting that BDCA-1⁺ cells should have greater antigen presenting capacity than BDCA-1⁻ cells. This discrepancy may be explained by BDCA-1⁻ cells maturing and gaining immunostimulatory capacity during emigration from the dermis. To support this theory, no CD11c⁺BDCA-1⁻ cells are DC-LAMP⁺ by *in situ* immunofluorescence, but FACS on CD11c⁺BDCA-1⁻ emigres shows 35% positivity for DC-LAMP. We are also aware that defining a cell subset using a negative marker (aka BDCA-1⁻) is not ideal, and ongoing work will focus on establishing antigens that are uniquely expressed on the inflammatory BDCA-1⁻ dermal DC population.

Double label immunofluorescence showed that many CD11c⁺ dermal cells produced TNF, and are thus likely targets of TNF neutralization therapies. In a clinical trial using the TNF-blocking drug etanercept, RT-PCR and gene array analysis showed an expected rapid downmodulation of myeloid genes. We also found that TH17 T cell gene products were rapidly downmodulated while Th1 T cell gene products were downregulated late in disease resolution. Previous clinical trials using gene array to probe a drug's mechanism of action include a cyclosporine (CSA) trial with the earliest treatment timepoint at 2 weeks (Haider *et al.*, 2008). Unlike etanercept which resulted in early and late gene modulation, CSA treated patients were globally immune suppressed by week 2.

While some of the genes downmodulated early by TNF inhibition are known TNF early genes, many have not been previously reported. Included in this early TNF-dependent gene list are lipid metabolism and osteoclastogenesis genes that may inform pathways of extracutaneous manifestations of psoriasis.

Although this thesis describes DC subsets and functions in psoriasis vulgaris, these principals may be applied to other inflammatory conditions. Non-MHC psoriasis susceptibility loci overlap with other inflammatory diseases such as atopic dermatitis (AD) (Cookson *et al.*, 2001) and inflammatory bowel disease (IBD) (Lee *et al.*, 2000), suggesting that these diseases may have overlapping inflammatory pathways. Future research comparing psoriasis DC and Th17/Th1 cells with those in AD and IBD would be interesting.

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