CONTRIBUTION OF CCR4 AND CXCR3 IN THE MIGRATION OF T CELLS TO INFLAMMATORY SITES IN THE SKIN, JOINTS AND LYMPH NODES

by

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To the source of my inspiration and perseverance,

To my parents, ***Aysha Ahmed Shawki*** *and* ***Ali Mahmoud Al-Banna***
To my sister, ***Fairouz*** *and to my brother,* ***Mahmoud***

……

Though I was continents away, your encouragement, your prayers,
and your words of wisdom guided my road

This thesis is dedicated with sincere gratitude to my dear family

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ABSTRACT

Chemokine receptors, CCR4 and CXCR3, found on T cells, are implicated in the inflammatory response in the skin, joint and lymph nodes (LNs), but whether they mediate T cell recruitment is unclear. Therefore, I examined the contribution of CCR4 and CXCR3 in T cell recruitment to inflammatory sites in the skin, joints and LNs.

I found that CXCR3 deficiency reduced the migration of ~50% of the CD4 cells, ~60% of Th1 cells and ~30% of Tc1 cells to inflamed skin. However, the dermal recruitment of memory CD4 and Th1 cells was associated with CCR4 expression, but did not require CCR4. Surprisingly, CCR4 deficiency increased the recruitment of memory CD4 cells and Treg cells to some inflamed skin sites, and reduced the infiltration of Th2 and Tc2 cells to ConA by ~30%, but did not affect TLR agonist sites.

Differing contributions of CXCR3 and CCR4 in the Th1 cell homing to LNs draining sites of CFA-immunization was demonstrated; the accumulation of $CXCR3^{-/-}$ Th1 cells was reduced, while the accumulation of $CCR4^{-/-}$ Th1 cells was increased. However, the deficiency of both CCR4 and CXCR3 did not affect recruitment of Th1 cells to inflamed paws of mice with collagen induced arthritis (CIA). Interestingly, $CCR4^{-/-}$ mice developed CIA with reduced incidence. While $CCR4^{-/-}$ Th1 cells migrated normally to inflamed paws of wild-type mice, $CCR4^{-/-}$ mice had fewer CD4 $CD25^{+}$ cells in the draining LNs and recruited fewer Th1 cells into the inflamed paws, suggesting a role for CCR4 on immune cells or stromal cells.

In summary, the contribution of CXCR3 varies in different tissues; it mediates part of Th1 cell recruitment to dermal inflammation and draining LNs, but not joint inflammation. The contribution of CXCR3 and CCR4 to dermal recruitment differs with T cell subsets. While CXCR3 mediates memory CD4, Th1 and Tc1 cell recruitment, CCR4 mediates Th2 and Tc2 cell recruitment to ConA sites. CCR4 is associated with, but does not mediate, memory CD4 and Th1 cell migration. Instead, the increased accumulation of $CCR4^{-/-}$ T cells was observed; memory CD4 and Treg cells in ConA, and Th1 cells in draining LNs.

LIST OF ABBREVIATIONS USED

-/-	Deficient
7-AAD	7-Amino-Actinomycin D
AA	Adjuvant Arthritis
Ab	Antibody
AD	Atopic Dermatitis
AIA	Antigen-Induced Arthritis
ALN	Axillary Lymph Node
BM	Bone marrow
BSA	Bovine Serum Albumin
CAIA	Collagen Antibody Induced Arthritis
CFA	Complete Freund's Adjuvant
CHS	Contact Hypersensitivity
CIA	Collagen Induced Arthritis
CK	Chemokine
CKR	Chemokine Receptor
CLA	Cutaneous Lymphocyte Antigen
CLE	Cutaneous Lupus Erythematosus
CLN	Cervical Lymph Node
ConA	Concanavalin A
DCs	Dendritic Cells
DMSO	Dimethyl Sulphoxide
DN	Double Negative
DNFB	Di-Nitro-Fluoro-Benezene
dsRNA	Double Stranded Ribonucleic Acid
DTH	Delayed Type Hypersensitivity
EBV	Epstein Barr Virus
ESL	E-selectin ligand
FACS	Fluorescence Activated Cell Sorter

FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
G6PI	Glucose-6-Phosphoisomerase
GAG	Glycosaminoglycan
GEF	Guanine exchange factor
GvHD	Graft Verus Host Disease
H&E	Hematoxylin and eosin
HEV	High Endothelial Venules
HSA	Human Serum Albumin
i.d.	Intradermal
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM	Intercellular Adhesion Molecule
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
ILN	Inguinal Lymph Node
IPF	Idiopathic Pulmonary Fibrosis
LFA	Leukocyte Function-Associated Antigen
LN	Lymph Node
LPS	Lipopolysaccharide
MACS	Magnetic Cell Sorter
mAb	Monoclonal Antibody
MDC	Macrophage-Derived Chemokine
MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph Node
NK	Natural Killer
NOD	Non-obese diabetic
OVA	Ovalbumin
OXA	Oxalazolone

PB	Peripheral Blood
PGN	Peptidoglycan
p.i.	Post-immunization
PLN	Popliteal Lymph Node
PMN	Polymorphonuclear cells
Poly-I:C	Poly-inosine poly-cytosine
PPD	Purified protein derivative of *Mycobacterium*
PSGL-1	P-selectin glycoprotein 1
PSL	P-selectin ligand
RA	Rheumatoid Arthritis
RAG	Recombination Activating Genes
RLN	Retroperitoneal Lymph Node
S1P	Sphingosine-1-Phosphate
s.c.	Subcutaneous
SCID	Severe Combined Immunodeficiency
SCW	Streptococcal Cell Wall
SF	Synovial Fluid
SP	Single Positive
ST	Synovial Tissue
T1	Tyrodes medium 1x
TARC	Thymus and Activation-Regulated Chemokine
Tc	Cytotoxic T
TCR	T Cell Recetor
TGF	Transforming Growth Factor
Th	Helper T
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TRAIL	Tumor Necrosis Factor Related Apoptosis Inducing Ligand
Treg	Regulatory T

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Chapter 1 Introduction

1.1 Activated T Cells

CD4 and CD8 T cells are activated in the presence of polarizing cytokines and differentiate into different phenotypes that vary in their production of cytokines and their function in the immune response (1-6). T cells are classically denoted by Th (T helper) cells for CD4 cells or Tc (T cytotoxic) cells for CD8 cells. Th1 and Tc1 cells are type 1 (IFN-γ producing) cells, while Th2 and Tc2 cells are type 2 (IL-4 producing) CD8 cells. Other T cell subsets have been identified, including Th17 and Tc17 cells that generate IL-17A, and Th9 cells that generate IL-9. It should be noted that most T cell subsets are plastic, in that they can be de-stabilized and can differentiate into other subsets (2, 3, 6).

Activated type 1 and type 2 cells have different properties (Figure 1). The polarizing cytokines involved in the development one cell type can inhibit the differentiation of other types: IL-12 *versus* IL-4 (7-11). Th1 cells activate the cell-mediated response and interact with macrophages and CD8 cells, while Th2 cells interact with B cells and induce most of the humoral responses. It should be noted that Th1 cells can influence the humoral responses induced by IgG2a subclass of antibodies (1, 11). Type 1 and type 2 cells participate in the immune responses to different pathogens: Th1 cells respond to intracellular pathogens while Th2 cells respond to helminth parasites (4, 12-16).

In addition, type 1 and type 2 cells participate in the development of different inflammatory diseases: autoimmunity such as arthritis (16-18) *versus* atopic and asthmatic reactions (19). There is an overlap between the function of Tc1 and Tc2 cells (1), such as cytotoxicity against tumors and viruses (7, 9, 11, 20), allograft rejection (10), and induction of DTH (21). However, their mechanisms might be different (1); Tc2 cells, but not Tc1 cells, are related to the recruitment of eosinophils (10, 21), the activation of Th2 cells (7) and the production of IL-4 (20). It should be noted that the other T cell subsets, such as type 17 cells, are also associated with development of autoimmune and inflammatory diseases, such as arthritis (22, 23), and dermal inflammation (24, 25).

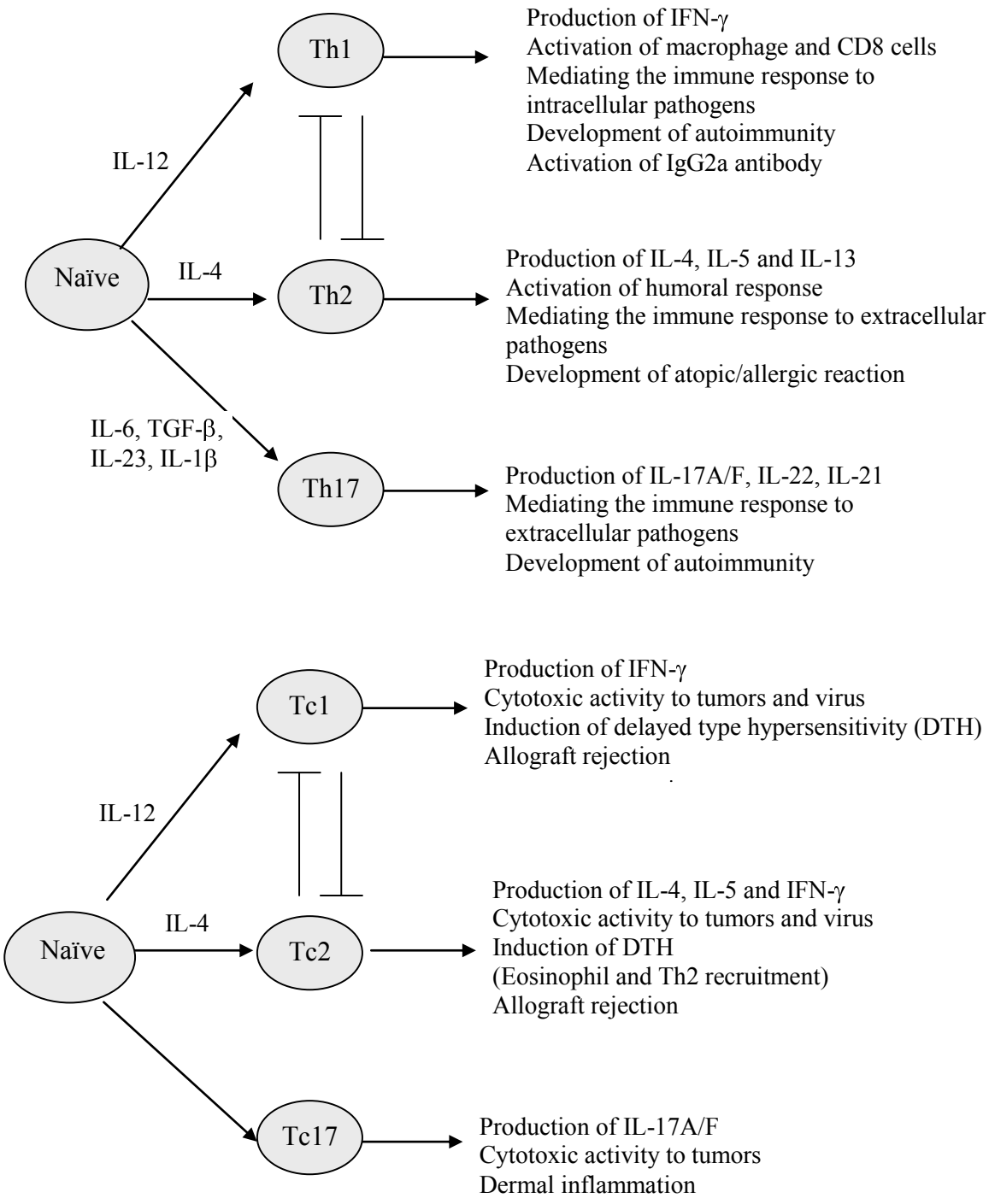

Figure 1. Overview of activated T cell subsets and their function

1.2 Regulatory CD4 T Cells

Another population of T cells are regulatory CD4 T (Treg) cells. CD4 Treg cells can be categorized into natural CD4 Treg cells, which develop in the thymus, or induced/adaptive CD4 Treg cells, such as Th3, Tr1 cells, which develop in the periphery (26, 27). Natural Treg cells constitute 5-10% of the CD4 cells, and are identified by their expression of IL-2 high affinity receptor CD25, transcription factor Foxp3 (28) and some other markers (29-35). Treg cells utilize different mechanisms in order to inhibit T cells (27, 29, 36), and control the immune response (37). The adoptive transfer of Treg cells was shown to reduce or delay the development of autoimmune diseases in mice (28, 30, 38-41). In contrast, the absence of functional Treg cells, such as in mice with a mutated Foxp3 (39) or after the depletion of $CD25^+$ cells (28), was related to the development of autoimmune diseases (28, 39, 42). This was also illustrated by biopsies of patients with inflammatory diseases that contained fewer Treg cells than control biopsies (43, 44).

Studies have reported the ability of tolergenic ($IL10^+$ $TGF\beta^+$) dendritic cells (DCs) (45), rapamycin and TGF-β cultured DCs (46), or TGF-β produced by tumor cells (47) to induce expression of Foxp3 in CD4 T cells *in vitro* (46). Indeed, several protocols have utilized TGF-β and/or rapamycin for the *in vitro* expansion and induction of $Foxp3^+$ T cells (48-50). TGF-β was found to maintain the expression of Foxp3 on natural Treg cells (51) and induce Foxp3 expression in $CD4^+$ $CD25^-$ cells (31, 51). Also, rapamycin was shown to expand the CD4 $CD25^+$ $Foxp3^+$ Treg cells (51), and induce Foxp3 expression in CD4 cells, when used in conjunction with IL-2 (50). *In vitro* expanded (52), TGFβ-induced (51, 53), or rapamycin treated Treg cells (49, 50, 53, 54) were shown to have suppressive activity, as assessed *in vitro* and *in vivo* by their inhibitory effect on the *in vitro* proliferation of naïve T cells (46, 49) and the development of inflammatory disease in mice (45, 46, 49, 52, 54).

The mechanism by which rapamycin induces Foxp3 expression and/or expands Treg cell populations is not fully determined, but it is thought to target Akt-mTOR pathway that is activated downstream of TCR/CD28 signalling. Rapamycin blocks the mTOR complex 1and 2 and therefore inhibits T cell polarization; it delays the induction of transcription factors T-bet and GATA3, and favors Foxp3 expression in CD4 T cells (55). In addition, rapamycin was shown to potentiate the TGF-β induced Foxp3

expression by blocking the signaling of Akt. It should be noted that activated Akt does not affect established Foxp3 expression in Treg cells (56). Rapamycin delays CD4 T cell proliferation (55), however its ability to expand the Treg cells may be related to the expression of pim2, a serine/threonine kinase that share downstream targets of Akt. The induction of pim2 by Foxp3 results in rapamycin resistance, thus allowing for the expansion of Treg cells in the presence of rapamycin (57).

1.3 Memory CD4 T Cells

Activated T cells are known to undergo a "contraction" phase to limit the duration of the immune response. At this stage, memory cells develop from effector cells so as to preserve the antigen-specificity and develop a recall response upon subsequent encounters with the antigen (58, 59).

Memory CD4 cells are defined as $CD45RA^{-}$ $CD45RO^{+}$ in humans (60, 61) or $CD44^{hi}CD45RB^{lo}$ in mice (62). They are classically described as central memory (T_{CM}) or effector memory (T_{EM}), based on their expression of CCR7 and L-selectin (59, 61). T_{CM} cells are $CCR7^{+}$ L-$selectin^{+}$ and are thought to be long lived memory cells that circulate the lymph nodes and proliferate into effector T cells when stimulated (61, 63). Whereas T_{EM} cells ($CCR7^{-}$ L-$selectin^{-}$) are thought to display effector functions, such as cytokine production (59, 61), and to recruit into peripheral tissues, as demonstrated in *M. tuberculosis* infected mice (63).

1.4 T Cell Recruitment Cascade: Chemokine Receptors and Adhesion Molecules

The transmigration of T cells is the process whereby T cells move across the endothelium to migrate from the circulation to the underlying tissue. This process is thought to involve chemokine receptors (CKRs) and adhesion molecules, which include selectins and integrins. Selectins include E- and P-selectin that are expressed on the endothelium, and L-selectin that is found on leukocytes. P-selectin can be constitutively expressed on the endothelium, however its expression can be increased by various stimuli; its transport from storage granules can be stimulated by histamine or thrombin

(64, 65) and its transcription can be induced by lipopolysaccharide (LPS) or by pro-inflammatory cytokines, such as TNF (66). E-selectin is not present on normal endothelium, and its expression requires activation by LPS or by pro-inflammatory cytokines, such as TNF (67). Several ligands of selectins have been characterized. P-selectin glycoprotein 1 (PSGL-1) is a ligand that can bind to P-selectin (68) as well as to E- and L- selectin (69, 70). L-selectin can also bind ligands like peripheral lymph node addressin (PNAd) and CD34 (71-73), so it may not necessarily depend on PSGL-1 (74). E-selectin binds to cutaneous lymphocyte antigen (CLA) which is thought to be expressed on CD43 (75). Still, the presence of E-selectin ligands was shown to depend on the expression of the enzyme fucosyl transferase VII, rather than on the presence of CLA (76). The function of selectins is mostly related to slowing the leuckocytes during the early stages of leukocyte recruitment (77), but they may also be involved in the activating the other participants to enhance the process of recruitment, as suggested for L-selectin and CXCR4 (78).

The other class of adhesion molecules is the integrins. These include α_4 integrins [e.g. VLA-4 ($\alpha_4\beta_1$, CD49/CD29), and $\alpha_4\beta_7$ (CD49d/B7)] and β2 integrins [e.g. mac-1 ($\alpha_M\beta_2$, CD11b/ CD18), and LFA-1 ($\alpha_L\beta_2$, CD11a/CD18)], which interact with their ligands, such as intercellular adhesion molecules (ICAMs) ICAM-1, -2, and VCAM-1 (79). The activation of integrins is induced by the binding of CK to CKRs, and therefore they are thought to contribute to the later stages of transmigration (Figure 2) (80, 81). Nevertheless, some of the integrins, such as VLA-4, may also participate in rolling and adhesion stage of transmigration (82, 83).

The recruitment of lymphocytes has been described as a process that requires CKRs and/or adhesion molecules in each of its sequential steps (Figure 2): tethering and rolling, activation, adhesion and extravasation/transendothelial migration (80, 84, 85). "Tethering" involves the transient interaction between the circulating leukocytes and the endothelium, which is sufficient to slow the cells down. "Rolling" is then initiated and requires the interaction between the E- and/or P-selectin and its ligands (e.g. PSGL-1 and CLA) (68). Then, chemokines (CKs) bind to glycosaminoglycans (GAGs) on the apical surface of the endothelium to be available for binding to the chemokine receptors (CKRs) that are expressed by rolling T cells. Changes in the conformation of cytoplasmic

domains of the integrin can be initiated by chemokine receptor signaling to result in "inside-out" activation of integrin. Then, the binding of the integrin to its ligands, intercellular adhesion molecules (ICAMs), induces conformational changes and the "outside-in" activation of the integrins. In addition, the ligand binding to integrin, and the activation by CKR signaling induces the mobilization and clustering (dimerization) of integrins, which increases their avidity, as suggested by studies with LFA-1 (86), and results in the " firm adhesion" of leukocytes to the endothelium. The upregulation of ICAMs on the endothelial surface, by pro-inflammatory cytokines and TLR agonists, further enhances the adhesion of the leukocytes (80, 87). Though α_4 integrins are important in mediating transendothelial migration (88), they can be involved in the tethering and rolling of leukocytes in mesenteric venules of inflamed tissues (82) and lamina propria venules under shear conditions, without the contribution of selectins (83).

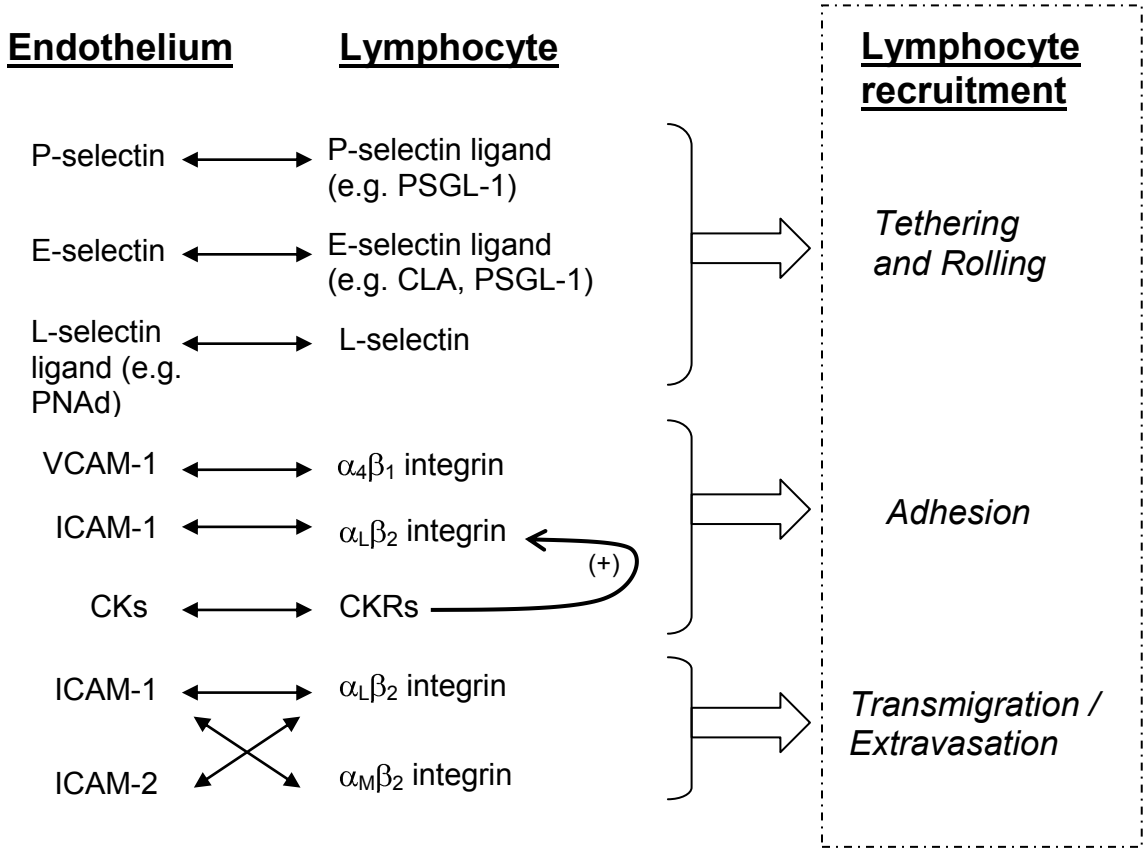

Figure 2. Overview of adhesion molecules and CKRs involved in different stages of leukocyte recruitment (adapted from (80)).

The process of transmigration involves a degree of specificity so as to guide the recruitment of T cells into different tissues. The expression of CCR9 and $\alpha_4\beta_7$ by the intestine-infiltrating memory CD4 cells (89), and the expression of CCR4 and CLA by the skin-infiltrating memory CD4 cells (89, 90) suggests the involvement of different CKRs and adhesion molecules for the recruitment to different tissues (85, 91). However, the CKRs required for the skin homing of memory and activated T cells are still unclear, as they express not only CCR4 (89, 90, 92), but also CCR5 (89), CCR10 (90) and CXCR3 (89, 93).

In addition, the requirement of adhesion molecules for T cell migration might vary for different T cell subsets (94). When the binding to E- and/or P-selectin was inhibited, by deficiency or antibody mediated blockade, fewer unstimulated T cells and activated CD4 cells migrated to dermal inflammation that was induced by ovalbumin / incomplete Freund's adjuvant (OVA/IFA) (95), delayed type hypersensitivity (DTH) (96, 97) and Concanavalin A (ConA) (98, 99). However, other studies demonstrated the requirement for L-selectin in addition to E- and P-selectin (100), or α_4-integrin in addition to E-selectin (92) for the migration of activated CD8 T cells to dermal inflammation.

1.5 Chemokines and Chemokine Receptors

An inflammatory response involves the recruitment of several innate and adaptive immune cells to the site of inflammation. Their subsequent interaction triggers different immune functions with the aim of eliminating or controlling the source of the inflammatory signal. This may also involve the egress of immune cells from the tissue to the draining lymph node (LN) or to the circulation. T cell recruitment into inflamed tissues is thought to be guided by mediators, such as chemokines that are produced by the structural cells and the innate immune cells in the tissue. CKRs required for T cell recruitment might differ depending on the tissue itself, the type of inflammatory stimuli or the type of infiltrating T cell.

Chemokines (CKs) are comprised of a group of proteins that are categorized according to the number and position of cysteine amino acids at their amino terminus into the CC-, CXC-, C- and CX3C families (101-103). Chemokines can be produced by

several cell types, as outlined in Figure 3. For instance, CCR4 ligands (CCL17 and CCL22) and CXCR3 ligands (CXCL9, 10, and 11) can be produced by endothelial cells (104-106), keratinocytes (104, 106-110), eosinophils (111), DCs (106, 112-114), monocytes/macrophages (112, 114, 115), and mast cells (116, 117). In addition to these cells, CXCR3 ligands can be produced by synovial fibroblasts (105), and CCR4 ligands can be produced by B cells (114, 118) and to lesser extent T cells (119, 120).

Some stimuli can induce the production of both CCR4 and CXCR3 ligands. It has been shown that CCL17, CCL22, CXCL9, 10, and 11 are induced in response to TNF, IFN-γ, IL-1β and TLR agonists (104-106, 108-110, 114). In addition to these stimuli, IL-4 and IL-13 were shown to induce the production of CCR4 ligands (107, 111-115, 119). Some stimuli were reported to have opposing effects on the production of CKs. For example, the production of CCL22 was shown to be inhibited by IFN-γ (113, 115). In contrast, one study demonstrated that the production of CCL22 by TNF and IFN-γ - stimulated keratinocytes was inhibited by IL-4 and IL-13 (108).

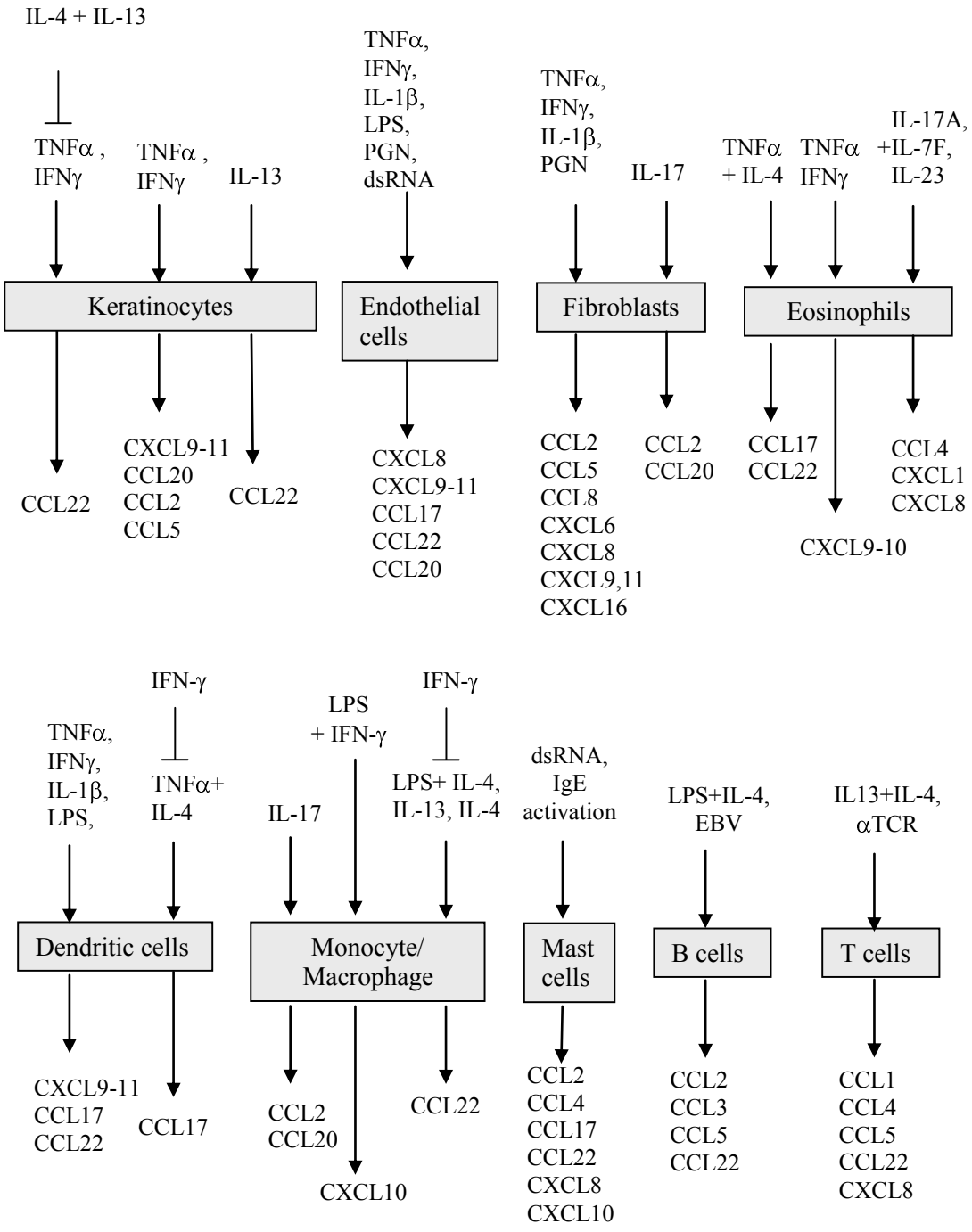

Figure 3. Schematic representation for the production of some of the chemokines by structural and immune cells in response to different stimuli, as per literature for keratinocytes (104, 106-110), endothelial cells (104-106), synovial fibroblasts (105, 121, 122), eosinophils (111, 123), DCs (106, 112-114), monocytes/macrophages (112, 114, 115, 121), mast cells (116, 117), B cells (114, 118) and T cells (112, 114, 119, 120).

Chemokine receptors (CKRs) are G-protein coupled receptors that are made of a single polypeptide chain with 7 transmembrane domains. CKRs are categorized into four families; CCR1-10, CXCR1-6, XCR1-2, CX3CR1 (101, 103).They can be expressed by multiple cell types (Table 1), such as CXCR4 that is expressed on naïve T cells, B cells, dendritic cells and neutrophils (103). Most CKRs can bind to more than one CK. For example, CXCR3 binds to CXCL9, 10 and 11, while CCR4 binds to CCL17 and CCL22 (124). The binding of a CK to its receptor results in the activation of a number of signaling pathways (125, 126). CKRs are G-protein coupled receptors, therefore in response to CK binding, the G-protein subunit (G_α) uncouples from the complex of two G-proteins (G_β and G_γ), which activate kinases, including phospholipase C and phosphoinositol-3-kinase. Signaling molecules, like inositol-3-phosphate and diacyl glycerol, are then formed and the signaling pathways, such as the Rac pathway, are activated (126, 127). Later, the formation of CKR-arrestin complexes targets the receptor for cathrin and/or lipid raft mediated endocytosis and initiates receptor internalization (124, 128). This is followed by the intracellular trafficking of the CKR and either its degradation or its turnover to the surface of the cell (128).

CKs can be functionally divided into "constitutive/homeostatic", "inducible/inflammatory" subsets or both (Table 1; in green, red and black respectively) (103). "Constitutive" CKs, such as CXCL12, are present in tissues during embryonic development (131), and during homeostatic circulation of cells through lymphoid and nonlymphoid tissues (131, 132). "Inflammatory" CKs are produced by structural and immune cells during an inflammatory process and are involved in the recruitment of innate and acquired immune cells to inflamed tissues (102, 132, 133). Although CKs are most known for their ability to recruit leukocytes, they have been implicated in several processes. These include the release of mediators, the induction or inhibition of angiogenesis, and the regulation of Th1/Th2 cell differentiation (129, 130).

TABLE 1. Summary of the expression of chemokine receptors on immune cells with their corresponding chemokines ("constitutive/homeostatic", "inducible/inflammatory" or both labeled in green, red or black respectively) (adapted from ref. (103)).

Chemokine Receptors (CKRs)	Chemokines (CKs)	Cells expressing CKRs										
		Naïve T cells	Memory T cells	Th1 cells	Th2 cells	Treg cells	Neutrophils	Natural Killer cells (NK)	Eosinophils	Monocytes	Plasmacytoid DCs	Myeoid DCs
CXCR1	CXCL6,7,8						✓					
CXCR2	CXCL1,2,3,5, 6,7,8						✓					
CXCR3	CXCL9,10,11			✓				✓			✓	
CXCR4	CXCL12	✓		✓	✓	✓		✓		✓	✓	✓
CXCR5	CXCL13											
CXCR6	CXCL16		✓									
CCR1	CCL3, 5, 7,13, 14, 15, 16, 23							✓		✓		✓
CCR2	CCL2, 7, 8, 13									✓	✓	✓
CCR3	CCL5, 7, 11, 15, 16, 24, 26				✓				✓			
CCR4	CCL17, 22				✓	✓			✓			
CCR5	CCL3, 4, 5, 8			✓				✓		✓	✓	✓
CCR6	CCL20											✓
CCR7	CCL19, 21	✓		✓								✓
CCR8	CCL1				✓	✓						
CCR9	CCL25		✓									
CCR10	CCL27, 28		✓									
XCR1	XCL1, 2							✓				
CX_3CR1	CX_3CL1			✓				✓		✓		

1.6 Expression of Chemokine Receptors on T Cells

The expression of CKRs on lymphocytes differs with the status of activation and differentiation. As naïve cells differentiate into memory and activated T cells, it is thought that they downregulate CCR7 and CXCR4, which are involved in homing to lymphoid tissues. They also upregulate CKRs such as CCR4, CXCR3, CCR5, CCR8, CCR6 and/or CCR10, enabling their migration to inflamed tissues where the corresponding CKs are present (60, 120). The expression of multiple CKRs on T cells is modified by the polarizing conditions, and interestingly, the same CKR can be expressed on several T cell subsets (60, 120). In order to demonstrate those two aspects, I will

outline literature of the expression of CKRs, including CCR4 and CXCR3, on T cell subsets, like memory CD4 cells, activated CD4 cells, activated CD8 cells, and Treg cells.

Memory CD4 cells are known to express several CKRs. They express $CXCR3^+$ (~10% in rats or ~30% in humans) (89, 93, 134, 135), $CCR4^+$ (~20% in humans) (136, 137), CCR6 (~30-45%) (134) and CCR10 (~30%) (90, 134). The co-expression of those CKRs has been demonstrated on the total CD4 population (135, 138), and on memory CD4 cells (60, 90, 134, 139), which explains why $CCR10^+$ memory CD4 cells can migrate *in vitro* to ligands of CCR4 and CXCR3 better than do $CCR10^-$ memory CD4 cells (134).

After their activation, CD4 T cells increase the expression of several CKRs (60, 120). CXCR3 and CCR5 are preferentially expressed on CD4 cells that are activated in type 1 polarizing conditions (93, 138, 140-143). Although CXCR3 was shown to be present on Th1 cells, but not Th2 cells (138, 141, 143), one study demonstrated the expression of CXCR3 on both antigen-activated transgenic Th1 and Th2 cells (140). CCR4 and CCR8 are preferentially expressed on CD4 cells that are activated in type 2 polarizing conditions (60, 137, 138, 141, 143-145). CCR4 was shown to be present on Th2 cells, but not Th1 cells (137, 138, 142), however one study reported that the activation of Th1 cell clones leads to an increase in their CCR4 expression (143). In fact, the *in vitro* activation of $CCR4^+$ CD4 T cells was found to increase their production of both IL-4 and IFN-γ (138). Still, CCR4 may remain lower on Th1 cells (~40%) than on Th2 cells (~50-60%) (60, 137, 142, 144, 145). Discrepancies in the expression of CCR4 on Th1 cells may be explained by differences in the downregulation of CCR4, which was observed after repeated activation of Th1 cells, but not Th2 cells (60, 142).

Activated CD8 T cells are known to increase their expression of CKRs, such as CXCR3 and CCR4. CD8 cells that are activated in type 2 polarizing conditions are ~50% $CCR4^+$ (144), but do not express mRNA for CXCR3 (142). In contrast, the expression of CXCR3 and CCR5 is increased on CD8 cells when activated in type 1 polarizing conditions *in vitro* (142) or *in vivo* (93, 146). $CXCR3^+$ Tc1 cells may co-express other CKRs, such as CCR2, CCR5, CCR8 and CCR10, as suggested by the transcript levels of these CKRs on *in vivo* generated $CXCR3^+$ CD8 cells that were ~60% IFN-γ^+ (146).

In addition, it is known that a high proportion of circulating Treg cells are $CCR4^+$ (75-90%) (147-152), $CCR5^+$ (~40%) (149-151), $CCR6^+$ (~70%) (149) and $CCR8^+$ (~80%) (148), and those CKRs can also be co-expressed (32, 153). Treg cells also express CXCR3 (~20-40%) and CCR7 (50-70%) and migrate *in vitro* to their ligands, but the expression is not higher than on CD4 cells (143, 147, 149-151, 155). The expression of other CKRs on Treg cells was shown using the transcript levels of CKRs (143, 150-155) and the *in vitro* migration to CKs (40, 148, 149). Interestingly, the expression of CKRs on Treg cells was suggested to affect to their ability to control the development of inflammatory disease, such as diabetes, in mice (40). This is thought to be related to the role of CKRs in guiding the Treg cell migration to the tissue or the lymph nodes. For instance, $CCR4^{-/-}$, $CCR5^{-/-}$, and $CCR7^{-/-}$ Treg cells were shown to have reduced recruitment into the tissue or draining LNs, and thus was related the reduced suppression of allograft rejection (154), or the increased development of inflammatory disease in tissues, such as the skin, lungs (156), and colon in mice (155).

It should be noted that CCR4, CXCR3 and other CKRs are expressed on innate immune and structural cells. Some of the related literature is summarized in Table 2.

Table 2. Summary of the expression of CXCR3 and CCR4 on innate immune and structural cells.

Cell type	**CKRs**			**Source of cells (reference)**
	CXCR3	**CCR4**	**Other examples**	
Innate immune cells				
Neutrophils	Yes		CCR5	BALF of patients of lung disease and SF of RA patients(157).
Eosinophils	Yes	Yes	CCR5, CCR8	BAL of allergic patients (158, 159), IL-5 transgenic mice (160).
$CD14^+$ Monocytes	No	Yes 30%	CCR5, CCR8*	SF and PB of RA patients (161). *intracellular (162)
Natural Killer cells	Yes	Yes	CCR1, CCR5, and CCR8	IL-2 activated human (163), but not normal PB (138).
Structural cells				
Keratinocytes		Yes **	CCR10	Primary and cell lines (164). **surface and intracellular
Endothelial cells	Yes #	Yes*		*dermal microvascular endothelial cells, # lung microvascular and bone marrow endothelial cell line (165).

1.7 CCR4 Is Related to Several Inflammatory Conditions

CCR4 has been associated with inflammatory conditions in several tissues, such as the skin, joints and lungs (119, 144, 156, 166-178).The presence of CCR4 ligands and/or $CCR4^+$ CD4 cells was demonstrated in biopsies of patients and mice with different inflammatory diseases. CCR4 was linked to the development of diabetes, the rejection of grafts, and the progression of tumors, as summarized in Table 3.

The broad expression of CCR4 on different cell types may explain why CCR4 was linked to inflammatory responses in various tissues. Studies examining the pulmonary inflammation and diabetes associated the relevance of CCR4 to effector CD4 cells; demonstrating the increased presence of $CCR4^+$ CD4 cells and CCR4 ligands, and their correlation to markers of disease severity (119, 144, 169, 170, 174, 179). On the other hand, other studies have associated CCR4 on Treg cells to their presence in tissues and hence the ability of Treg cells to control allograft rejection (154, 180, 181), and tumor progression (182, 183), though CCR4 can also be expressed by the tumor itself (184-186).

Finally, studies examining lethal peritonitis in CCR4 deficient mice have demonstrated a function for CCR4 on innate immune cells, like macrophages. In these models, $CCR4^{-/-}$ mice were shown to have improved survival and/or enhanced bacterial clearance (173, 187-189). This was associated to the effect of CCR4 deficiency on macrophages, in terms of their reduced peritoneal infiltration (173), reduced production of pro-inflammatory cytokines (189) or skewed development into the alternatively activated phenotype (187). It is also possible that other innate immune cells are influenced by the deficiency of CCR4, since the cytokine production of $CCR4^{-/-}$ $CD11b^+$ cells was reduced in some of these studies (187).

The effect of CCR4 on the infiltration of one cell type was highlighted in studies such as those examining the effect of deficiency or blockade of CCR4 on pulmonary inflammation. However, it is possible that the observations reflect the effect of CCR4 on activated T cells, regulatory T cells as well as on innate immune cells. The deficiency of CCR4 reduced the infiltration of Treg cells into the lungs, and hence an inflammatory response developed in the lungs (156).Yet, some reports showed that the lack of CCR4 reduced the number of T cells found in lungs during inflammation (166, 168, 170), but

this was not reported by others (172, 173). Freeman et al (171) demonstrated the reduced levels of IFN-γ and IL-4 in the lungs of $CCR4^{-/-}$ mice after the induction of type 1 and type 2 granulomatous responses respectively, but this effect may not have been related to T cells, since it could not be reconstituted with WT CD4 cells (171). Since CCR4 deficiency influences the development of macrophages (187), the effect observed after CCL17 blockade may have been caused by its effect on macrophages, whose numbers were reduced (168). It should be noted most studies enumerated the presence of T cells in the tissue after blockade or deficiency of CCR4, but only a few examined the *in vivo* migration of $CCR4^{-/-}$ T cells, namely for Th2 cells (170) and Treg cells (156).

Table 3. Summary of literature related to the effects of CCR4 in inflammatory diseases in different tissues.

Tissue	**Name of disease or model**	**Presence of CCR4 ligand**	**Presence of $CCR4^+$ CD4**	**Effect of blockade or deficiency**	***Reference***
Lung and Broncho-alveolar lavage	Eosinophilia pneumonia, atopic asthmatics, idiopathic pulmonary fibrosis (IPF).	Yes *#	Yes #	* Correlated to IL-5, IL-13 # Correlated to number of T cells	(119, 144, 174-178)
	Mouse models = Aspergillus fumigatus challenged neutropenic, bleomycin treated, Antigen (OVA) sensitization and challenge, Type 1 and 2 granuloma lung model	Yes	Yes (in draining LN)	<u>Various</u> Reduced inflammatory response, improved survival (166, 167, 190) Reduced number of T cells (166, 168) Reduced migration of OT2 Th2 cells (170) No effect on lymphocyte no. or Ab levels (172, 173) Reduced formation of type 1 granuloma (171) Reduced IFN-γ and IL-4 production (171) Reduced Treg accumulation in normal lung (156)	(156, 166-173)
Diabetes	NOD mice	Yes	Yes	Reduce incidence and severity	(179)
Tumors	Lymphoma, Leukemia cell lines and biospsies (BM, LN, skin lesions)	Yes	Yes ($Foxp3^+$, $CD25^+$)	Improve survival of SCID mice	(182, 183, 185, 186)
Transplants	Cardiac and pancreatic allograft model	Yes	Yes	Delayed rejection : $CCR4^{-/-}$ have normal number of T cells, reduced $Foxp3^+$ or NKT cells accumulation	(154, 180, 181)
Lethal peritonitis	LPS intraperitoneal challenge, CpG/D-gal induced shock, Colon ascendens stent peritonitis, cecal ligation and puncture	Yes	Not demonstra-ted	Improved survival, improved bacterial clearance: Reduced inflammatory cytokines in the serum, and reduced macrophage numbers.	(173, 187-189)

1.8 Chemokine Receptors in Dermal Inflammation

Different immune cells can participate in the inflammatory response in skin diseases, depending on the triggering stimuli. Dermal inflammation can be instigated by a number of different infections, such as those induced by *Staphylococcus aureus*, *Mycobacteria*, pox viruses, *Candida albicans*, or parasites (191). Acute inflammatory disease in the skin can be associated with type 2 cell - and mast cell - mediated responses, as in urticaria and atopic dermatitis (192). Other acute diseases, including contact dermatitis, may involve T cell mediated responses induced by a sensitizing antigen (192). Chronic skin diseases can be related to type 1 T cells and be autoimmune in nature, such as in psorasis, Graft versus host disease (GvHD), and discoid lupus erythematosus (192, 193). Therefore, no one model can represent the breadth of skin inflammatory diseases.

1.8.1 Models of Dermal Inflammation

Mouse models of dermal inflammation were used to examine the immuno-pathogenesis of skin diseases. The most commonly used model of dermal inflammation is the model of contact hypersensitivity (CHS) (194-201), though other stimuli, including DTH (92, 96) and ConA (98, 99, 202, 203), were used to induce inflammatory responses in the skin. Also, studies have used irritants like tape stripping to induce or enhance dermal inflammation (197, 204, 205).

Models of contact hypersensitivity that are extensively used in mice, are induced by skin painting with di-nitro-fluoro-benezene (DNFB), tri-nitro-chloro-benzene, oxazolone (OXA), trimellitic anhydride or fluorescein isothiocyanate (FITC) (194-201). These are thought to acts as haptens and modify the endogenous proteins, that are then recognized and presented to T cells by Langerhans cells, resulting in the activation of T cells during the sensitization phase. T cells are known to be recruited to the skin site during the elicitation phase, but it is not clear whether this relies on Th1 or Th2 cells (206). The model is associated with swelling (195, 197, 207), the expression of chemokines, such as CXCL10 (106, 208) and cytokines, such as IFN-γ (195, 197, 201, 207), IL-1β (195, 201, 208) and IL-6 (195). The concurrent induction of IFN-γ and IL-4 transcripts was demonstrated in CHS (195, 197). However, the induction of IFN-γ may likely precede that of IL-4; progression to the chronic phase is associated with an

increased IL-4 transcript and a reduced IFN-γ transcript (201, 209). Moreover, the cellular infiltrates appear to vary with the phase of CHS. In the acute phase, infiltration of neutrophils, eosinophils and lymphocytes was demonstrated (195, 197, 207), while in chronic phase, mast cells were present as well (197, 201). Other studies demonstrated that CD8 T cells infiltrate before the onset of symptoms, while CD4 cells and IL-10 producing Treg cells infiltrate during the resolution of symptoms (200, 207). CHS is considered to be a cell-mediated response that involves DCs and different subsets of T cells (206), however the importance of antibodies was also demonstrated; the transfer of serum enhanced the development of CHS, and so the severity of CHS was reduced in the B cell deficient mice (209).

In addition, delayed type hypersensitivity has been used extensively studied. Though CHS can be regarded as a subclass of the DTH responses, the more classical forms of DTH involve the tuberculin DTH and the protein-adjuvant DTH. The presence of *M. tuberculosis* acts as an antigen source to elicit the immune response in "tuberculin DTH", or as an adjuvant to enhance the immunogenicity of co-administered antigen in "protein-adjuvant DTH" (191). DTH responses are based on the macrophage-Th1 cell axis that involves: the activation of Th1 cells by antigen presenting cells, including macrophages, the recruitment of neutrophils, monocytes and T cells to DTH site, the production of inflammatory cytokines, such as IFN-γ by the activated T cells (191), which then activate the macrophages that accumulate at the DTH site (210). DTH responses and the persistence of the antigen results in the accumulation of activated macrophages and Th1 cells, thus forming a chronic "granulomatous" inflammatory response (191, 210). DTH models have been used to examine the recruitment of T cells and the relevance of adhesion molecules, like E- and P-selectins (96) and α_4-integrin (92, 211) to this process. The ability of DTH reactions to activate T cell responses was utilized to induce arthritis after the transfer anti-collagen antibodies in mice (212). While IL-4 was shown to reduce the development of DTH reactions (213), some studies demonstrated that the recruitment of eosinophils (211) and basophils are related to DTH (214). These cells are not as abundant in DTH as cutaneous hypersensitivity sites, but their presence in DTH correlated with an increase in the metabolism of histamine in DTH

in humans (214), so they may be involved in the development of DTH as suggested by studies in mice (215).

In addition, concanavalin (ConA) has been used to induce hemorrhagic skin lesions in mice (202). ConA is known to induce the activation of different immune cells. For instance, ConA treated monocytes were shown to form multinucleated giant cells, which was further enhanced by the addition of IFN-γ (216). Also, ConA can induce the production of a mediator for eosinophil recruitment into the skin of guinea pigs (203), and the production of histamine from mast cells or T cells (217, 218). ConA has been used as a model of hepatitis in mice, where it induces the recruitment of lymphocytes and eosinophils, and the production of several cytokines; IL-5, IL-10, IFN-γ and IL-17 (219, 220). In response to ConA, CD4 and CD8 T cells are known to produce not only IL-2, and IFN-γ, but also IL-4 and IL-10 (217). Though the exact mechanism of its activity is not understood, ConA can stimulate T cells by binding and crosslinking glycosylated proteins (221), like TCR, which would explain its ability to activate pyruvate oxidation (222) and increase the levels of intracellular cAMP and calcium in lymphocytes (223).

A number of models of dermal inflammation are based on the use of allergens such as house dust mite, or the use of irritants, like croton oil, tape stripping and acetone rubbing (106, 197, 204, 205). Allergens can induce both IL-4and IFN-γ. Their ability to induce ligands of CCR4 and CXCR3 was also shown, but this may vary between the different allergens (106, 204). The application of irritants was shown to induce lymphocyte infiltration, but they differ in their ability to induce CKs. For instance, tape-stripping was shown to induce more CCL5, CCL17 and CCL22 compared to acetone rubbing of skin in BALB/c mice (197). Studies have used tape-stripping in combination with allergen sensitization to enhance the induction of IL-4, and the infiltration of eosinophils into the skin (204). However, the ability of tape-stripping to induce eosinophil recruitment may be affected by mouse strains; being observed in BALB/c mice, but not in C57Bl/6 mice (205).

Furthermore, toll-like receptors (TLRs) were used as stimuli of acute inflammation in the skin. TLR 3 and 4 are known to respond to LPS and dsRNA respectively and to induce the production of cytokines and the activation of innate immune cells (224). *In vitro* studies have shown that several cell types, including

macrophages, endothelial cells and fibroblasts respond to TLR ligands, activate the NF-κB pathway and produce cytokines, like IL-1β and IL-6, and chemokines, such as CXCL9, 10 and 11 (105, 225). As a result, the intradermal administration of TLR agonists, such as LPS and poly I:C, was shown to recruit T cells into rat skin (93, 226), and promote the rejection of skin allografts in mice (227).

1.8.2 Contribution of Chemokine Receptors in Dermal Inflammation

The expression of CKRs by skin-infiltrating T cells and the presence of their ligands in biopsies of patients and animals with dermal inflammation (89, 90, 104, 110, 196, 198, 199, 228-236) suggests their contribution to the migration of T cells to inflamed skin. Studies have examined the effect of CKR blockade on the accumulation of T cells or the development of dermal inflammation (93, 194, 198, 236, 237). Yet, the expression of multiple CKRs, including CXCR3 and CCR4, on skin-infiltrating T cells makes it challenging to identify their contribution to T cell recruitment. Also, the ability of different T cell subsets to migrate to dermal inflammation has been shown (145, 238), but it is not clear if CXCR3 or CCR4 contributes to the infiltration of different T cells.

The expression of CXCR3 on skin-infiltrating T cells and the effect of CXCR3 blockade on the development of dermal inflammation suggest an association between CXCR3 and dermal inflammation. CXCR3 is found on ~50% of the skin-infiltrating CD4 and CD8 T cells (89, 104, 229, 230) and on a proportion of CD4 and CD8 T cells that express a skin-homing marker, namely CLA, in patients with psoriasis vulgaris (104, 229), atopic dermatitis (AD) (229) and allergic contact dermatitis (230, 239). Also, CXCR3 is found on most of unstimulated T cells migrating to skin sites injected with pro-inflammatory cytokines and TLR agonists in rats (93), and on most of the CD4 and CD8 T cells infiltrating skin grafts of mice (231). In addition, the presence of CXCR3 ligands in the lesions and serum of patients with skin inflammation (230, 232) and in skin grafts of mice (231) suggests a role for CXCR3 in T cell recruitment to inflamed skin. In fact, when CXCL10 was injected into human skin grafted on SCID mice, the adoptively transferred human CD4 cells were recruited to the grafts (238).

Blockade of CXCR3 was shown to reduce the development of inflammatory responses in the skin. Swelling and/or cell infiltration in CHS (240) and DTH (140), and

the rejection of skin grafts (231) was reduced in mice after blockade of CXCR3 or in mice that are deficient in CXCL10. Also, $CXCR3^{-/-}$ mice were found to have reduced infiltration of CD4 and CD8 T cells in *Leishmania major* infected skin (241). Though CXCR3 blockade inhibited most of the migration of *in vivo* activated T cells to dermal inflammation induced by proinflammatory cytokines and TLR agonists, the migration of memory CD4 cells was only reduced by 50% (93). Thus, CXCR3 is associated to dermal inflammation, but other CKRs are likely to be involved.

CCR4 is thought to be associated to skin inflammation, based on the increased expression of CCR4 in skin lesions, as in patients with atopic dermatitis (AD) (110), cutaneous lupus erythematosus (CLE) (233), cutaneous T cell lymphoma (CTL) (235), *Candida albicans* infected grafts (234), and *Candida* extract induced DTH (90), in which CCR4 ligands were also found (110, 233, 234). Several studies demonstrated the expression of CCR4 on 60-90% of the skin-infiltrating CD4 T cells (89, 90, 235), though in some cases, CCR4 was expressed only on ~30% of the CD4 T cells, as in *C. albicans* infected skin grafts (234). Interestingly, CCR4 is not only expressed on CD4 cells, identified as memory CD4 cells (89, 229), but it is also present on $Foxp3^+$ CD4 Treg cells. CCR4 is present on 60-90% of the $Foxp3^+$ T cells in *Paracoccidioides brasiliensis* induced lesions or in normal skin (242, 243), as well as on the CLA^+ Treg cells in the circulation (147, 149, 150). In addition to the expression of CCR4 on 30-60% of the circulating CD4 T cells (229, 233), CCR4 is expressed on 30-50% of the circulating CD8 T cells in patients with skin inflammation (233). Therefore, the increased expression of CCR4 in inflamed skin may reflect the infiltration of several T subsets.

The expression of CCR4 or the levels of its ligands in atopic patients was correlated to markers of disease severity, such as the number of skin-infiltrating T cells (144), the number of eosinophils in blood (110, 229, 232) or the levels of IgE in serum in patients with AD (229, 232). Since CCR4 ligands were co-localized with DCs (112) and keratinocytes in the lesional skin of AD patients (109), it is likely that that the production of CCL22 would recruit $CCR4^+$ CD4 cells *in vivo*. This notion is supported by the co-localization of $CCR4^+$ CD4 T cell with CCL22 producing DCs (234) and by the proximity of $Foxp3^+$ cells to CCR4 ligands in skin lesions (242). *In vitro* studies have also shown the migration of $CCR4^+$ CD4 cells to stimulated keratinocytes (107) and the

migration of Treg cells to CCR4 ligands derived from mature DCs (148) or from langerhans and macrophages from skin lesions (244) .

The expression of CCR4 and the infiltration of $CCR4^+$ CD4 cells was demonstrated in mouse models of CHS (196, 236), and this was shown to be further increased after the transgenic overexpression of CCL17 in keratinocytes (197). The intradermal administration of CCR4 ligands in human skin grafts on mice were found to recruit not only CD4 cells, but also CD8 cells (145, 238). Yet, CCR4 ligands induced the recruitment of Th2 cells, but not Th1 cells, to skin grafts, though both Th1 and Th2 cells can migrate to CCR4 ligands *in vitro* (145, 245). So the presence of CCR4 ligands and $CCR4^+$ T cells in skin may be related, but the role of CCR4 in skin homing may vary for different T cell subsets.

In addition, it was suggested that the deficiency of CCR4 may influence the skin homing of several T cells. Antigen specific $CCR4^{-/-}$ CD4 cells were shown to have reduced accumulation to antigen-injected sites in the skin (246, 247). However, the migration of *in vivo* activated $CCR4^{-/-}$ CD4 cells to CHS in mice was unaffected (237) or was increased in $CCR4^{-/-}$ mice (195). Studies also demonstrated the reduced migration of $CCR4^{-/-}$ Treg cells into normal skin (156). The inability of adoptively transferred $CCR4^{-/-}$ Treg cells to control the development of inflammatory skin disease (156) may be due to their reduced migration, given that their suppressive ability is normal when locally administered into islet allografts (154). Yet, there were more $Foxp3^+$ T cells in the CHS of $CCR4^{-/-}$ mice (195). Therefore, several T cell subsets express CCR4, but it is difficult to assess whether they require CCR4 for their migration to dermal inflammation.

Most of the studies that have examined the contribution of CXCR3 and CCR4 in the skin homing of T cells were based on histological studies (104, 233, 234) or measurements of CKs and CKR^+ T cells in the circulation of patients (32, 104, 110, 144, 147, 149, 150, 229, 232, 233). The expression of CCR4 on skin-infiltrating T cells was reported for several inflammatory diseases (90, 228, 234, 242), except for psoriatic lesions where only a few skin-infiltrating T cells express CCR4 (104, 228). These discrepancies may reflect differing contributions of CCR4 in response to different inflammatory stimuli, but this has not been examined. Most studies that examined the *in vivo* migration of T cells rely on the CHS model (195, 237, 246). In addition, in some

studies, the identification of skin-infiltrating CD4 cells was not sufficiently specific so as to understand the contribution of CCR4 to memory CD4, Th1, Th2, or Treg cells; all of which are CD4 cells that express CCR4 and migrate *in vitro* to CCR4 ligands (90, 145, 148, 242, 243). The increased transcript levels of CCR4, IL-4 and IFN-γ was concurrently observed in the OXA-induced CHS skin (196) or granulomatous inflammation in the lungs (171). Yet, the contribution of CCR4 in the migration of type 1 T cells and type 2 T cells is not clearly demonstrated.

In addition to CXCR3 and CCR4, CCR10 is expressed by skin-infiltrating T cells. CCR10 is found in the 30-40% of the T cells in skin biopsies and circulation of patients with inflammatory skin disease (90, 198, 228, 239, 248), and 20-30% of skin-infiltrating CD4 T cells in CHS in mice (195, 236). Its ligand, CCL27, is present in lesions of patients with AD, psorasis (198), GvHD (248), and in the skin of mice with CHS (198, 199), and was located on fibroblasts, endothelial and keratinocytes in these lesions (198). CCL27 treated endothelial cells were shown to enhance the adhesion of CLA^+ CD4 T cells *in vitro*, and CCL27 injected skin sites were shown to recruit T cells (198). Some studies demonstrated that blocking CCL27 was sufficient to reduce the recruitment of *in vivo* activated T cells to CHS by ~40% (198). While others observed that $CCR10^{-/-}$ CD4 cells can accumulate normally in antigen-injected skin sites (247), and that the absence of both CCR10 and CCR4 was necessary to reduce the number of T cells and/or CD4 T cells in CHS (194, 236, 237).

Interestingly, skin-infiltrating T cells may co-express different CKRs. CXCR3 blockade was shown to inhibit the migration of memory CD4 cells to dermal inflammation in rats (93), though most of these are $CCR4^+$ (89, 92). Also, a proportion of circulating $CCR4^+$ memory cells (90) or $CCR4^+$ CD4 cells in patients with GvHD (248) also express CCR10. Skin-infiltrating memory CD4 cells were also shown to express CCR5, as well as CCR4 and CXCR3 (89). In addition, CCR5 and CCR6 were shown to be expressed on 50-70% of the Treg cells that are present in normal skin (243), infected lesions (242) or in the peripheral blood of atopic patients (32). Since $CCR4^+$ T cells may express other CKRs like CXCR3, CCR5, CCR6 or CCR10, the presence of $CCR4^+$ CD4 cells in dermal lesions does not definitely show a requirement for CCR4. Thus, the

contribution of CXCR3 and CCR4 on the migration of T cells to dermal inflammation is not clear.

1.9 Chemokine Receptors in Arthritis

Arthritis encompasses a wide range of inflammatory diseases in the joints. They may be autoimmune in nature, like rheumatoid arthritis (RA) and juvenile RA (16). In another form of arthritis, osteoarthritis, the inflammatory response in the joints is thought to be initiated by mechanical factors, rather than by autoimmunity (192, 249). In addition, arthritis can be secondary to an inflammation in another tissue, such as psoriatic arthritis, which develops in some patients with psoriasis (250), or reactive arthritis, which may occur after infections in gastrointestinal or urogenital tracts by bacteria including *Salmonella, Campylobacter,* and *Chlamydia* (251). Another class of arthritis is the infectious arthritis, which involves a local infection of the joints by viruses or bacteria, including *Mycobacteria, Streptococcus* or *Staphylococcus aureus* (192, 251), as opposed to reactive arthritis whereby the inflammatory response in the joints results from an extra-articular infection (251). Therefore, a variety of different stimuli can induce the inflammatory response in the joints of patients with arthritic diseases.

1.9.1 Models of Arthritis

Several models of arthritis are used to examine the immunopathogenesis of joint inflammation. The most commonly used models of arthritis include the collagen-induced arthritis (CIA) in mice (252-258) and adjuvant arthritis (AA) in rats (259-263). However, several other models are utilized for the study of arthritis, such as K/BxN arthritis (264-266), collagen antibody induced arthritis (CAIA) (267-272), and streptococcal cell wall (SCW) or proteoglycan induced arthritis (273-277).

Collagen induced arthritis was developed in mice in 1980, and utilized the immunization with collagen type II and Complete Freund's adjuvant (CFA) for the development of paw swelling and joint inflammation with inflammatory cell infiltrate, pannus formation, and cartilage destruction (252). Histopathological changes in the inflamed joints of mice and rats with CIA have been shown to resemble that of human

RA (252, 278). Differences in strain and gender susceptibility that were observed in earlier studies (252-254) were overcome by modifying the immunization protocols, in terms of the source of collagen II (254, 255), dose of *Mycobacterium tuberculosis* (279), and the use of CFA during immunization and booster injections (256, 257).This enabled the development of CIA in 60-70% of the less susceptible strains like C57Bl/6 mice (255-257), and accelerated the development of CIA (258). Additional treatments were shown to accelerate the onset, potentiate the severity and/or maintain the progression of CIA. These included the administration of lipopolysaccharide (LPS) of *E. coli* and other bacterial species (258, 280), Staphylococcal enterotoxin B (SEB) (281, 282) and cytokines such as IL-1β (258, 283) and GM-CSF (284). These aimed at improving the T cell and/or B responses, as shown by the production of pro-inflammatory cytokines (280, 285) and the serum levels of anti-collagen II IgG2a/b antibody (280, 283, 285).

Other antigen-induced models in rodents used components of CFA, but not collagen II, to induce arthritis. Adjuvant arthritis (AA) was shown to develop after administration of *Mycoplasma arthritidis* (261) and *Mycobacterium butyricum* (259, 260). This involved the activation of T and B cell responses, as shown by increase in antibody levels, and the antigen - stimulated proliferation of lymphocytes (261). There is some overlap in the mechanisms by which AA and CIA are induced, since rats with AA had increased levels of antibodies to collagen II, the soluble fraction of *M. butyricum* and to IgG (259, 260), and they developed DTH responses to collagen II and *M. butyricum* (259). It was also shown that the administration of collagen prior to immunization delayed the onset of AA, while that of *M. butyricum* almost completely abrogated development of AA (259). The transfer of disease by lymphoid cells from rats with AA to normal rats was further potentiated by the transfer of anti-collagen II serum (262). Therefore, the development of AA and CIA may occur via though overlapping, but different mechanisms.

Another model, named antigen-induced arthritis (AIA), is also used to induce arthritis after the immunization of bovine serum albumin (BSA) and CFA (285). Since the co-administration of anti-collagen II monoclonal antibody (mAb) mixture was shown to enhance the duration of arthritic symptoms after challenge with the antigen (i.e. BSA), it is thought that the mechanisms involved in the development of AIA and CIA models

might differ. The model of AIA appears to involve type 1 cytokines, since IL-12$^{-/-}$ mice resisted the development of AIA (286). This model was also suggested to involve B cells and CD8 T cells, but not CD4 T cells, as shown by the transfer of disease into reconstituted SCID mice (212).

Many other models have been developed to examine different aspects of the immune response in arthritis. For instance, SCW or proteoglycan induced arthritis are induced using the cell wall of *Streptococcus pyogenes* alone or with IL-1β injection (273-275) or using the proteoglycan of *S. aureus* or *Lactobacillus* (275, 276). These mouse models of acute arthritis or repeated acute arthritis appears to involve TNF, IL-18, IFN-γ, IL-17, T cells and B cells (274, 277), but not neutrophils, NK cells, or monocytes (276). In addition, collagen antibody-induced arthritis (CAIA) was developed based on the ability of anti-collagen antibodies to induce arthritis that persist for up 3 weeks (267) and has been modified with the administration of collagen II or LPS (268, 269). Based on the development of CAIA in mice with different genetic backgrounds, CAIA is thought to depend on TNF and IL-1β (270) neutrophils (269, 271), complement (271, 272), but not T cells and/or B cells (268-270). Several other models of arthritis, such as K/BxN model, were developed with the use of transgenic mice, in which TCR recognizes a peptide of glucose-6-phosphoisomerase (G6PI) in the context of MHC II in NOD mice. The spontaneous development of antibodies against G6PI results in the induction of arthritis in these transgenic mice. The progression of arthritis involves not only CD4 T cells, and B cells (264, 266), but also complement factors and mast cell degranulation (265).

1.9.2 T Cells in Arthritis

Studies have shown that the development of arthritis involves both B and T cells. B cells are required for development of collagen induced arthritis (CIA), as shown by the reduced development of CIA in B-cell deficient mice (257, 287). Indeed, the levels of anti-collagen IgG2a antibody in immunized mice that do not develop arthritis are lower than in arthritic mice (253), indicating the importance of the CII-specific antibodies in the induction of arthritis. Yet, the transfer of antibodies was not sufficient to induce severe disease, but with the co-transfer of lymphoid cells from arthritic mice, the severity of the resultant arthritis was enhanced (254, 288). The importance of collagen-specific T cells

was first suggested by the proliferation of T cells from arthritic mice in response to collagen II (289, 290). The ability of lymphoid cells from immunized mice that were *in vitro* cultured with collagen II or were injected *in vivo* with collagen II to transfer disease also established the relevance of T cells in the development of arthritis (254, 290, 291).

Earlier studies examined the importance of CD4 and CD8 T cells in the development of arthritis. The depletion of CD4 T cells, but not CD8 T cells, was shown to inhibit the transfer of arthritis (254, 291). The involvement of CD4 cells in the early stages of arthritis development was suggested by their infiltration of CD4 T cells into the joints of DBA/1 mice during the early phase, but not later phase of CIA (292). This explains why anti-CD4 antibody inhibited the development of CIA only if it was given early during immunization (257, 293), and why the severity of CIA in $CD8^{-/-}$ mice was normal (294).

It was shown that CD4 and CD8 T cells in synovial fluid (SF) of patients with RA produce IFN-γ rather than IL-4; the proportion of Th1 and Tc1 cells were higher than the Th2 or Tc2 cells (17, 295). In fact, more Th1 cell clones migrated *in vitro* to supernatants derived from ST of patients with RA than did Th2 clones (296). It is known that the level of pro-inflammatory cytokines are increased as arthritis develops. The expression of IL-6, IL-1β and TNF in arthritic paws of mice with CIA was shown to reach maximum on days 29-35 post-immunization, which correlates to the time of maximal lymphocyte infiltration (297). The expression of IFN-γ, IL-12 and IL-18 were also shown to be increased in the draining LNs and the joints of mice with CIA or SCW (274, 279). CD4 T cells of arthritic mice are known to produce IFN-γ, which was shown to be increased in mice as the dose of *Mycobacterium* used in the immunization increases (253, 255, 279).

In addition, the presence of IL-17 in patients and mice with arthritis is reported, and it was related to the disease severity (277, 298, 299), though the proportion of $IL17^{+}$ T CD4 T cells is not as high as the $IFN\gamma^{+}$ CD4 T cells. The involvement of Th17 or Tc17 cells in arthritic disease was suggested by studies showing the inhibitory effect of anti-IL17 on the development of arthritis in IFN-$\gamma^{-/-}$ mice (300). In fact, IL-17 was shown to induce the production of pro-inflammatory cytokines, IL-6 and IL-18, by synovial fibroblasts (301). Though Th2 cytokines are thought to contribute to the resolution of the inflammatory disease, studies using IL-2Rβ/IL-4Rα transgenic mice suggest otherwise.

The development of CIA in these mice was enhanced, and was associated with an increased inflammatory infiltrate of eosinophils (302).

It should be noted that the differentiation of T cells in arthritis is affected by dendritic cells (DCs). The dose of *M. tuberculosis* used in the immunization influenced the maturation of DCs (279), and hence DCs that expressed more MHC II and CD80 improved the transfer of disease by CD4 T cells (303). This effect may be due to the role of mature DCs in the proliferation and cytokine production of collagen II-stimulated CD4 T cells (303). Also, their effect may be apparent through B cells responses; IL-12 treatment of immunized mice was shown to increase the serum levels of anti-collagen II IgG2a antibody and enhance the development of CIA (304).

1.9.3 Contribution of Chemokine Receptors in Arthritis

The upregulation of CKRs in biopsies of patients and animals with arthritis (89, 135, 151, 263, 297, 305-308) suggests the importance of CKRs in the recruitment of T cells into inflamed synovium. Attempts to identify the CKRs required for T cell infiltration were made by determining transcript levels of CK and CKRs by cells in synovial tissue (ST) or peripheral blood (PB), or by examining the effect of CKR blockade. Still, it is difficult to relate the requirement of CKRs to a certain T cell subset, since CKR can be expressed by different T cells that are present in the inflamed synovium, such as activated, memory and regulatory CD4 cells and CD8 cells. Also, the expression of multiple CKRs by T cells adds to the challenges of identifying the CKR required for T cell infiltration to inflamed joints.

CXCR3 is present on ~45% of CD4 T cells in the synovial fluid (SF) and 80-90% of the CD4 and CD8 T cells in the synovium of patients with RA or JRA (135, 305-307), while those in the circulation were 20-30% $CXCR3^+$ (135, 305, 307). The presence of CXCR3 ligands (306) and $CXCR3^+$ CD4 T cells in the SF or synovium of patients with RA (89, 309) suggests a contribution of CXCR3 in T cell recruitment. In fact, the *in vitro* migration of Th1 cell clones to ST cell derived supernatants from RA patients was shown to be partially inhibited using anti-CXCL10 (296). CXCR3 blockade was also shown to reduce the recruitment of *in vivo* activated T cells to inflamed joints in rats with AA by

~60% (310). When *in vivo* activated T cells were used to transfer disease, the severity of the arthritis in the recipients was also reduced by CXCR3 blockade (310).

In addition, CCR4 is expressed on a higher proportion of CD4 T cells in the SF (20-40%) than in the PB of patients with arthritis (5-20%) (89, 305, 311). The proportion of $CCR4^+$ CD4 T cells was shown to correlate to the disease duration in children with JRA (311) or the severity of arthritis in adult patients (312). Studies have shown that CCR4 ligands are present in the SF and plasma (311, 313), and that they are produced by mature DCs from patients with arthritis (314). Indeed, the proximity of $CCR4^+$ cells to $CCL22^+$ monocyte-like cells in the synovial tissue of patients with RA (313), may suggest the contribution of CCR4 ligands in recruiting T cells of inflamed synovium.

The expression of CXCR3 and CCR4 on CD4 T cells in the SF and synovium of patients with arthritis is described, yet some discrepancies are reported; CCR4 is present on as high as ~45% (305) or, in other reports, on 5-10% of the CD4 T cells (307, 315). Likewise, CXCR3 is found on 80-90% (89, 135, 307) or, in alternate studies, on 30-45% of the CD4 T cells in these tissues (305, 309). Differences in the proportion of $CCR4^+$ T cells appears to reflect differences in the proportion of CD4 and CD8 T cells in the SF of patients with JRA (311). Literature is also unclear on the identity of the $CCR4^+$ cells. Studies have reported that Treg cells or IL-4 producing CD4 cells in SF of patients with arthritis are $CCR4^+$ (151, 311). Thus, the transcript levels of CCR4 in arthritic rats (308) or the expression of CCR4 on $CD45RO^+$ CD4 T cells in patients (89, 309, 311, 313) may not sufficiently identify the different $CCR4^+$ cells. Therefore, the relationship between the expression of CXCR3 and CCR4 and the migration of different T cell subsets to inflamed joints is not established.

In addition to CXCR3 and CCR4, $CCR5^+$ T cells are present in the SF of patients with arthritis and so CCR5 may contribute to T cell recruitment. CCR5 is present on the majority of CD4 cells (60-85%) and CD8 cells (70-90%) in the SF and ST of patients with RA (89, 135, 306, 307, 309, 315, 316). Interestingly, CCR5 is co-expressed on 66-80% of the $CXCR3^+$ CD4 cells (135, 305) and on ~55% of the $CCR4^+$ CD4 cells (305), and ~12% of the CD4 T cells were found to express all three CKRs in patients with JRA (305). Therefore, the increased presence of CCR5 ligands in SF of patients with RA (306, 315) and in rats with AA (308) may result in the recruitment of $CCR5^+$ T cells that co-

express CXCR3 or CCR4. Studies have shown the efficacy of blocking of CCR5 or CCL5 in inhibiting ~70% of the *in vitro* migration of Th1 cell clones to RA ST cell derived supernatants (296). CCR5 antagonists were also found to reduce the development of CIA in mice (317, 318) and in rhesus monkeys (319). However, different observations were reported on the development of arthritis in $CCR5^{-/-}$ mice. The deficiency of CCR5 was found to reduce the incidence, but not the severity of CIA (320), or resulted in normal development of CIA (321). In contrast, $CCR5^{-/-}$ mice were shown to develop proteoglycan-induced arthritis with increased severity (322). It is possible that these observations reflect the effect of CCR5 deficiency on B cells rather than an effect on T cells, since the levels of anti-collagen antibodies were reduced in $CCR5^{-/-}$ mice (320) in contrast to the normal ability of $CCR5^{-/-}$ splenocytes to transfer disease to SCID mice (322). Deficiency of CCR5 may have also affected monocytes/macrophages, which express CCR5 in ST of rats with AA (263), or in SF of patients with RA (161, 316).

Furthermore, it is suggested that other CKRs, like CXCR4 and CXCR6, may contribute to the recruitment of T cells to inflamed synovium. A proportion of CD4 and CD8 T cells in SF of patients and mice with arthritis express CXCR4 (~60%) (323) and CXCR6 (30-60%) (324). The blockade of CXCL12 reduced the migration of *in vitro* activated CD4 and CD8 T cells across fibroblast-like synoviocytes (323), and the *in vitro* migration of Th1 cell clones to RA ST cell derived supernatants (by 40-60%) (296). Interestingly, when the T cell specific CXCR4 deficient mouse was generated, the deficiency of CXCR4 on *in vivo* activated T cells was shown to inhibit their infiltration into CIA-inflamed joints (325). This study demonstrated a role for CXCR4 on T cell infiltration, but it did not indicate if the effect was related to CD4 or CD8 T cell subsets.

In addition, CCR2 is thought to contribute to the T cell recruitment, due to its expression on ~20% of the circulating T cells in patients with RA (309), and on a proportion of memory CD4 cells and Treg cells in mice (326). In fact, the blockade of CCR2 was found to reduce the *in vitro* migration of Th1 cell clones to ST cell derived supernatants of patients with RA by 60-75% (296). Yet, *in vivo* studies demonstrated that CCR2, due to its expression on multiple T cell subsets, may play opposing roles as arthritis progresses. Blocking CCR2 during the initiation phase (day 0-15) was shown to reduce the severity of CIA, possibly due to the expression of CCR2 on memory CD4

cells (326). While CCR2 blockade during later stages (day 21-36) enhanced the severity of CIA in mice, demonstrating that the expression of CCR2 on Treg cells may have influenced their tissue migration and therefore their contribution to resolving the inflammatory response induced by CIA in mice (326). It is worthy to note that a contrasting observation was made in $CCR2^{-/-}$ mice. They were shown to have an increased severity or prolonged progression of CIA or CAIA, which was associated with increased infiltration of multiple cells, including both CD4 and CD8 T cells (321, 327).

Therefore, the presence of CXCR3 and CCR4 expressing T cells in SF of patients with RA or JRA (89, 135, 305-307, 311) does not demonstrate their relevance to T cell migration. T cells express other CKRs, such as CCR5, CXCR4, and CCR2, that might play a role in this process. Given that the same CKR can be expressed by several T cell subsets, it is important to determine the effect of CKR on *in vivo* migration of different T cell subsets in isolation.

1.10 Chemokine Receptors in Draining Lymph Nodes

Most of the knowledge on the function of CKRs in T cell migration to lymph nodes (LNs) is based on the migration of naïve T cells in normal LNs and the migration of the developing T cells in the thymus (94, 328-335). However, CKRs that regulate the migration of T cells to LNs during homeostasis might not necessarily reflect their migration into tertiary lymphoid structures that develop during chronic inflammation or into reactive LNs that drain inflammatory sites. Lymphoid tissues in different states of activation might differ in their chemokine profile (169, 196, 199, 336-339) and hence the involvement of CKRs for T cell infiltration is likely to differ. Though CXCR3 and CCR4 are suggested to contribute to this process (338-340), there is limited literature related to the CKR requirement for Th1 cell recruitment to LNs draining a site of immunization.

The classical view of T cell migration to lymphoid tissues involves the adhesion molecules, L-selectin and LFA-1, and the CKRs, CCR7 and CXCR4 (94, 328-330). The presence of CCR7 ligands on high endothelial venules (HEVs), the T cell area, and lymphatic endothelium has been demonstrated (331, 332). Deficiency of CCR7 or its ligands was shown to influence the structural organization of LNs, ands this lead to reduced accumulation of T cells in LNs (329), which may be due to the reduced adhesion to and migration across HEVs (332) and/or reduced motility of T cells (341). The ability of CCR7 to induce transmigration may also be related to its ability to trigger integrin dependent adhesion, as shown *in vitro* (331) and *in vivo* (332). Intra vital microscopy studies showed that the administration of CCR7 ligands induced the adhesion of T cells to HEVs (332), and enhanced the motility of T cells in the LNs (342).

The role of CCR7 is also highlighted by studies examining the thymic development of T cells The migration of developing T cells through the thymus involves a set of sequential changes in the expression of several G-protein coupled receptors; CXCR4, CCR9, CCR4, CCR7 and S1P receptor (334, 335). CCR7 is found on $CD4^-$ $CD8^-$ phenotype (DN1-2) and single positive (SP) T cells (333). Mice that lack CCR7 (CCR7 deficient) or lack the ability to express CCR7 ligands (*plt/plt*) were shown to have altered thymic architecture. They also had reduced numbers of DN cells in the subcortical zone and increased numbers of their progenitors in the cortico-medullar junction (333).

This deficiency also resulted in the inability of SP cells to enter medulla, which resulted in their accumulation in the cortex (343).

Nevertheless, T cells may possibly utilize other CKRs for this process, in that CCR7 may not be required by all T cells. CCR7 was shown to account for only ~40% of the $G_{\alpha i}$-dependent T cell motility in the LNs (341), or ~50% of the recruitment of Th1 cells to LNs, as demonstrated when CCR7 or its ligands were deficient (344). Other CKRs, such as CXCR4, were suggested to mediate part of the migration of $CCR7^{-/-}$ CD4 T cells, since the *in vitro* migration of IFN-γ producing CD4 cells to CXCL12 was unaffected by the CCR7 deficiency (344). Indeed, the adherence of T_{CM} to *plt/plt* HEVs, which do not produce CCR7 ligands, was diminished by the blockade of CXCR4 (345).

However, the CKRs involved in migration to LNs during normal conditions may differ from those involved in the migration to lymphoid tissue during inflammatory process. For instance, tertiary lymphoid tissues are present in chronic inflammatory diseases, including arthritis (346, 347) and chronic infectious diseases, such as *Helicobacter* induced hepatitis (348). These structures are morphologically similar to LNs and spleen, in that they are organized into T cells area and follicular dendritic cell area containing B cell follicles (349). They were shown to be induced by "inducer" $CD4^{+}CD3^{-}$ cells (350) or by mesenteric lymph node cells (351), and their organization was further developed in the presence of inflammatory stimuli, such as oxazolone on the skin (351), pristine (hydrocarbon oil based compound) in the peritoneum (349), or autoimmune gastritis (352).

It is known that the tertiary lymphoid structures recruit DCs, B cells and CKR^{+} T cells (353, 354), and that these cells interact to generate immune responses (355, 356). Studies have demonstrated the presence of ligands of CCR4, CCR5, CCR7, CXCR3, CXCR4 and CXCR5 in tertiary lymphoid tissues (349, 352). It should be noted that there is no consensus on the CKs induced in tertiary lymphoid tissue and in fact, ligands of CCR4 may be reduced in activated gastric LNs (352). Still, it is thought that as tertiary LNs develop their HEV-like structures and T and B-cell compartments (346), T cells are recruited because of their expression of CKR. For instance, the relevance of CXCR5 and CCR7 was shown by of the reduced development of tertiary lymph nodes found in $CXCR5^{-/-}$ and $CCR7^{-/-}$ mice (357). The effect of CKRs in the migration of T cells to

tertiary LNs may in turn influence the development of immune response. Therefore, the reduced development of tertiary LNs in CXCR5$^{-/-}$ and CCR7$^{-/-}$ mice (357) may explain the reduced development of antigen induced arthritis in these mice (354).

In addition, CKRs required for the recruitment of T cells to LNs may differ during an active inflammatory response. Reactive LNs that drain sites of inflammation are known to have an increased recruitment of lymphocytes (358). The subsequent recirculation of the lymphocytes through the efferent lymphatics contributes to the induction of immune responses, as reported with DTH responses in the skin (359) and tumor rejection in mice (360). In addition, reactive LNs are known to have other properties of inflammatory sites, such as the upregulation of ligands of E- and P-selectin (96, 196, 361) which are present on the accumulating T cells (246). Reactive LNs also have increased transcripts of CKs, such as ligands of CCR4, CCR5, CCR10 and CXCR3 (169, 196, 199, 337-339), and reduced expression of ligands for CCR7 and CXCR4 (362, 363). The expression of CKRs in draining LN was shown to differ depending on the inflammatory stimuli; the transcript levels of CCR4 and CCR10 were increased in draining LNs after OVA sensitization (340), while the expression of ligands of CXCR3 (CXCL9, 10 and 11) are increased in draining LNs after CFA immunization in mice (338). In some cases, as in LNs draining hepatic inflammation, the production of both CXCL10 and CCL22 was shown to be increased (339).

Th1 cells were previously shown to be recruited into LNs draining sites of skin DTH (361) or tumors (360), but the CKRs responsible for Th1 cell recruitment have not been determined. The production of CXCR3 ligands by TNF-stimulated endothelial venules was shown to increase the binding of monocytes (336) and the *in vitro* recruitment of IFN-γ producing CD11c^{+} DCs (364). *In vivo* blockade studies demonstrated the role of CXCR3 in mediating part of the recruitment of CXCR3^{+} tumor cells through afferent lymphatics to the draining LNs (338). The presence of CXCL10 in the LNs was also shown to mediate the retention of *in vivo* activated T cells in the LNs draining hepatic inflammation (339), and these observations implicate CXCR3 in the migration of Th1 cells to reactive LNs. However, other CKRs, like CCR5, may be involved; since blocking either CCL3 or CCL4 was shown to reduce ~60% of the recruitment of CD4 T cells to draining LNs during DNFB sensitization (337). Therefore,

it is unclear whether CCR4 or CXCR3 contributes to the recruitment of Th1 cells to reactive LNs.

It is important to examine the recruitment of activated T cells to draining LNs, as it may assist in the understanding the CKRs required for activated T cell migration to lymphoid tissue during acute and chronic inflammatory responses. Though IFN-γ producing CD4 T cells can migrate to ligands of CCR7 or CXCR4 *in vitro*, the migration of $CCR7^{-/-}$ Th1 cells into LNs in DNFB sensitized mice was reduced only by ~50% (344). The ligands of other CKRs that were found in the activated LNs does not indicate the contribution of their corresponding CKRs, such as CXCR3 and CCR4, to the recruitment of Th1 cells to activated LNs.

1.11 Rationale And Objectives

The chemokine receptors that mediate the migration of T cells to inflammatory sites are not well characterized. CXCR3 and CCR4 are related to inflammatory diseases in the skin and joints, but their contribution to T cell migration is not clear. The literature does not define the extent by which these chemokine receptors are required for the different T cell subsets. In addition, the relationship between the expression of CXCR3 and CCR4 on T cells and their migration to activated lymph nodes is not known.

In order to examine the contribution of CXCR3 and CCR4 in the migration of T cells to inflammatory sites in the skin, joints and lymph nodes, specific objectives were defined to examine the following:

1. Migration of T cells to dermal inflammation, by determining the:
    a. Effect of CCR4 expression on memory and activated CD4 cell migration to dermal inflammation.
    b. Effect of CXCR3 deficiency on the *in vivo* migration of T cells to dermal inflammation.
    c. Effect of CCR4 deficiency on the *in vivo* migration of T cells to dermal inflammation.
2. Effect of CXCR3 and CCR4 deficiency on the *in vivo* migration of activated T cells to joint inflammation, and the effect of CCR4 deficiency on the development of collagen-induced arthritis.
3. Effect of deficiency of CXCR3 and CCR4 on the accumulation of Th1 cells in lymph nodes draining a site of immunization

Chapter 2. Materials and Methods

2.1 Animals

Inbred male Lewis rats, 6 - 8 weeks old weighing 200 – 250 g, were purchased from Charles River Canada (Lasalle, PQ, Canada), and Brown Norway rats, weighing 150 – 200 g, were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN, USA). C57Bl/6 mice, 6 - 8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and Charles River Laboratories (Lasalle, PQ, Canada). CCR4 deficient mice ($CCR4^{-/-}$) on C57Bl/6 background were kindly provided by Dr. Steven Kunkel (University of Michigan, Ann Arbor, MI, USA). CXCR3 deficient mice ($CXCR3^{-/-}$) on the C57Bl/6 background were supplied by the Jackson labs. All animals were housed in the IWK Animal Care Facility and maintained on standard chow and water supplied *ad libitum*.

2.2 Culture Medium, Reagents and Antibodies

RPMI 1640 (RPMI) medium (Sigma-Aldrich, Oakvile, ON, Canada) was supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone Laboratories, Burlington, ON, Canada), 50 U/ml Penicillin, 50 μg Streptomycin (Invitrogen, Montreal, QB, Canada), and 0.05 mM 2-β-mercaptoethanol (Sigma-Aldrich).

Tyrodes medium (T). A 10x stock solution of tyrodes medium (T) was prepared by NaCl (80 g), KCl (2 g), NaH_2PO_4.H2O (0.5 g), $NaHCO_3$ (10 g) and Dextrose (D-glucose) (10 g) per 1000ml total volume.

Table 4. Reagents used.

Reagents	**Purchased from**
7-AAD (7-amino-actinomycin D)	eBioscience, San Diego, CA, USA
Acetic Acid	BDH
Annexin V – FITC	BD Pharmingen, Mississauga, ON, Canada
Arlacel ® A	Acros Organics, Ceel, Belgium
Brefeldin A solution	eBioscience
CFSE (5,6- Carboxyfluorescein diacetate)	Sigma-Aldrich
Chicken sternum collagen type II	Axxora, San Diego, CA, USA or Cedarlane laboratories, Ontario, Canada
Concanavalin A	Sigma, Saint Louis, Missouri, USA
Dulbecco's phosphate buffered saline (PBS)	Invitrogen, Montreal, QB, Canada
DiD (1,1'-dioctadecyl-3,3,3',3'-tetra-methyl indodicarbocyanine, 4-chlorobenzenesulfonate salt)	Invitrogen
Dimethyl Sulphoxide (DMSO)	Sigma-Aldrich
Anhydrous Ethyl Alcohol (Ethanol)	Commercial Alcohols Inc., Brampton, Ontario, Canada
Foxp3 staining buffer set (fixation / permeabilization and permeabilization buffers)	eBioscience
FTY720	Cayman Chemicals Co., Ann Arbor, MI, USA
Hanks buffered saline solution (HBSS) (with or without $NaHCO_3$, Calcium chloride, and Magnesium sulphate are denoted $HBSS^{++}$ or $HBSS^{--}$ respectively)	Invitrogen
Heavy mineral oil	Fisher Chemical, Ottawa, ON, Canada
HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])	Sigma-Aldrich
Human serum albumin (HSA)	Canadian Blood Services
Incomplete Freund's adjuvant	Difco laboratories (Detroit, MI, USA)
Ionomycin calcium salt	Sigma-Aldrich
Lipopolysaccharide (LPS) of *Escherichia coli* 0111	List Biologics (Cambell, CA, USA)
Heavy Mineral oil	Fisher Chemical, Fair Lawn, NJ, USA
Mycobacterium butyricum - Non-viable, dessicated	Difco laboratories
Mycobacterium tuberculosis H37 RA - Non-viable, dessicated	Difco laboratories
Ovalbumin	Sigma-Aldrich
PMA (Phorbol 12-myristate 13-acetate)	Sigma-Aldrich
Poly inosine:cytosine (poly I:C)	Sigma-Aldrich
Rapamycin	Cayman Chemicals
Trypan Blue	Sigma-Aldrich

Table 5. Primary antibodies used.

Specificity	Description	Host	Isotype	Clone	Source
CD3	α-mouse CD3	Hamster	IgG_1	145.2C11	Hybridoma, ATCC (American type culture collection)
CD4	α-mouse CD4 (L3T4)	Rat	IgG_{2a}, κ	RM4-5, GK1.5	eBioscience
CD4	α-rat CD4	mouse	IgG_1	W3/25	Hybridoma, ATCC
CD8a	α-mouse CD8a (Ly-2)	Rat	IgG_{2a}, κ	53-6.7	eBioscience
CD8	α-rat CD8	mouse	IgG_1, κ	Ox-8	Serotec (Raleigh, NC)
CD11b	α-mouse/human CD11b (Mac-1α, integrinα_M)	Rat	IgG_{2a}	M1/70	Hybridoma, ATCC
CD19	α-mouse CD19	Rat	IgG_{2a}, κ	eBio1D3	eBioscience
CD25	α-mouse CD25 (IL-2Rα)	Rat	IgG_1, λ	PC61.5	eBioscience
CD28	α-mouse CD28	Golden Syrian Hamster	IgG	37.51	eBioscience
CD28	α-rat CD28	Mouse	IgG_1	JJ319	eBioscience
CD44	α-mouse / human CD44	Rat	IgG_{2b}, κ	IM7	eBioscience
CD45RB	α-mouse CD45RB	Rat	IgG_{2a}, κ	C363.16A	eBioscience
CD45RC	α-rat CD45RC	Mouse	IgG_1, κ	Ox-22	Immunotools, Friesoythe, Germany
CD49d	α-mouse α_4 integrin	Rat	IgG_{2b}	PS/2	Hybridoma, ATCC
CD49d	α-rat α_4 (anti-CD49d)	Mouse	IgG_1	TA-2	Hybridoma, Developed in our lab(*)
CD62L	α-mouse CD62L (Ly-22, L-selectin, LECAM-1)	Rat	IgG_{2a}, κ	MEL-14	eBioscience
CD161a	α-rat CD161a (NKR-P1A)	Mouse	IgG_1, κ	10/78	BD Pharmingen
CCR4	α-rat CCR4	Hamster	$IgG_{2,3}$	4F8.1B7 (CR4.1)	Hybridoma, Developed in our lab(*)

Specificity	Description	Host	Isotype	Clone	Source
CXCR3	α-mouse CD183 (CXCR3)	Armenian Hamster	IgG	CXCR3-173	eBioscience
Foxp3	α-mouse/rat Foxp3	Rat	IgG_{2a}, κ	FJK-16s	eBioscience
Goat-a-mouse IgG	α-mouse IgG	Goat	IgG		Sigma-Aldrich
Goat-a-rat IgG	α-rat IgG	Goat	IgG		Sigma-Aldrich
HRL3	α-rat L-selectin	Hamster			Hybridoma, ATCC
IgG kappa light	α-mouse IgG kappa light chain	Rat	IgG_1	187.1	Hybridoma, ATCC
IFN-γ	α-mouse IFN-γ	Rat	IgG_1, κ	XMG1.2	eBioscience
IL-4	α-mouse IL-4	Rat	IgG_1, κ	11B11	eBioscience
IL-10	α-mouse IL-10	Rat	IgG_{2b}, κ	JES5-16E3	eBioscience
IL-12	α-mouse IL-12/IL-23 (p40 subunit)	Rat	IgG_{2a}, κ	C17.8	eBioscience
NK-1.1 (NKR-P1C, Ly-55)	Anti-mouse	mouse	IgG_{2a}, κ	PK136	eBioscience
TCRα/β	α-rat α/β TCR	mouse	IgG1, κ	R7.3	eBioscience

(*) each of these blocks the adhesion function of its respective antigens based on *in vitro* and *in vivo* studies (365-367).

Also, mouse E-selectin and mouse P-selectin chimera constructs were fused to human μ-chain (kind gift from Drs. J. Lowe and L. Stoolman, University of Michigan, Ann Arbor, MI, USA). E-selectin (RME-1; IgG_1) and P-selectin (RMP-1;IgG_{2a}) chimeras were used to detect expression of ligands of E- and P-selectin on lymphocytes.

Table 6. Secondary and tertiary antibodies used.

Antibody	**Source**
Goat-α-rat IgG	Jackson ImmunoResearch Labs, West Groove, PA, USA
Goat-α-rat Alexa Fluor 647	Invitrogen - Molecular Probes Inc. (Eugene, OR, USA)
Goat-α-mouse Alexa Fluor 647 (Mouse IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3)	Invitrogen - Molecular Probes Inc.
α-human IgG PE	Jackson ImmunoResearch Labs
Mouse-α-hamster IgG1/2/3 biotin (Mouse IgG_1, κ and IgG_{2b}, κ reacts with Armenian hamster IgG_1, IgG_2, and IgG_3, and Syrian hamster IgG)	BD Pharmingen
Goat-α-human IgM biotin	Jackson ImmunoResearch Labs
Streptavidin PE	BD Pharmingen
Streptavidin PE-Cy5	BD Pharmingen

Chemokines and cytokines were purchased from PeproTech Inc (Rocky Hill, NJ, USA) and contained very low endotoxin (<0.01 ng/ mg; 1 EU/mg). These were:

1. CCL17: Recombinant human TARC.
2. CCL22: Recombinant human or murine MDC.
3. IFN-γ: Recombinant murine or rat IFN-γ.
4. IL-2: Recombinant human IL-2.
5. IL-4: Recombinant mouse IL-4.
6. IL-12: Recombinant murine IL-12.
7. TGF-β2: Recombinant human TGF-β2.
8. TNF-α: Recombinant murine TNF-α.

2.3 Collagen Induced Arthritis

An emulsion was prepared from chicken collagen type II and Complete Freund's adjuvant (CFA) as previously described (256, 257). Chicken collagen II (Biocol, Cedarlane laboratories) was dissolved in 10 mM acetic acid at 4 mg/ml overnight with continuous mixing at 4 °C. CFA was prepared by combining 100 mg of heat killed *M. tuberculosis* (H37Ra, Difco laboratories, Detroit, MI, USA) with 20 ml of incomplete Freund's adjuvant that was made of 85% heavy mineral oil (Fisher Scientific, Ottawa, ON, Canada), and 15% Arlacel ® A (Acros Organics, Ceel, Belgium). The emulsion was prepared by mixing equal volumes of CFA and chicken collagen type II with a hand homogenizer (Fisher Scientific).

Mice were immunized with the prepared emulsion to induce collagen-induced arthritis. Each mouse was injected subcutaneously at the base of the tail with 100 μl of the emulsion (50 μl per side). Mice were boosted after 21 days; an emulsion of collagen II and CFA was freshly prepared and was injected subcutaneously at the base of the tail at a site close to the site of primary injection. Mice were weighed and clinically scored on alternate days starting day 21 till day 31, using a scoring system that was modified from other references (256, 303). Three areas were scored in each paw. For example, a separate score was given to ankle area, midfoot area, and toe area in each hindpaw. For ankle and midfoot areas, the scoring was as follows: 0 for no symptoms, 1 for redness, 2 for mild swelling, and 3 for severe swelling. For each digit in the toe area, the scoring was as follows: 0 for no symptoms, 1 for swelling and/or redness. This gives a maximum total score of 11 for each frontal paw and 10 for each hind paw, therefore the maximum possible total score is 42 in each mouse.

2.4 T Cell Isolation from Rats

2.4.1 Isolation of CD4 Cells from Rat Splenocytes

Rats were humanely euthanized by cardiac embolism while anesthetized. The spleen was aseptically removed, and the splenocytes were isolated by finely mincing the spleen to produce a single cell suspension. The stroma was allowed to settle for 5 min at 1 *g*. The resultant cell suspension was resuspended in RPMI medium and washed. The red blood cells were lysed with 3 – 4 ml of warm ammonium chloride (0.8%, pH 7.4)

(Fisher Scienific) for 60 seconds, and the splenocytes were washed twice in RPMI. To purify spleen T cells, the splenocytes were resuspended in RPMI plus 10% heat-inactivated FCS and applied onto a nylon wool column (Polysciences Inc., Warrington, PA, USA). After 60 min. of incubation at 37 °C, the unbound lymphocytes were eluted, washed, and resuspended in fresh RPMI + 10% FCS (366). Negative selection was used to isolate CD4 cells. Nylon-wool enriched rat lymphocytes (at 1 $x10^8$ cells/ml) were treated with mouse anti-rat CD8 mAb (100 μg/ml) and mouse anti-rat CD161a mAb (25 μg/ml) in RPMI + 5% FCS for 40 minutes on ice. The treated lymphocytes were washed twice with HBSS containing 2% FCS to remove excess antibody. Cells were resuspended in HBSS containing 10% FCS and panned using a bacteriological petri plate that was coated with 10 μg/ml goat anti-mouse IgG (Sigma-Aldrich) for 40 minutes at 4°C to remove CD8 cells, B cells and NK cells. Non adherent cells were collected and washed in RPMI. The resultant population was > 95% CD4 cells as determined by flow cytometry.

2.4.2 Isolation of Memory CD4 Cells from Rat Splenocytes

In order to isolate memory CD4 cells, CD4 splenocytes were isolated as in section 2.4.1, except that anti-CD8, anti-rat CD161a and anti-CD45RC (100 μg/ml) were used.

2.4.3 Isolation of CD4 and CD8 Cells from Rat Lymph Nodes

Rats were euthanized as mentioned in section 2.4.1, and the cervical, auxiliary, and mesenteric lymph nodes (LNs) were aseptically removed. Lymphocytes were obtained by gently mincing the LNs to produce a cell suspension. The stroma was allowed to settle for 5 min at 1*g*. The resultant cell suspension was resuspended in RPMI + 10% FCS, washed and passed through nylon wool column; with a maximum of 1 x 10^8 cells per 10 ml column. After 60 min of incubation at 37 °C, the unbound lymphocytes were eluted, washed, and resuspended in fresh RPMI + 10% FCS (366). CD4 cells were isolated using negative selection, as outlined in section 2.4.1. In order to isolate CD8 cells, the same procedure was followed, except that anti-CD4 mAb (25 μg/ml) was used for negative selection instead of anti-CD8 mAb.

2.4.4 *In Vitro* Activation of Rat CD4 T Cells

To induce polyclonal T-cell activation, CD4 T cells were isolated from lymph nodes of rats, as outlined in section 2.4.3. Cells were cultured in RPMI + 10% FCS on 25 cm^2 flasks (Nunc; Fisher Scientific) coated with 2 μg/ml anti-rat TCR αβ mAb (R7.3). T cells were cultured at 1.5 x 10^5 cells/ml in media supplemented with anti-CD28 mAb (0.4 μg/ml; JJ319), human IL-2 (20 U/ml for CD4 cells or 200 U/ml for CD8 cells), and murine IL-12 (10 ng/ml). After 3 days, the cells were replated and expanded at 3 x 10^5 cells/ml, in the presence of 20 U/ml IL-2 for another 2 days. Cell counts and cell viability was determined using trypan blue exclusion assay

2.4.5 Isolation of $CCR4^+$ and $CCR4^-$ Memory or Activated Rat CD4 T Cells

To obtain $CCR4^+$ and $CCR4^-$ cells, memory CD4 cells were isolated as outlined in section 2.3.2 or activated CD4 cells were obtained as in section 2.3.4. CD4 T cells were resuspended at 10^8 cells/ml in Calcium and Magnesium deficient HBSS ($HBSS^{--}$) + 10% FCS, and were treated with anti-CCR4 mAb CR4.1 (80 μg/ml) for 15 min at 10°C. Cells were washed and treated with biotinylated mouse anti-hamster mAb (BD Biosciences) for 15 min at 10°C. Cells were washed and treated with streptavidin magnetic beads for 15 min at 10°C (Miltenyi Biotec, Auburn, CA, USA). The cells were washed, resuspended in degassed $HBSS^{--}$ + 10% FCS and passed through a MACS column in a magnetic field. Flow-through cells were >98% $CR4.1^-$, while the adherent cells washed through in the absence of magnetic field were ~85-95% $CR4.1^+$. Purities of flow-through and adherent cells were determined by flow cytometry.

2.5 T Cell Isolation from Mice

2.5.1 Isolation of CD4 Cells from Mouse Splenocytes

Mice sedated with intraperitoneal (i.p.) injection of xylazine and ketamine were humanely euthanized by cervical dislocation. The spleen was aseptically removed and the T cells were prepared from the splenocytes, as outlined in section 2.4.1. This was followed by negative selection to isolate CD4 cells from splenocytes. Mouse splenocytes (at 1 x10^8 cells/ml) were treated with rat anti-mouse CD8 mAb (30 μg/ml), anti-CD11b mAb and anti-mouse IgGκ mAb (50 μg/ml) in RPMI + 5% FCS for 40 minutes on ice.

The treated splenocytes were washed twice with HBSS containing 2% FCS to remove excess antibody. Cells were then were resuspended in HBSS containing 10% FCS and panned using a bacteriological petri plate that was coated with 10 μg/ml goat anti-rat IgG (Sigma-Aldrich) for 40 minutes at 4°C to remove CD8 cells, B cells and $CD11b^{+}$ cells. Non adherent cells were collected and washed in RPMI. The resultant population was ~ 95% CD4 cells as determined by flow cytometry.

2.5.2 Isolation of Memory CD4 Cells from Mouse Splenocytes

In order to isolate memory ($CD44^{hi}$ $CD45RB^{lo}$) or naïve ($CD44^{lo}$ $CD45RB^{int}$) CD4 cells from mouse splenocytes, memory and naïve cells were sorted using FACS ARIA cell sorter (BD Bioscience). Isolated splenocytes (section 2.5.1) were resupended at 1 $x10^{8}$ cells/ml and treated with flurorochome labeled antibodies: anti-mouse CD8 mAb (5 μg/ml), anti-mouse NK mAb (2 μg/ml), anti-mouse CD44 (10 μg/ml) and anti-mouse CD45RB (20 μg/ml) in RPMI + 5% FCS for 40 minutes on ice. Then, $CD44^{hi}$ $CD45RB^{lo}$ cells and $CD44^{lo}$ $CD45RB^{int}$ cells were sorted by gating on the $CD8^{-}$ $NK1.1^{-}$ cells (which were CD4 cells as determined by flow cytometry). Cells were collected in conical tubes pre-coated with 4% BSA in PBS for at least 1 h at 4 °C.

2.5.3 Isolation of CD4 and CD8 Cells from Mouse Lymph Nodes

Mice were euthanized as mentioned in section 2.5.1, and the cervical, auxiliary, inguinal and mesenteric LNs were aseptically removed. Lymphocytes were obtained by gently mincing the nodes to produce a cell suspension. The stroma was allowed to settle for 5 min at 1*g*. The resultant cell suspension was resuspended in RPMI + 10% FCS and washed. CD4 cells were isolated using negative selection as outlined in section 2.5.1. In order to isolate CD8 cells, the same procedure was followed, except that anti-CD4 mAb (25 μg/ml) was used instead of anti-CD8 mAb for negative selection.

2.5.4 *In Vitro* Activation of CD4 and CD8 Cells in Type 1 or Type 2 Polarizing Conditions

To induce polyclonal T-cell activation, T cells were isolated from LNs of mice, as explained in section 2.5.3. Cells were cultured in RPMI + 10% FCS on 24 well- flat bottom tissue culture plates (Costar, Corning Incorporated, NY, USA) coated with 2 μg/ml anti-mouse CD3 mAb (145.2C11). The culture medium was supplemented with anti-CD28 mAb (2 μg/ml, 37.51, eBioscience), and human IL-2 (10 U/ml for CD4 cells or 200 U/ml for CD8 cells) (9, 368).

In order to induce type 1 polarization, T cells were cultured at 1.5 x 10^5 cells/ml in media supplemented with mouse IL-12 (1 ng/ml). After 3 days, the cells were expanded at 3 x 10^5 cells/ml, in the presence of 20 U/ml IL-2 for another 3 days. To induce type 2 polarization, cells were cultured at 5 x 10^5 cells/ml in media supplemented with 10 ng/ml mouse IL-4 and 5 μg/ml anti-mouse IL-12 mAb (C17.8, eBioscience) for 3 days. Cells were then expanded at 5 x 10^5 cells/ml in the presence of 20 U/ml IL-2 and 10 ng/ml IL-4 for another 3 days. Cell counts and cell viability was determined using trypan blue exclusion assay. The production of IFN-γ and IL-4 by *in vitro* activated T cells was determined by intracellular staining (as explained in section 2.10) to confirm the polarization of type 1 CD4 or CD8 T cells (denoted as Th1 or Tc1 respectively) or type 2 CD4 or CD8 T cells (denoted as Th2 or Tc2 respectively).

2.5.5 Isolation of $CCR4^+$ and $CCR4^-$ from Activated Mouse CD4 Cells

To obtain $CCR4^+$ and $CCR4^-$ cells, activated CD4 cells were generated as outlined in section 2.5.4. CD4 T cells were resuspended at 1 x 10^8 cells/ml in sort buffer [PBS^{--} +1mM EDTA + 15 mM HEPES + 0.5% bovine serum albumin (BSA) at pH 7.0]. Cells were incubated with anti-CCR4 mAb CR4.1 (75 μg/ml) for 15 min in the dark at 14°C, and were washed twice with sort buffer. Cells were incubated with biotinylated mouse anti-hamster IgG Ab (BD Biosciences), were washed twice and incubated with Streptavidin-PE or streptavidin PE-Cy7 for 15 min in the dark at 14 °C. Cells were washed once and resuspended at 3 – 4 x 10^7 cells/ml in sort buffer, in preparation for cell sorting with a FACS ARIA cell sorter (BD Bioscience). $CCR4^+$ and $CCR4^-$ cells were collected in conical tubes pre-coated with 4% BSA in PBS for at least 1 h at 4 °C.

2.5.6 Isolation and *In Vitro* Culture of Regulatory CD4 Cells from Mouse Splenocytes

In order to isolate regulatory CD4 cells, CD4 splenocytes were isolated from mice as mentioned in section 2.5.1. Cells were resuspended at 5 x 10^7 cells/ml in sort buffer, and incubated with fluorochrome labeled anti-CD4 mAb (2 µg/ml,GK1.5, eBioscience) and anti-CD25 mAb (1.3 µg/ml, PC61.5, eBioscience) for 30 min in the dark at 14 °C. The cells were then washed once and resuspended at 3 – 4 x 10^7 cells/ml in sort buffer in preparation for cell sorting with a FACS ARIA (BD Bioscience). $CD4^+$ $CD25^+$ cells were collected in conical tubes pre-coated with 4% BSA in PBS for at least 1 h at 4 °C. Cells were expanded *in vitro* as follows. They were cultured at 1.5 x 10^5 cells/ml in RPMI + 10% FCS on 24 well-flat bottom tissue culture plates (Costar, Corning Incorporated, NY, USA) that were coated with 2 µg/ml anti-mouse CD3 mAb (145.2C11). The culture medium was supplemented with 1 µg/ml anti-mouse CD28 mAb, 500 U/ml human IL-2 for 3 days. Then, cells were expanded at 3 x 10^5 cells/ml in the presence of 500 U/ml human IL-2 for another 2-3 days.

In addition, CD4 T cells were cultured in conditions that allow for the *in vitro* expansion and induction of $Foxp3^+$ T cells (48-50). CD4 T cells were cultured at 1.5 x 10^5 cells/ml in RPMI + 10% FCS on 25 cm^2 flasks (Nunc Sigma-Aldrich) or in 24 well-flat bottom tissue culture plates (Costar, Sigma-Aldrich) that were coated with 2 µg/ml anti-mouse CD3 mAb (145.2C11). The culture medium was supplemented with 1 µg/ml anti-mouse CD28 mAb, 500 U/ml human IL-2, 5 ng/ml TGF-β, and 100nM Rapamycin (Cayman Chemicals) for 3 days. Then, the cells were cultured at 3 x 10^5 cells/ml and expanded further in the presence of 500 U/ml human IL-2, 5 ng/ml TGF-β, and 100 nM Rapamycin for another 2-3 days. On a daily basis, cell counts and cell viability was determined by trypan blue exclusion assay. Also, Foxp3 expression in CD4 cells was determined by the intracellular staining of on CD4 cells, and >80% were $Foxp3^+$.

2.6 *In Vivo* Migration of Labeled T Cells to Dermal Inflammation

2.6.1 *In Vivo* Migration of Labeled T Cells to Dermal Inflammation in Rats

The migration of T cells to dermal sites in rats was measured using $Na_2{}^{51}CrO_4$ or ^{111}In oxine (Amersham, Biosciences Corporation, Piscataway, NJ) radioisotope-labeled T cells (93, 366). T cells resuspended at 5 x 10^7 cells/ml were incubated with 50 μCi/ml $Na_2{}^{51}CrO_4$ for 45 min in $HBSS^{--}$ + 10% FCS + 15mM HEPES, or T cells at 1 x 10^8 cells/ml were incubated with 10 μCi/ml ^{111}In-labeled oxine for 10 min in RPMI. Labeled cells were washed twice, once with RPMI + 10% FCS, then with RPMI. Cells were resuspended in RPMI for injections. A volume of 20 μl of labeled cells were sampled and the radioactive counts was determined prior to their injection into the animals.

Rats were anesthetized with aerosolized halothane/nitric oxide and injected i.v. with 5 – 10 x 10^6 T cells having 1- 5 × 10^5 cpm were injected i.v. in volume of ~300 μl. Immediately afterward, the skin on the backs of the animals was shaved and inflammatory agents or vehicle alone were injected intradermally at different sites using 30-gauge needles in volume of 50 μl. Inflammatory stimuli included: TNF-α (10 ng), IFN-γ (300 U), LPS (10 ng), and Poly I:C (200 ng). Each of these stimulants were previously shown to recruit T cells to dermal sites over 6 – 20 hours (93, 226). Control sites were injected with diluent (RPMI + 0.1% HSA). Animals were sacrificed after 20 h, the skin on the backs was removed and blood in the superficial veins was squeezed out. Dermal sites were collected with a 12-mm leather punch. In addition to the skin sites, blood, lymphoid tissues, liver, and lung were collected, weighed and their radioisotope content determined by Wallac Wizard III gamma radioisotope counter (Wallac, Gaithersburg, Germany). Results are expressed as counts per minute (cpm) of radioisotope accumulated over the migration period per mg tissue or per 0.3 ml blood normalized to 10^5 cpm labeled cells injected.

For DTH reactions, rats were sensitized by subcutaneous injection with 100 μl of an emulsion containing 50 μg ovalbumin and 12.5 μg *M. butyricum* in mineral oil. After 9-11 days, DTH was elicited by injecting ovalbumin (10 μg, 50μl in diluent) in 2 dermal sites. After 20 h , tissues were processed as mentioned above.

2.6.2 *In Vivo* Migration of Labeled T Cells to Dermal Inflammation in Mice

The migration of T cells to dermal sites in mice was measured using radioisotope-labeled T cells, as outlined above in section 2.6.1. Animals were anesthetized with isofluorane and injected i.v. with 1 – 4 x 10^6 T cells. Immediately afterward, the skin on the backs of the animals was shaved and inflammatory agents or vehicle alone (10 µl) were injected intradermally using 30-gauge needles. Inflammatory stimuli included LPS (5 ng), Poly I:C (200 ng) and Concanavalin A (ConA, 10µg). Each of these stimulants were previously shown to recruit T cells to dermal sites over 6 – 20 hours (93, 98, 226). Control sites were injected with diluent (RPMI + 0.1% HSA). After 20 h, animals were sacrificed, the skin on the backs of the animals was removed, blood in the superficial veins was squeezed out, and dermal sites were collected with a 8-mm leather punch. In addition to the skin sites, blood, lymphoid tissues, liver, and lung were collected, weighed and their radioisotope content determined by Wallac Wizard III gamma radioisotope counter (Wallac, Gaithersburg, Germany). Results are expressed as counts per minute (cpm) of radioisotope accumulated over the migration period per mg tissue or per 0.3 ml blood normalized to 10^5 cpm labeled cells injected.

For DTH reactions, mice were sensitized by subcutaneous injection with 50 µl of an emulsion containing 25 µg ovalbumin and 6.25 µg *M. butyricum* in mineral oil. After 9 – 11 days, DTH was elicited by injecting ovalbumin (10 µg, 10 µl in diluent) in 2 dermal sites. After 20 h , tissues were processed as mentioned above.

2.7 *In Vivo* Migration of Labeled T Cells to Inflamed Paws in Arthritic Mice

The migration of T cells to mouse paws was measured as follows. Mice were intravenously (i.v.) injected with 1 – 4 x 10^6 T cells that were labeled with radioisotopes (as outlined above in section 2.6). After 5 hours, mice were euthanized, and the blood, normal and inflamed paws, lymphoid tissues, liver and lung were collected, weighed and their radioisotope content determined by Wallac Wizard III gamma radioisotope counter. Lymphoid tissues collected include spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), mesenteric LN (MLN), popliteal LN (PLN) and retroperitoneal LN (RLN). Results are expressed as counts per minute (cpm) of radioisotope accumulated over the migration period per mg tissue or per 0.3ml blood normalized to 10^5 cpm labeled

cells injected. In order to assess vascular permeability, 0.5 – 1 x 10^5 counts of ^{125}I-albumin in 100 μl of RPMI + 0.1% HSA was intravenously injected into the mice 1 hour prior to the time of their sacrifice. The counts per minute (cpm) of ^{125}I-albumin accumulated in paws over one hour period was determined. This was used to estimate the volume of ^{125}I-albumin (μl) present in the paws, which was a representative of the vascular permeability.

2.8 *In Vivo* Migration of Labeled T Cells to Lymph Nodes of Immunized Mice

2.8.1 Adoptive Transfer of Cells by Intravenous Injection

The migration of T cells from blood to LNs draining a site of immunization was measured using fluorochrome-labeled T cells. T cells were washed in 1x Tyrodes (T1) medium and incubated with the fluorescent dyes, CFSE or DiD (Table 4). Cells (1 x 10^7 cells/ml) were incubated with 0.4 μg/ml CFSE in T1 medium for 30 min at room temperature in the dark, or cells were incubated with 6.25 μg/ml DiD in T1 + 0.2% HSA medium for 10 min at 37°C in the dark. Labeled cells were washed three times with T1 + 0.2% HSA. Cells were then resuspended at 3 – 5 x 10^7 cells/ml in RPMI + 0.1% HSA for injections. Cell counts were performed using a hemocytometer prior to injection.

Mice were immunized at the base of the tail with 50 μl of an emulsion containing 25 μg ovalbumin and 6.25 μg *M. butyricum* homogenized in mineral oil or an emulsion of ovalbumin in Alum. After 3 days, labeled T cells (2 – 5 x 10^6 T cells) were intravenously injected into the immunized mice. At different time points, mice were sacrificed, and spleen, draining LN (inguinal LN) and nondraining LN (axillary and cervical LNs) were collected. Cell suspensions were prepared from these tissues and were stained with fluorescent labeled antibodies (Table 5 and 6) in order to quantify the number of labeled cells recovered in these tissues, as per section 2.9. The number of labeled cells in the tissue was determined using the percent of labeled cells and the total number of cells in the tissue. Then, results are expressed as ((number of labeled cells/10^6 cells in the tissue) / 10^5 labeled cells injected).

2.8.2 Adoptive Transfer of Cells By Subcutaneous Injection

The migration of T cells from subcutaneous site of injection to LNs draining a site of immunization was measured using fluorochrome-labeled T cells T cells are washed in Tyrodes (T1) medium, and incubated with CFSE or DiD (Table 4), as outlined in section 2.8.1. Mice were immunized at the hock with 200 μg *M. butyricum* in mineral oil emulsified with PBS (1:1) in a total volume 40 μl. After 3 days, labeled T cells (0.5 – 1 x 10^6 T cells) were subcutaneously injected into the same hock of immunized mice. It should be noted that the immunization at the hock of mice was shown to be more humane than footpad immunization (369). At different time points, mice were sacrificed, and spleen and draining LNs (popliteal and inguinal LNs) were collected. Cell suspensions were prepared from the tissues and were stained with fluorescent labeled antibodies (Table 5 and 6) in order to quantify the number of labeled cells recovered in the tissues as in section 2.9. The number of labeled cells in the tissue was determined using the percent of labeled cells and the total number of cells in the tissue. Then, results are expressed as ((number of labeled cells/10^6 cells in the tissue) / 10^5 labeled cells injected).

2.9 Immunofluoresence Staining of T Cells

To determine the expression of cell surface markers, *in vitro* activated T cells or freshly isolated cells from spleen or lymph nodes were suspended at a concentrations of 0.5 – 2 x 10^7 cells / ml in immunofluoresence buffer [Dulbecco's PBS (Invitrogen) + 0.5% bovine serum albumin (BSA) (Roche Diagnostics, Laval, QC, Canada) + 0.01% sodium azide (Sigma-Aldrich)]. Cells were sequentially incubated with primary mAb at an appropriate concentration (e.g. 10 μg/ml of CR4.1) on ice (at 4°C) for 30 minutes, and were washed twice. The cells were then incubated with an appropriate secondary antibody (such as biotinylated mouse anti-hamster IgG Ab) on ice for 30 minutes. Cells were washed twice, and then were incubated with fluorochrome labeled Streptavidin on ice for 30 minutes and/or directly conjugated mAbs against T cell markers (such as anti-mouse CD4 Alexa 647). Cells were then washed twice and resuspended in ~300 μl of 1% paraformaldehyde (Fisher Scientific) in PBS.

The expression of ligands for E-selectin and P-selectin on T cells was determined as previously described (99) using mouse E-selectin and mouse P-selectin chimera

constructs fused to human μ-chain (kind gift from Drs. J. Lowe and L. Stoolman, University of Michigan, Ann Arbor, MI, USA) as reported previously (370). Briefly, cells were incubated (45 min at 4°C) with either E- or P-selectin chimera constructs in immunofluoresence buffer. Binding was detected by using sequential incubation with biotin-labeled mouse antihuman μ-chain (BD Biosciences Pharmingen) followed by washing and incubation with streptavidin-conjugated PE (BD Biosciences Pharmingen). Controls included chimera plus 10 mM EDTA used so as to eliminate cation-dependent binding. Cells were then washed twice and resuspended in ~300 μl of 1% Paraformaldehyde (Fisher Scientific) in PBS.

Stained cells were acquired in a FACS ARIA flow cytometer (BD Biosciences, Mississauga, ON, Canada) with appropriate compensation, and analyzed using Winlist-5 and -6 program (Verity Software Inc. Topsham, ME, USA). 10^5 - 10^6 events were analyzed per sample.

2.10 Intracellular Cytokine Staining of T Cells

For intracellular detection of cytokines, cells at $5x10^5$ cells/ml were incubated in RPMI + 10% FCS, Brefeldin A (used at 1x, BD Biosciences), PMA (25 ng/ml, Sigma) and ionomycin (1 μM, Sigma) at 37°C for 5 hours in 24 well plates. PMA and ionomycin are used to restimulate cytokine production from activated cells, and Brefeldin A is added to block protein secretion (368). Control cells were incubated in RPMI + 10% FCS without any stimulation at 37 °C for 5 hours in 24 well plates. Cells were suspended in immunofluoresence buffer and stained with anti-mouse CD4 or CD8 for 30 minutes at 4 °C and then washed with immunofluorescence buffer. They were spun down and fixed by resuspending the cells in 200 μl of 4% paraformaldehye in PBS for 20 min in the dark at room temperature. Cells were washed twice with 1 ml permeabilization buffer (eBioscience) and were spun for 5 minutes each time. The cells were then incubated with antibodies against IL-4 labeled with Alexa 488 (clone 11B11; eBioscience), IFN-γ labeled with PE (clone XMG1.2; eBioscience) and/or IL-10 labeled with Alexa 700 (clone JES5-16E3; eBioscience) for 20 minutes in permeabilization buffer in the dark at room temperature. Cells were washed with permeabilization buffer then fixed with 1% paraformaldehye in PBS and analyzed using a flow cytometer.

2.11 Intracellular Staining for Foxp3 on T Cells

For intracellular detection of Foxp3, T cells were resuspended at a concentration of 1 x 10^7 cells/ml in immunofluoresence buffer. Cells were incubated with anti-mouse CD4 and anti-mouse CD25 on ice for 30 minutes, and were washed twice. They were incubated with 1ml of freshly prepared fixation/permeabilization working solution (eBioscience) in dark conditions at 4 °C for 30 – 60 min. This was followed by 2 washes with 2 ml of permeabilization buffer. Cells were incubated with 10 μg/ml of anti-Foxp3 labeled with PE (FJK-16s, eBioscience) or an isotype control antibody in dark conditions at 4 °C for for 30 min, and the cells were washed twice with permeabilization buffer and fixed with 1% paraformaldehyde in PBS and analyzed using a flow cytometer.

2.12 Measurement of Chemokine-Induced Downregulation of CCR4

T cells were either pre-treated or left untreated with varying concentrations of mouse CCL22 (PeproTech Inc) at 37 °C for 30 min, and then they were stained with anti-CCR4 mAb CR4.1 as outlined in section 2.9.

2.13 Apoptosis Induced in Th1 Cells after *In Vitro* IL-2 Deprivation

In order to determine the proportion of cells undergoing apoptosis in the absence of IL-2, *in vitro* activated Th1 cells were deprived of IL-2 for up to 72 h while in culture. At different time points, the proportion of cells undergoing apoptosis was determined by staining for Annexin V (BD biosciences) and 7AAD (BD biosciences) according to the instructions of the manufacturer. Cells were washed twice in cold Dulbecco's PBS and resuspended at 1 x 10^6 cells/ml in 1x binding buffer provided in the Annexin V Apoptosis Detection Kit (BD Biosciences). 1 x 10^5 cells were stained using 5 μl Annexin V FITC and 5 μg/ml 7AAD in volume of 100 μl for 30 min in the dark at room temperature. Binding buffer (400 μl) was added to each tube and cells were analyzed by flow cytometer within 1 h. Also, cell counts and cell viability were determined using trypan blue exclusion assay.

2.14 Histology

In order to examine the presence of infiltrating leukocytes in inflammatory skin sites, punch biopsies were fixed in buffered formalin and embedded in paraffin for histological analysis. 5 μm sections were cut and stained with hematoxylin and eosin (H & E). Sections were visualized using Nikon Eclipse E600 Microscope (Spach Optics, Rochester, NY, USA).

2.15 Statistics

Data were expressed as mean ± S.E.M of multiple assays, and Student's unpaired *t-test* was used for analysis. Also, Mann Whitney U test was used for analysis of nonparametric data. ANOVA with Tukey post-test was used for multiple comparisons unless otherwise noted. P values less than 0.05 were considered as statistically significant. * denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, while NS denotes not significant with a $P > 0.05$.

Chapter 3. Effect of CCR4 Expression on Memory and Activated CD4 Cell Migration to Dermal Inflammation in Rats

The relationship between the expression of CCR4 on memory and activated CD4 T cells and their migration to dermal inflammation is not clear. In addition, the association between CCR4 expression and migration of T cells to inflammation induced by various stimuli, such as cytokines, TLR agonists and DTH, has not been previously compared. Therefore, the recruitment of memory and activated CD4 cells to dermal inflammation was analyzed. In order to examine the expression of CCR4 on CD4 T cells and the recruitment of $CCR4^+$ and $CCR4^-$ CD4 T cells to dermal inflammation, our laboratory has developed a monoclonal antibody against rat CCR4 (clone CR4.1) (92).

3.1 Migration of Memory CCR4^{+} and CCR4^{-} CD4 T Cells to Sites of Dermal Inflammation

In order to examine the expression of CCR4 on memory CD4 cells, CD4 T cells were isolated from cell suspensions of spleen and lymph nodes of normal rats by negative selection. They were stained using antibodies against CD4, CCR4 and CD45RC. CD45RC is a useful marker to differentiate between naïve and memory CD4 cells, since it is expressed on naïve cells but not memory cells in the rat. As shown Figure 4A, CCR4 was expressed on ~2% of CD4 T cells in the LNs and ~7% of the CD4 T cells in the spleen. The expression on CD4 T cells was mostly restricted to the memory CD45RC^{-} T cells with ~5 and 20% of these cells being CCR4^{+} in LNs and spleen respectively.

Amongst CD4 cells, naïve cells are known to migrate poorly to dermal sites of inflammation, while memory cells migrate well to these sites (93). In order to examine the association between the CCR4 expression and the migration of memory CD4 cells to sites of dermal inflammation, the recruitment of CCR4^{+} and CCR4^{-} memory CD4 cells to dermal inflammation was compared. First, CCR4^{+} and CCR4^{-} cells were sorted from memory CD4 cells that were isolated from splenocytes by negative selection followed by MACS separation. Then, CCR4^{+} and CCR4^{-} memory CD4 cells were radiolabeled and intravenously injected into rats that was treated subcutaneously with inflammatory stimuli on the back skin. As shown in Figure 4B-C, ~5-7 fold more CCR4^{+} memory CD4 cells were found in the inflamed skin sites than CCR4^{-} cells. The number of CCR4^{+} and CCR4^{-} cells was comparable in the blood and spleen, but 3 times more CCR4^{-} cells were found in LNs than CCR4^{+} cells.

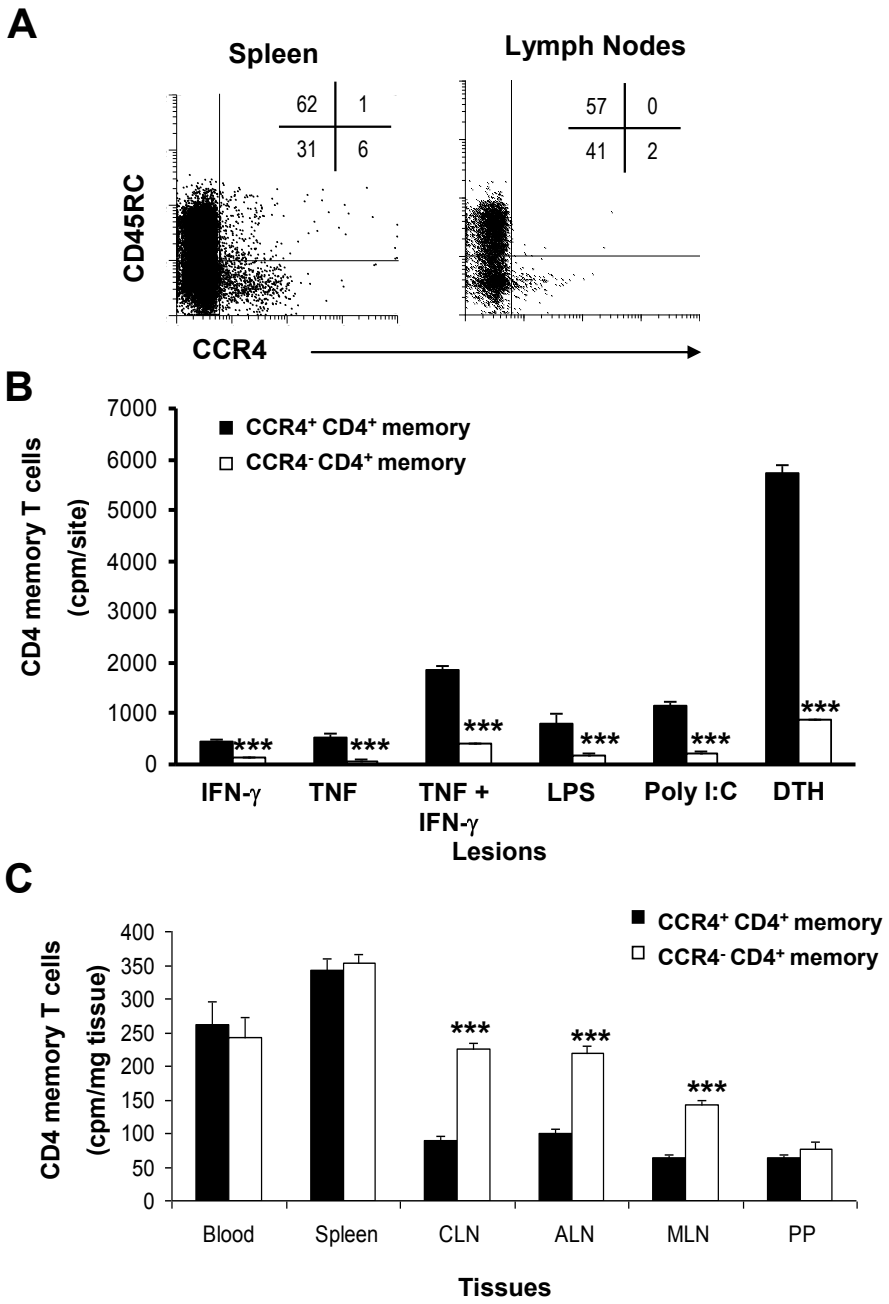

Figure 4. Expression of CCR4 on CD4 T cells and migration of memory CCR4$^+$ and CCR4$^-$ CD4 cells to sites of dermal inflammation and lymphoid tissues in the rat. CD4 T cells were isolated from spleens and LNs of rats and stained for CD4, CD45RC and CCR4.Representative dot plots show the expression of CCR4 on CD4 cells in spleen (on left panel) and LNs (on right panel) of normal rats (n=2) (A). Spleen CD4 T cells were separated into CCR4$^+$ and CCR4$^-$ CD45RC$^-$ T cells, radiolabeled and injected i.v. (with 5 – 10 x 10^6 T cells having 1- 5 $\times$ 10^5 cpm). Each animal received i.d. injections of cytokines, TLR agonists, control diluent, and DTH was induced in response to OVA. After 18 h, skin sites (B), blood, spleen, cervical LN (CLN), axillary LN (ALN), mesenteric LN (MLN), and peyer's patches (PP) (C) were collected, and their radioactivity content was determined as a measure of labeled cell migration. Bars represent mean increase in cpm ± SEM over control sites (117 ± 25 for CCR4$^+$, 61 ± 15 for CCR4$^-$) or the mean cpm ± SEM in tissues, n=10-13. *** $P < 0.005$ by t-test compared to CCR4$^+$ CD4 memory cells.

3.2 Migration of Activated CCR4^{+} and CCR4^{-} CD4 T Cells to Sites of Dermal Inflammation

In order to examine the expression of CCR4 on activated CD4 T cells, CD4 cells were isolated from cell suspensions of lymph nodes by negative selection, and activated *in vitro* using antibodies to the αβ-TCR and CD28 in the presence of IL-2. Then, on a daily basis, CD4 cells were stained for CCR4 expression. As shown in Figure 5A, the expression of CCR4 on CD4 T cells increased steadily during *in vitro* activation, and ~40% of the activated CD4 T cells expressed CCR4 after 5 days of *in vitro* activation.

In order to examine the association between the CCR4 expression and the migration of activated CD4 cells to dermal inflammation, the recruitment of *in vitro* activated CCR4^{+} and CCR4^{-} CD4 cells to dermal inflammation was compared. Activated CCR4^{+} and CCR4^{-} CD4 cells were radiolabeled and intravenously injected to rats that had received stimuli of inflammation on the back skin. As shown in Figure 5B-C, there was no difference in the migration of CCR4^{+} and CCR4^{-} cells to sites injected with IFN-γ and/or TNF. There was ~35% more migration of CCR4^{+} cells to the TLR agonists compared to CCR4^{-} CD4 cells. Migration of CCR4^{+} cells to the DTH reaction was twice as great than CCR4^{-} cells, but was a smaller difference than observed with resting memory CD4 cells (Figure 4B). There was no difference in the circulation of CCR4^{+} and CCR4^{-} T cells in the blood, but CCR4^{-} T cells accumulated in significantly greater numbers in the spleen and mesenteric LN than CCR4^{+} T cells. CCR4^{-} T cells and CCR4^{+} T cells accumulated similarly in the peripheral cervical and axillary LNs.

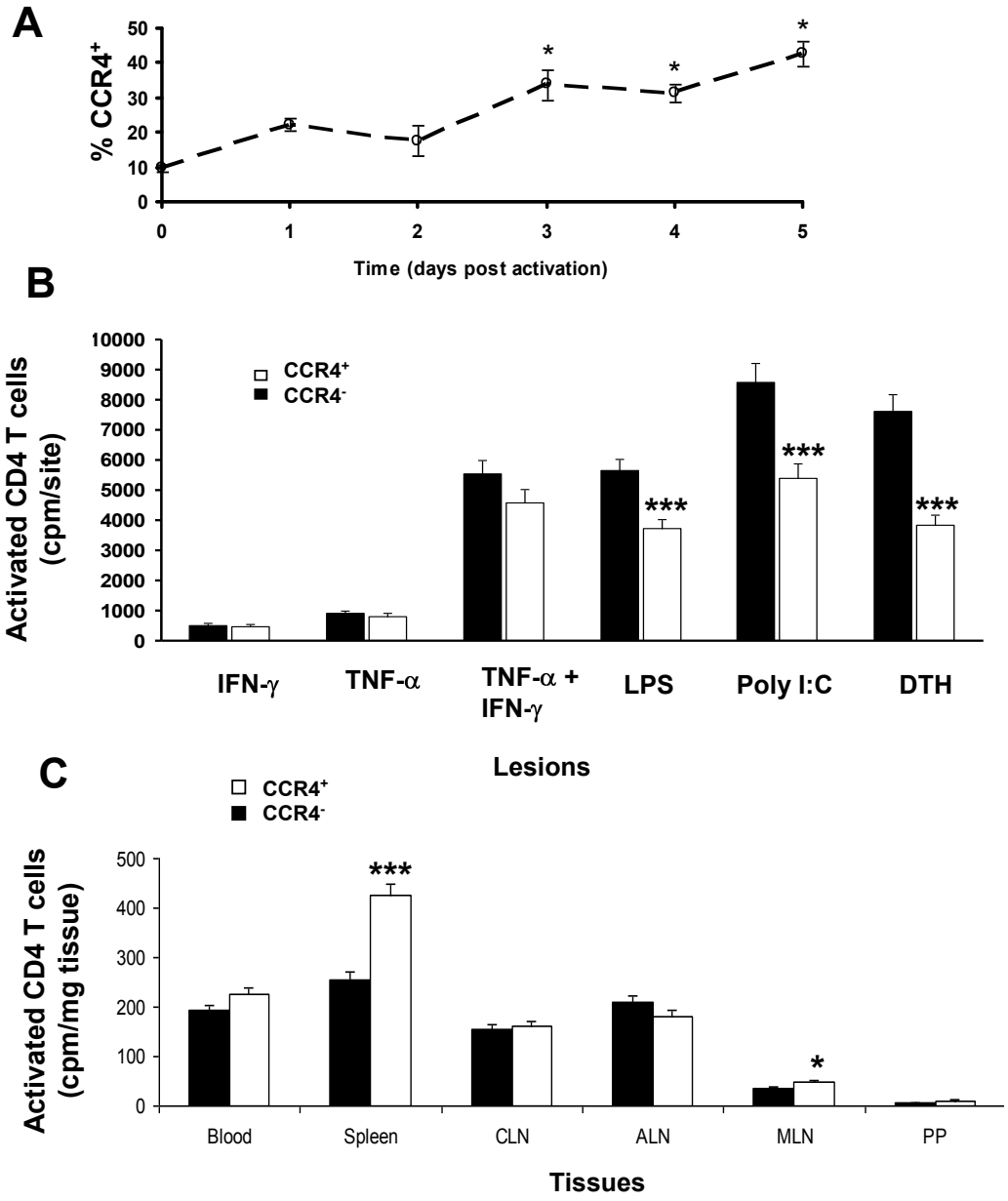

Figure 5. Expression of CCR4 on activated CD4 cells and migration of activated CCR4$^+$ and CCR4$^-$ CD4 cells to sites of dermal inflammation and lymphoid tissues in the rat. CD4 T cells were activated *in vitro* using plate bound anti-TCR αβ and soluble anti-CD28 mAbs in the presence of IL-2. Expression of CCR4 on CD4 cells was determined on a daily basis during *in vitro* activation, n=5-21, * $P < 0.05$ by ANOVA and Dunnett's test compared to day 0 (A). Anti-TCR activated CD4 T cells were separated into CCR4$^+$ and CCR4$^-$ T cells, radiolabeled and injected i.v. (with 5 – 10 x 10^6 T cells having 1- 5 × 10^5 cpm). Each animal received i.d. injections of cytokines, TLR agonists, control diluent, and DTH was induced in response to OVA. After 18 h, skin sites (B), blood, spleen, cervical LN (CLN), axillary LN (ALN), mesenteric LN (MLN), and peyer's patches (PP) (C) were collected, and their radioactivity content was determined as measure of labeled cell migration. Bars represent mean increase in cpm ± SEM over control sites (118 ± 15 for CCR4$^+$, 87 ± 14 for CCR4$^-$) or the mean ± SEM in tissues, n = 18-19. *** $P < 0.005$ by Student's t-test compared to CCR4$^+$ CD4 activated cells.

3.3 Summary

CCR4 is thought to be related to the recruitment of T cells to skin during inflammation, however this is mostly based on the detection of CCR4 on CD4 cells from patients with skin inflammation (229) or from mice with CHS in the skin (196), and on the ability of CD4 cells to migrate towards to CCL22 injected *in vivo* (245). In fact, the relationship between the expression of CCR4 on memory and activated CD4 T cells and their migration to dermal inflammation induced by various stimuli, such as cytokines, TLR agonists and DTH has not been previously examined. The development of anti-CCR4 antibody by our laboratory has enabled the expression of CCR4 on memory and activated CD4 T cells and the recruitment of $CCR4^+$ and $CCR4^-$ CD4 cells to dermal inflammation to be examined (92).

The expression of CCR4 on unstimulated CD4 cells was found to be mostly restricted to memory cells and steadily increased during *in vitro* activation. Also, memory $CCR4^+$ CD4 cells accounted for nearly all of the recruitment to inflamed skin *in vivo*, though most memory cells are not $CCR4^+$ (Figure 4B). However, activated $CCR4^+$ CD4 cells did not follow the same pattern of enhanced migration observed with memory $CCR4^+$ CD4 cells, in that activated $CCR4^+$ and $CCR4^-$ CD4 cells migrated to a similar extent to the cytokines. Relatively high expression of CCR4 on *in vitro* activated CD4 cells was observed, yet the activated $CCR4^+$ CD4 cells were only modestly greater than CCR4- cells in TLR agonists and DTH (Figure 5). Thus, the expression of CCR4 on CD4 cells does not necessarily lead to increased recruitment to dermal inflammation; activated CD4 T cells that lack CCR4 expression can still migrate to dermal sites. This has not been specifically studied previously *in vivo.*

Whether CD4 cells require CCR4 for their recruitment to dermal inflammation remains unclear. Memory and activated CD4 cells express multiple CKRs, such as CXCR3, and therefore their requirement of CCR4 for skin homing can be better examined in the absence of CCR4 expression; by using CCR4 deficient mouse CD4 cells.

Chapter 4. Effect of CXCR3 Deficiency on the *In Vivo* Migration of T Cells to Dermal Inflammation

Previous studies in rats using a blocking monoclonal antibody (mAb) have shown that the inhibition of CXCR3 reduces the ability of T lymphoblasts from antigen-stimulated LNs to recruit into sites of dermal inflammation by ~80% (93). Memory CD4 cell recruitment into inflamed skin was reduced partially by CXCR3 blockade, but there was a substantial CXCR3-independent component (93).

As shown in the previous chapter, the expression of CCR4 was associated with memory CD4 cell, and less so with activated CD4 cell, migration to inflamed skin. In order to determine the requirement for CXCR3 and CCR4 in the recruitment to dermal inflammation, the migration of T cells to inflamed skin was evaluated in mice using $CXCR3^{-/-}$ and $CCR4^{-/-}$ T cells. A mAb to CXCR3 was commercially available, and a mAb to mouse CCR4 was identified for these studies. In addition, techniques for measurement of radiolabeled T cell migration were developed in order to enable the *in vivo* migration of T cells to the skin to be examined in a highly quantitative manner in normal mice.

4.1 Determining the Cross-Reactivity of Anti-Rat CCR4 Antibody to Mouse CCR4

The absence of commercially available monoclonal antibodies to mouse CCR4 during the start of the experiments, necessitated that we determine whether the monoclonal antibody developed in our laboratory towards rat CCR4 could also detect mouse CCR4. For this purpose, CD4 and CD8 cells were isolated from lymph nodes of normal (WT) and CCR4 deficient ($CCR4^{-/-}$) mice, and activated *in vitro* using antibodies against CD3 and CD28 in the presence of IL-12 and IL-2. The expression of CCR4 on activated Th1 and Tc1 cells was then determined by immunofluorescence staining using the anti-rat CCR4 mAb (CR4.1). As shown in Figure 6A, CR4.1 detected CCR4 on WT Th1 and Tc1 cells, but not on $CCR4^{-/-}$ Th1 and Tc1 cells. A higher proportion of Th1 cells expressed CCR4 than did Tc1 cells (Figure 6A). The differential expression of CCR4 on activated CD4 and CD8 cells is in concordance with results from *in vitro* culture of human PBMC in the presence of IL-2 (144).

To further confirm that CR4.1 was specifically detecting mouse CCR4, activated Th1 cells were pre-treated with CCL22, a CCR4 ligand which would induce the downregulation (124). An antibody specific to CCR4 would be expected to detect less CCR4 on the surface of the Th1 cells that were pre-treated with CCL22 than on untreated Th1 cells. As shown on Figure 6B, pretreatment with CCL22 downregulated the expression of CCR4 on the surface of Th1 cells, which was reflected by a reduction in the positive staining by CR4.1, but not by an isotype control antibody.

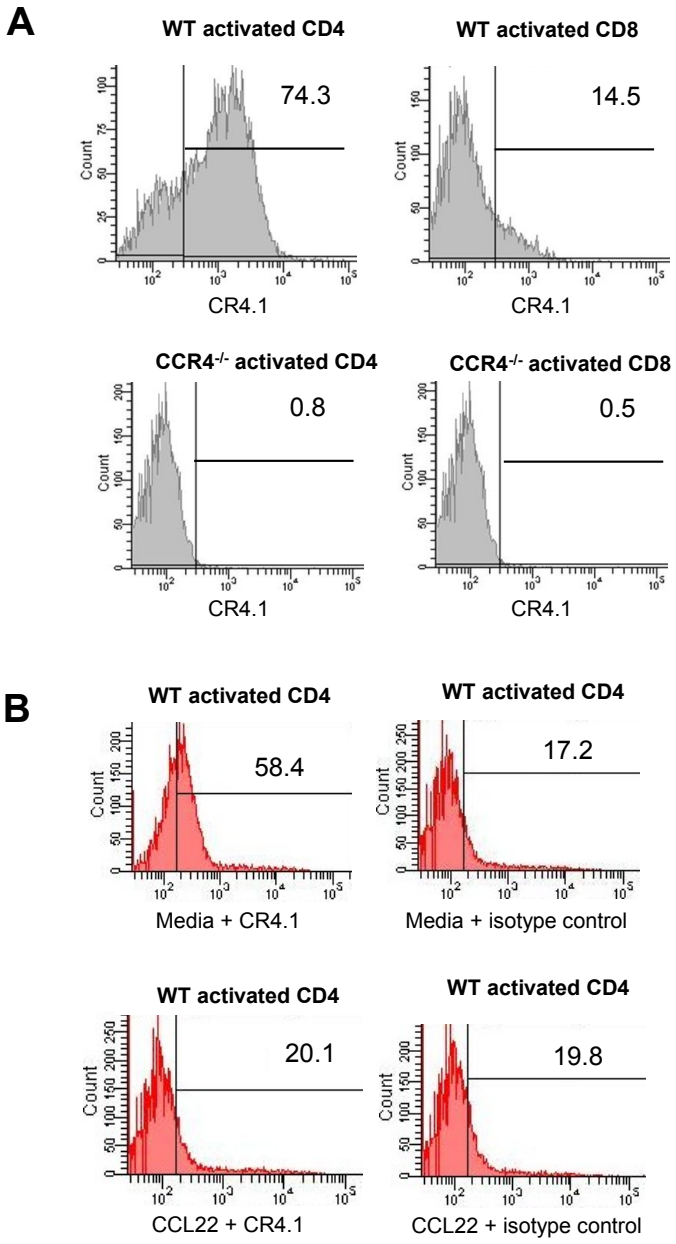

Figure 6. Flow cytometry staining demonstrating the binding of anti-rat CCR4 mAb (CR4.1) to mouse activated T cells. CD4 and CD8 T cells were isolated from WT and CCR4$^{-/-}$ mice and activated *in vitro* using anti-CD3 and anti-CD28, IL-2 and IL-12. Representative histograms show the expression of CCR4 on activated Th1 and Tc1 cells as determined by anti-CCR4 mAb (CR4.1) (n=10-18 for CD4, n=3-5 for CD8) (A). Activated WT Th1 cells were untreated or pretreated with CCL22 and the surface expression of CCR4 was determined using CR4.1 compared to an isotype control (B). Representative histograms shown, n=3.

4.2 *In Vivo* Migration of T Cells to Sites of Dermal Inflammation in Mice

Since T cells express multiple CKRs, mice deficient in CKRs (e.g. CCR4 or CXCR3) would be useful for studying the contribution of individual CKRs (e.g. CCR4) to the migration of T cells to dermal inflammation. An assay to measure the *in vivo* migration of T cells to dermal inflammation was established by intradermally injecting stimuli, such as ConA, that would recruit memory and activated T cells. First, when ConA was injected i.d. or DTH was induced in the skin, both ConA and DTH treatments were found to recruit immune cells to the skin, as shown by H & E staining of the skin sections (Figure 7A-C).

In order to quantify the migration of T cells to these sites, memory and naïve CD4 T cells were isolated from mouse spleen radiolabeled, and injected i.v. into recipient mice. Recipients also received i.d. injections of ConA and DTH was induced on the back skin. As shown in Figure 7D, ConA and DTH sites had marked increase in radioactivity compared to diluent control, demonstrating an increase in the labeled T cell accumulation. About ~10 times more memory CD4 cells were found in the inflamed skin sites than naïve CD4 cells, as seen with rat memory CD4 T cells (93).

It was important to optimize the assay so that it would measure the migration of both activated CD4 and CD8 T cells to dermal inflammation, therefore CD4 and CD8 T cells were activated in type 1 polarizing conditions for 5 and 6 days. They were radiolabeled and injected i.v. to mice that were also injected i.d. with stimuli of inflammation on the back skin. In order to optimize the assay, the migration of day 5 and day 6 activated T cells to dermal inflammation was compared. ConA was found to recruit ~4 times more day 6 activated Th1 cells than day 5 activated Th1 cells, and ~70% more day 6 activated Tc1 cells than day 5 activated Tc1 cells. The accumulation of day 5 and day 6 activated T cells was found to be comparable in the LNs of these mice as shown in Figure 8A and 8C.

When the migration of day 6 activated T cells to dermal inflammation induced by various stimuli (Poly I:C, LPS, ConA and DTH) was examined, poly I:C and LPS sites were found to recruit 3-4 times more Th1 and Tc1 cells than control site. DTH recruited 15-25 times more Th1 and Tc1 cells than the control site, and ConA recruited 25-30 times more Th1 and Tc1 cells than the control site (Figure 8B, D).

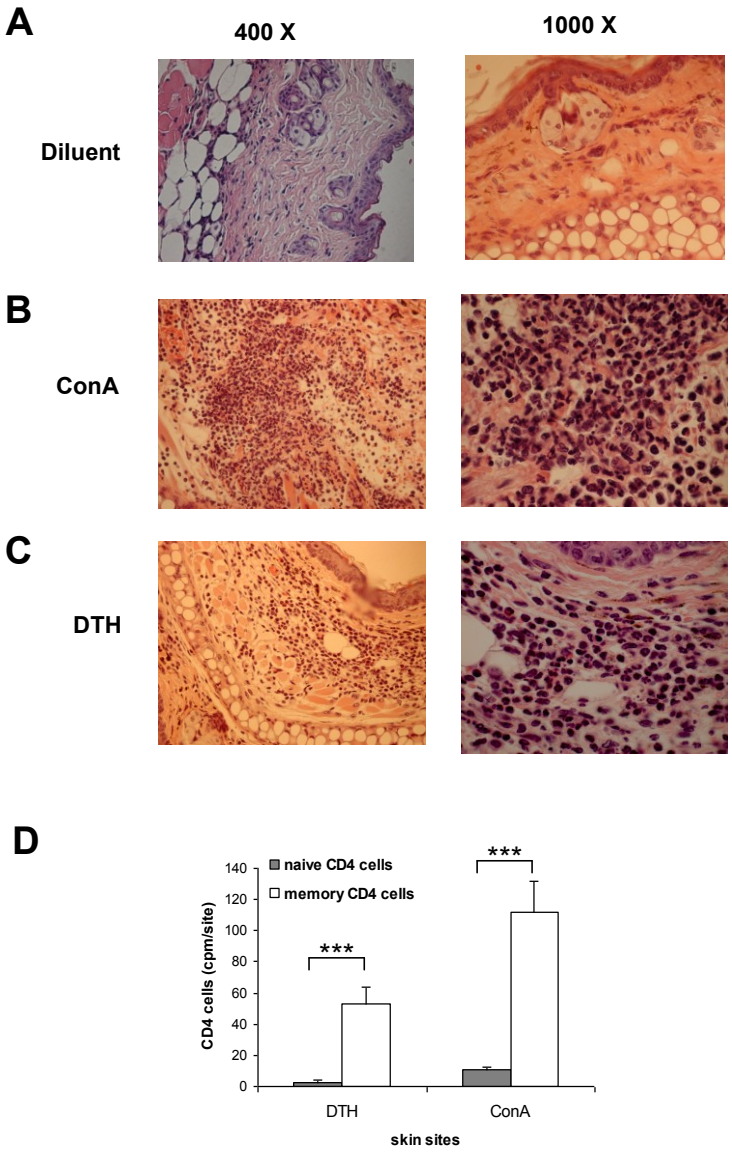

Figure 7. Histology of dermal inflammatory sites and the migration of labeled memory CD4 cells to inflamed skin sites in mice. H & E stained sections of skin sites that were injected with diluent (A), ConA (B), or where DTH was induced (C) (400x on left panel and 1000x on right panel). (D) Memory and naïve CD4 cells were radiolabeled and injected into mice that also had ConA and DTH sites on the back skin. The content of radioactivity in the skin was determined as a measure of the migration of labeled memory or naïve CD4 cells. Bars represent mean cpm ± SEM over control sites (1 ± 1), n=5-13 mice in 2-4 exps. *** $P < 0.005$ by Student's t-test.

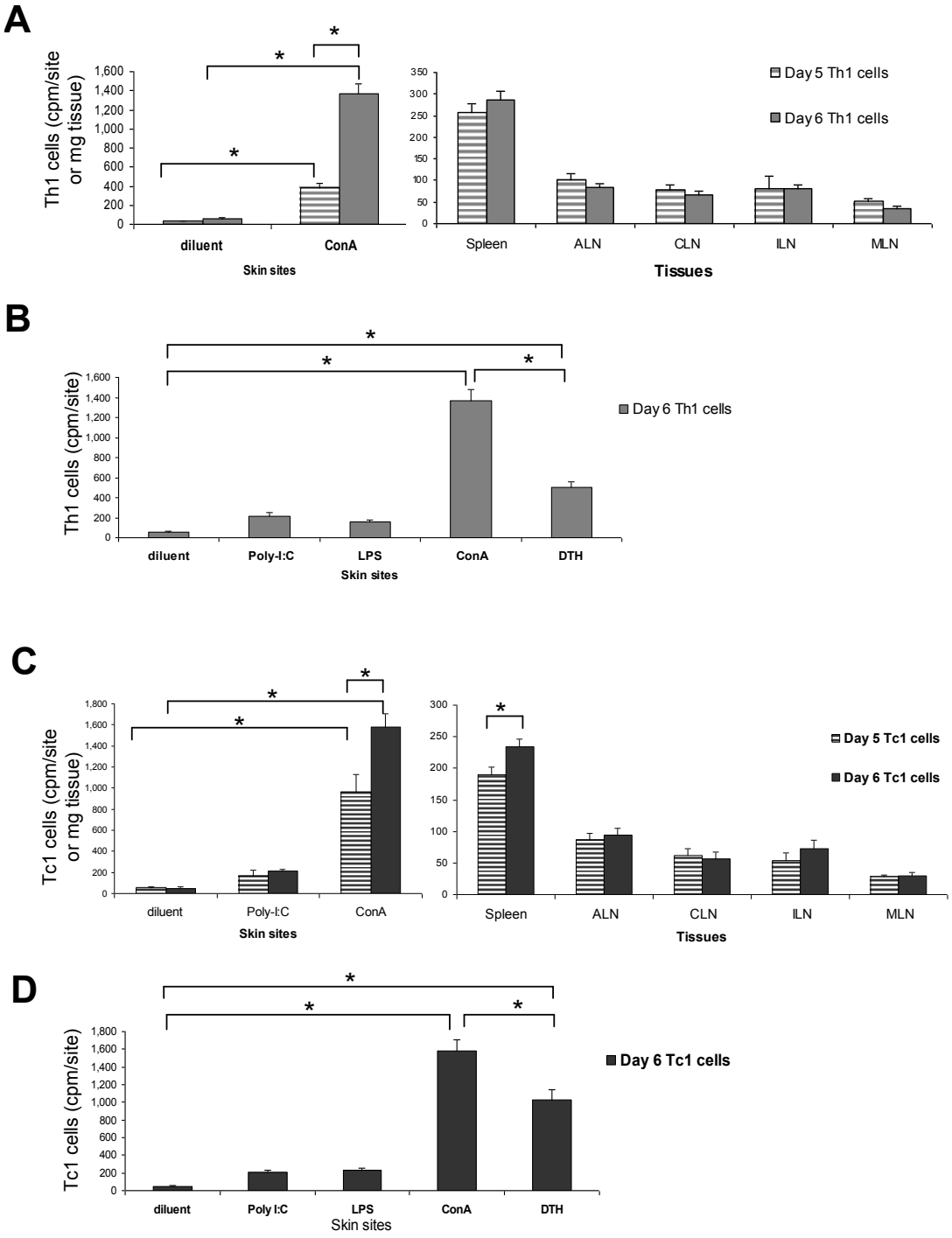

Figure 8. Measurement of activated Th1 and Tc1 cell migration to sites of dermal inflammation and lymphoid tissues. T cells, activated in type 1 polarizing conditions for 5 or 6 days, were radiolabeled and injected i.v. into mice that received i.d. inflammatory stimuli. The radioactivity content was determined in the skin sites, spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), and mesenteric LN (MLN) as a measure of labeled cell migration. (A-B) Accumulation of day 5 or day 6 activated Th1 cells. (C-D) Accumulation of day 5 or day 6 activated Tc1 cells. Bars represent mean cpm ± SEM, n (day 5)=6-12 mice in 2-4 exps, n (day 6)= 10-24 mice in 4-9 exps. * $P < 0.05$ by ANOVA.

4.3 Proliferation and Expression of Adhesion Molecules and CCR4 on *In Vitro* Activated CD4 and CD8 T Cells from CXCR3$^{-/-}$ Mice

CD4 cells from the spleen of wild-type (WT) and CXCR3 deficient (CXCR3$^{-/-}$) mice were stained to determine the proportion of memory cells and the CXCR3 expression on memory CD4 cells. Memory CD4 cells (CD44^{hi}CD45RB^{lo}) constitute 14.1 ± 3.6% of the CD4 cells in the spleen of the WT mice and 15.1 ± 0.4% of CD4 cells in spleen of CXCR3$^{-/-}$ mice. CXCR3 was expressed on 11.2 ± 5.8% of total CD4 T cells. Most of the CXCR3 expression on CD4 cells was restricted to memory cells; 30 ± 10% of memory CD4 cells express CXCR3, while only 3.3 ± 4% of naïve cells express CXCR3.

In order to examine the contribution of CXCR3 to the *in vivo* migration of activated T cells, CD4 and CD8 T cells from CXCR3$^{-/-}$ and WT mice were activated in type 1 polarizing condition. As shown in Figure 9A, CXCR3$^{-/-}$ CD4 cells proliferated well, but only 40 fold while the WT CD4 cells proliferated 60 fold by day 6. The expression of the activation marker CD25 on activated CXCR3$^{-/-}$ and WT CD4 cells was similar after 6 days of *in vitro* activation; ~94% of the CD4 cells expressed CD25. These activated cells had a Th1 profile in that 64.4 ±7.1% of the WT CD4 cells and 64.8 ±5.3% of the CXCR3$^{-/-}$ CD4 cells expressed IFN-γ but not IL-4 (Table 7). Also, the expression of adhesion molecules on CXCR3$^{-/-}$ Th1 cells appeared to be normal; both WT and CXCR3$^{-/-}$ Th1 cells expressed 98% CD44, 50% α_4-integrin, 35% L-selectin, 58% ESL and 38% PSL (Table 7).

WT Th1 cells expressed both CCR4 and CXCR3 (Table 7; Fig 9B-C). Most Th1 cells expressed CXCR3 and 60% of the Th1 cells expressed CCR4. In fact, CCR4 and CXCR3 were co-expressed on 54% of the Th1 cells. About 30% of the Th1 cells expressed CXCR3 alone, while 9% of the Th1 cells expressed express CCR4 alone. (Figure 9B). It should be noted that the expression of CCR4 was unaffected on CXCR3$^{-/-}$ Th1 cells (Figure 9C).

Table 7. Expression of adhesion molecules, cytokines, and CCR4 by WT and CXCR3$^{-/-}$ CD4 cells after 6 days of *in vitro* activation in type 1 polarizing conditions.

	WT Th1 cells			**CXCR3$^{-/-}$ Th1 cells**		
Markers	N	% expression mean (±SEM)	MFI mean (±SEM)	N	% expression mean (±SEM)	MFI mean (±SEM)
CD25	5	93.2 (±2.6)	146.2 (±72.6)	5	94.5 (±1.1)	126.3(±68.3)
Adhesion molecules						
CD44	1	98.1	327.8	1	98.1	269.3
α_4-integin	3	48.4 (±10.2)	14.0 (±0.6)	2	51.9 (±9.8)	16.1 (±2.1)
L-selectin	4	35.1 (±5.9)	36.0 (±11.3)	4	32.8 (±7.4)	41.1 (±6.4)
ESL	5	58.7 (±8.9)	326.0 (±175.0)	5	58.7 (±10.2)	449.1 (±184.5)
PSL	4	40.6 (±11.4)	487.9 (±360.8)	4	32.7 (±11.2)	531.5 (±377.6)
CKRs						
CXCR3	21	91.0 (±1.9)	229.2 (±56.6)			
CCR4	8	60.6 (±6.1)	25.2 (±2.2)	9	60.6 (±4.8)	28.4 (±3.5)
Cytokines						
IFN-γ	9	64.4 (±7.1)	135.7 (±74.5)	3	64.8 (±5.3)	158.5 (±81.6)
IL-4	3	2.3 (±1.3)	11.1 (±2.5)	1	0.6	6.7

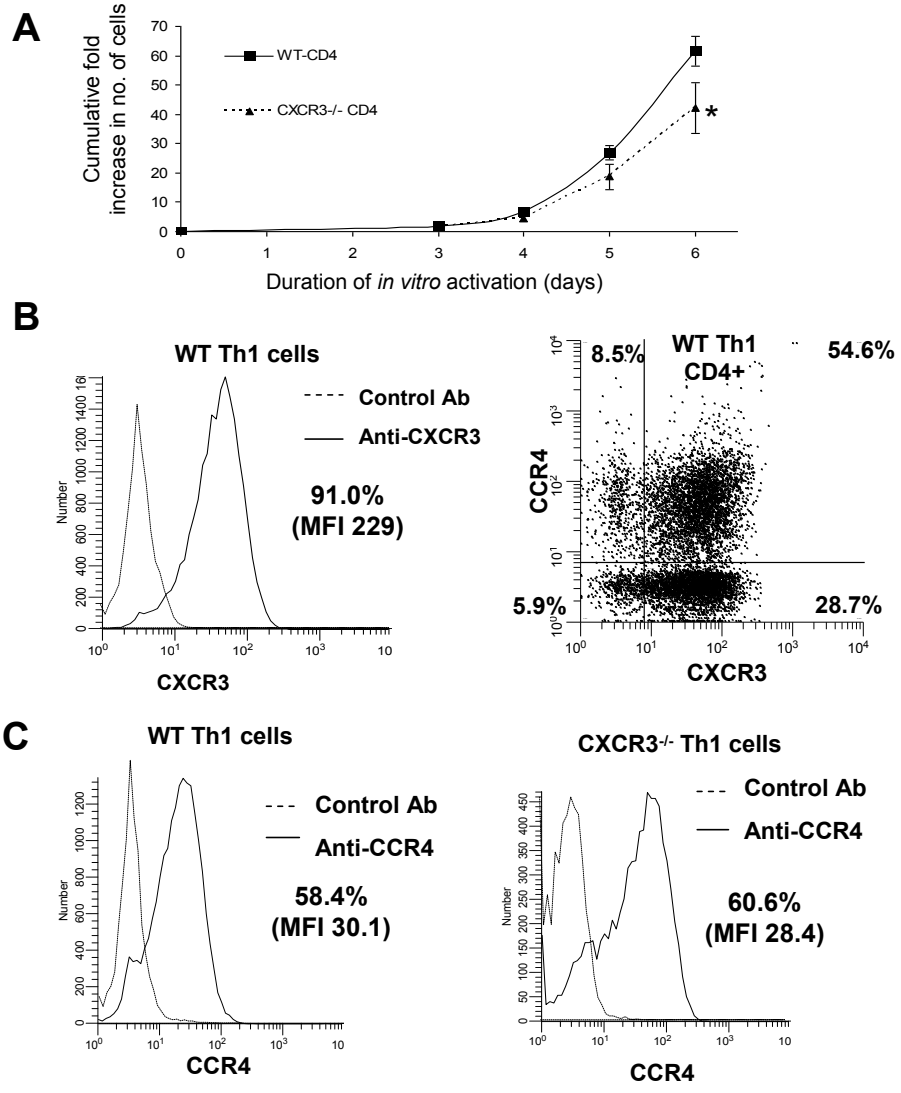

Figure 9. Proliferation and expression of CCR4 and CXCR3 on *in vitro* activated Th1 cells from WT and CXCR3$^{-/-}$ mice. (A) WT and CXCR3$^{-/-}$ CD4 cells were activated in type 1 polarizing conditions. The cumulative fold increase in the number of cells during *in vitro* proliferation of CXCR3$^{-/-}$ and WT Th1 cells was determined using trypan blue exclusion assay (n=22-58). (B) Expression of CCR4 and CXCR3 on WT Th1 cells. (C) Expression of CCR4 on CXCR3$^{-/-}$ and WT Th1 cells (n=9-21). Error bars represent mean ± SEM. * $P < 0.05$ compared WT cells by Student's t-test assuming unequal variances. Representative histograms with average frequency of expression (mean MFI).

When CD8 cells were activated in type 1 polarizing conditions, 79.6 ±3.2% of the WT CD8 cells and 88.3 ±1.7% of the CXCR3$^{-/-}$ CD8 cells express IFN-γ (Table 8). The proliferation of CXCR3$^{-/-}$ Tc1 cells was not different from WT cells (Figure 10A). As shown in Figure 10B, only 20% of Tc1 cells expressed CCR4, while CXCR3 was expressed by most of the Tc1 cells. Also, the deficiency of CXCR3 did not affect the expression of adhesion molecules; both WT and CXCR3$^{-/-}$ Tc1 cells expressed 65% α_4-integrin, 30% L-selectin, 67% ESL and 40% PSL (Table 8).

Table 8 Expression of adhesion molecules, cytokines and CCR4 by WT and CXCR3$^{-/-}$ CD8 cells after 6 days of *in vitro* activation in type 1 polarizing conditions.

	WT Tc1 cells			**CXCR3$^{-/-}$ Tc1 cells**		
Markers	N	% expression mean (±SEM)	MFI mean (±SEM)	N	% expression mean (±SEM)	MFI mean (±SEM)
Adhesion molecules						
α_4-integin	6	66.5 (±5.1)	26.0 (±1.5)	4	64.0 (±1.4)	21.8 (±1.1)
L-selectin	5	28.8 (±3.8)	44.5 (±6.9)	4	32.3 (±5.3)	40.8 (±6.7)
ESL	6	67.4 (±6.1)	108.3 (±22.7)	4	67.8 (±5.3)	142.0 (±24.2)
PSL	6	42.5 (±9.4)	612.8 (±5.3)	4	37.5 (±3.9)	639.3 (±9.8)
CKRs						
CXCR3	3	96.8 (±1.3)	60.8 (±4.3)		ND	ND
CCR4	8	20.6 (±4.5)	37.0 (±13.5)	5	19.0 (±9.6)	12.6 (±2.2)
Cytokines						
IFN-γ	3	84.8 (±2.8)	134.6 (±20.7)	4	88.3 (±1.7)	160.0 (±18.9)
IL-4	3	3.7 (±1.4)	11.1 (±3.2)		ND	ND

ND=not determined

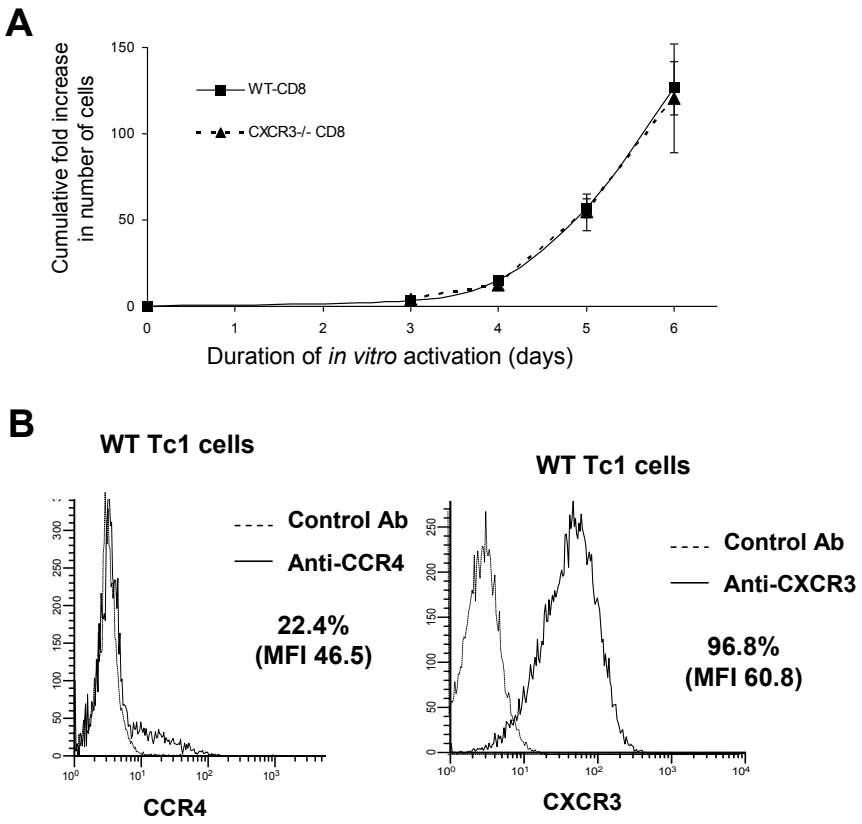

Figure 10. Proliferation of *in vitro* activated Tc1 cells from CXCR3$^{-/-}$ and WT mice, and expression of CCR4 and CXCR3 on WT Tc1 cells. (A) WT and CXCR3$^{-/-}$ CD8 cells were activated in type 1 polarizing conditions. The cumulative fold increase in the number of cells during *in vitro* proliferation of CXCR3$^{-/-}$ and WT Tc1 cells was determined using trypan blue exclusion assay (n=7-29). (B) Expression of CCR4 and CXCR3 on WT Tc1 cells (n=3-5). Error bars represent mean ± SEM. Representative histograms with average frequency of expression (mean MFI).

4.4 Effect of CXCR3 Deficiency on the *In Vivo* Migration of T Cells to Sites of Dermal Inflammation

CXCR3 is expressed on most of the CD4 and CD8 T cells activated in type 1 polarizing conditions, but whether the expression of CXCR3 is associated with a requirement for this CKR in the recruitment of Th1 or Tc1 cells to dermal inflammation is unclear. In order to study this, CD4 (or CD8 cells) were isolated from $CXCR3^{-/-}$ and WT mice and were activated *in vitro* in type 1 polarizing condition. T cells were radiolabeled and injected i.v. into mice that were injected i.d. with inflammatory stimuli.

First, the accumulation of unstimulated $CXCR3^{-/-}$ and WT CD4 cells into ConA sites was assessed. About 50% fewer $CXCR3^{-/-}$ CD4 cell were found in ConA sites than WT cells (Figure 11), but ~2 times more $CXCR3^{-/-}$ CD4 cell were found in the lymphoid tissues than WT cells (Figure 11). This was expected given the previous work in our laboratory demonstrating that CXCR3 blockade inhibits ~50% of the migration of memory $CD45RC^{-}$ CD4 cells to inflamed skin, and that $CXCR3^{-}$ splenocytes do not accumulate as well as $CXCR3^{+}$ cells in dermal inflammation (93).

In order to determine whether CXCR3 is required for the migration of Th1 cells to site of dermal inflammation, the recruitment of $CXCR3^{-/-}$ and WT Th1 cells into inflamed skin was examined. As shown in Figure 12A, ~60% fewer $CXCR3^{-/-}$ Th1 cells are found in skin sites treated with ConA, TLR agonists, or DTH compared to WT Th1 cells. Even though the recruitment of Th1 cells to the skin was reduced 50-60% in the absence CXCR3, the accumulation of $CXCR3^{-/-}$ Th1 cells in LNs was either unaffected as in normal mice (Figure 12B), or was increased in some LNs of OVA CFA-immunized mice (Figure 12C).

Most Tc1 cells, like Th1 cells, expressed CXCR3 (Figure 10B), but it is not clear whether the expression of CXCR3 on Tc1 cells is required for their migration to dermal inflammation. As shown in Figure 13A, ~ 25-35% fewer $CXCR3^{-/-}$ Tc1 cells were found in ConA and DTH skin sites than WT Tc1 cells. Compared with Th1 cells (Figure 13A), CXCR3 played a smaller role in the recruitment of Tc1 cells to inflamed skin. The accumulation of $CXCR3^{-/-}$ Tc1 cells in the LNs was unaffected (Figure 13B), except in immunized mice, where more $CXCR3^{-/-}$ Tc1 cells were found in the LNs than WT Tc1 cells (Figure 13C).

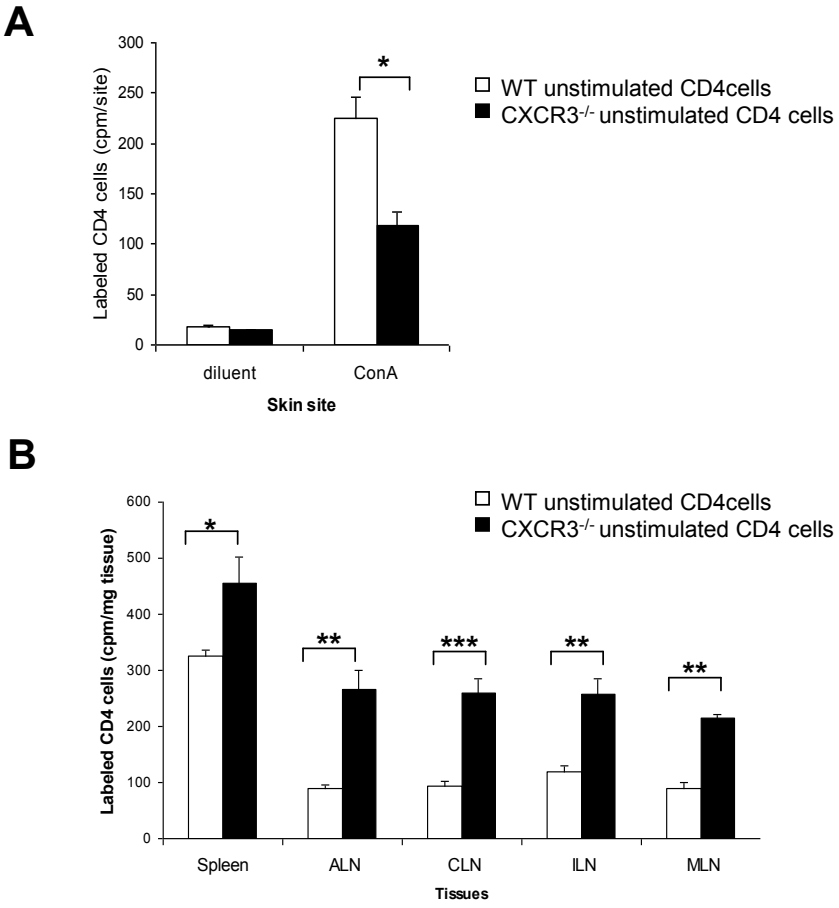

Figure 11. Migration of unstimulated CD4 T cells from CXCR3$^{-/-}$ and WT mice into sites of dermal inflammation and lymphoid tissues. CD4 isolated from the spleens of CXCR3$^{-/-}$ and WT mice were radiolabeled and injected i.v. into mice with i.d. injections of ConA and diluent on the back skin. After 20 h, the content of radioactivity of skin sites, spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), and mesenteric LN (MLN) was determined to measure CD4 T cell accumulation. n = 5 mice in 2-3 exps. Bars represent mean cpm ± SEM for skin and lymphoid homing. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ by Student's t-test compared to WT cells.

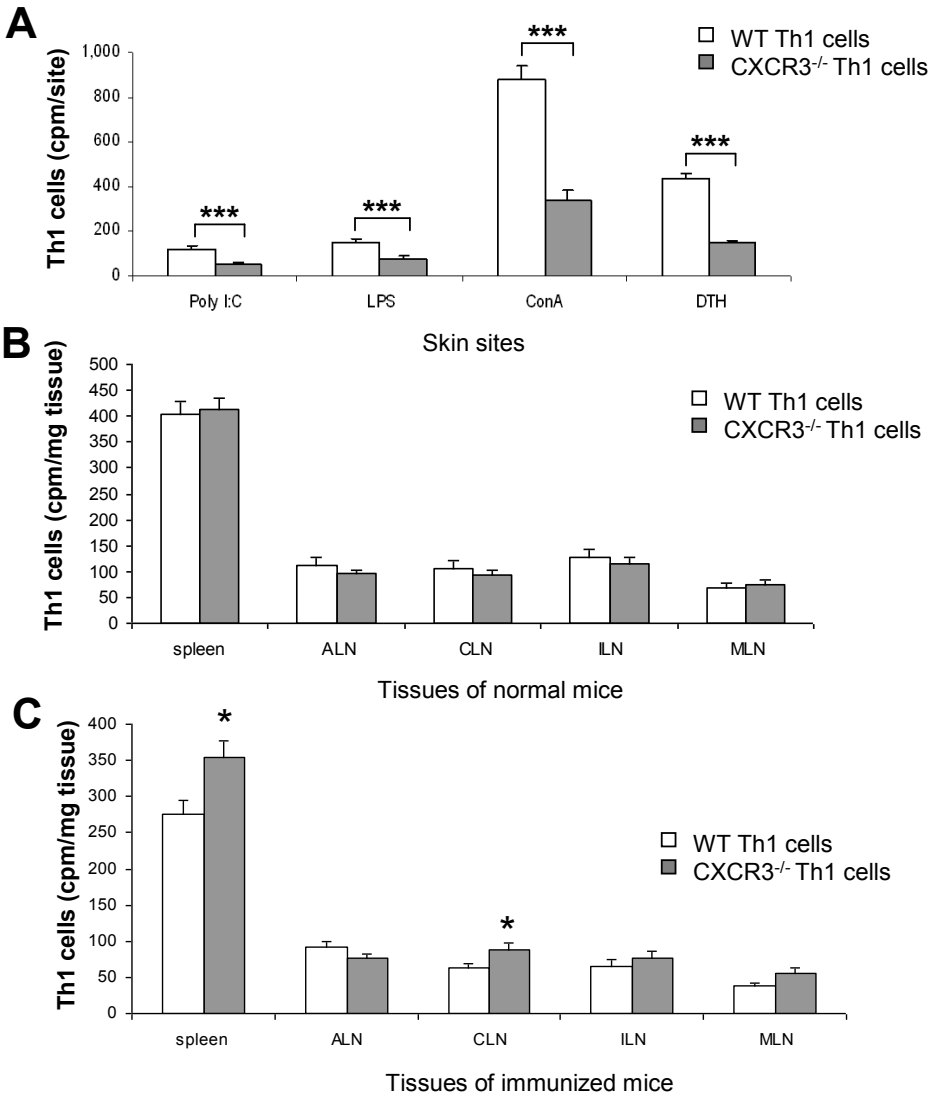

Figure 12. Migration of activated Th1 cells from CXCR3$^{-/-}$ and WT mice into sites of dermal inflammation and lymphoid tissues. CXCR3$^{-/-}$ and WT Th1 cells were radiolabeled and injected i.v. into mice that received i.d. injections of inflammatory stimuli. Accumulation of labeled Th1 cells in the skin sites (A), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), and mesenteric LN (MLN) of normal mice, which received i.d. injections of Poly I:C, LPS and ConA (B) and of OVA/CFA immunized mice, in which DTH was induced (C) was determined. n=7-21 mice in 3-7 exps. Bars represent mean increase in cpm ± SEM over control sites (27±5 for WT Th1, 25±7 for CXCR3$^{-/-}$ Th1) for skin homing or the mean cpm ± SEM for lymphoid homing. * $P < 0.05$, *** $P < 0.005$ by Student's t-test compared to WT Th1 cells.

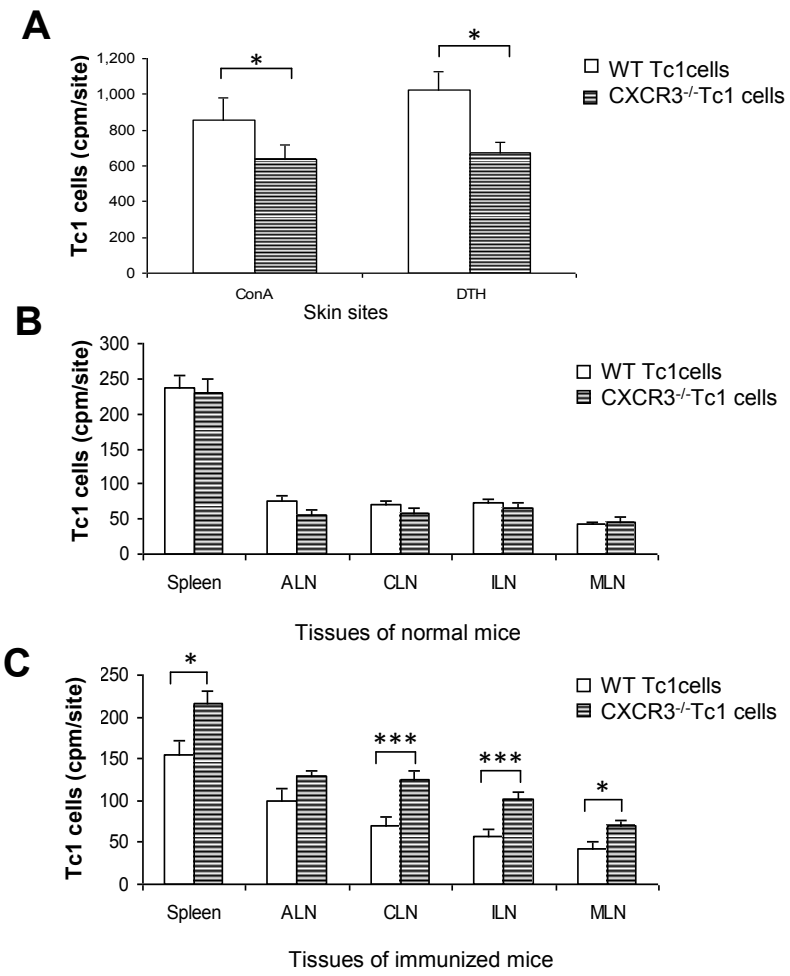

Figure 13. Migration of activated Tc1 cells from CXCR3$^{-/-}$ and WT mice into sites of dermal inflammation and lymphoid tissues. CXCR3$^{-/-}$ and WT Tc1 cells were radiolabeled and injected i.v. into mice that received i.d. injections of inflammatory stimuli. Accumulation of labeled Tc1 cells in the skin sites (A), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), and mesenteric LN (MLN) of normal mice, which received i.d. injections of ConA (B) and of OVA/CFA immunized mice, in which DTH was induced (C) was determined. n=9-13 mice in 3-5 exps. Bars represent mean increase in cpm ± SEM over control sites (45±5 for WT Tc1, 39±4 for CXCR3$^{-/-}$ Tc1) for skin homing or the mean cpm ± SEM for lymphoid homing. * $P < 0.05$, *** $P < 0.005$ by Student's t-test compared to WT Tc1 cells.

4.5 Effect of CXCR3 Deficiency and CCR4 Expression on the Migration of Th1 Cells to Sites of Dermal Inflammation

Th1 cells express CCR4 and CXCR3, and ~50% of the Th1 cells co-express both CKRs (Figure 9). In previous studies in the rat, the association between CCR4 expression and the migration of activated CD4 cells to dermal inflammation was observed with TLR agonists and DTH (Section 3). Whether CCR4 expression affects the recruitment of Th1 cells in mice is not known. In the absence of CXCR3, the recruitment of Th1 cells was reduced by ~50%, but whether CXCR3 deficiency affects the recruitment of $CCR4^+$ and/or $CCR4^-$ cells is not clear.

For this purpose, WT and $CXCR3^{-/-}$ Th1 cells were sorted into $CCR4^+$ and $CCR4^-$ cells, using CR4.1. The $CCR4^+$ and $CCR4^-$ cells were radiolabeled and injected i.v. into mice that had received inflammatory stimuli on the back skin. When the recruitment of $CCR4^+$ or $CCR4^-$ WT Th1 cells to dermal inflammation was examined, $CCR4^+$ Th1 cells were found in greater numbers in the skin sites injected with TLR agonists or ConA compared with $CCR4^-$ cells, while they were comparably found in DTH (Figure 14).

The increased accumulation of $CCR4^+$ Th1 cells in the ConA and TLR agonists that was found with WT cells, was also observed with $CXCR3^{-/-}$ cells (Figure 14). About 2 fold more $CCR4^+$ $CXCR3^{-/-}$ Th1 cells are found in the dermal inflammation induced by ConA and TLR agonists than $CCR4^-$ cells, but they were found in comparable numbers in DTH (Figure 14).

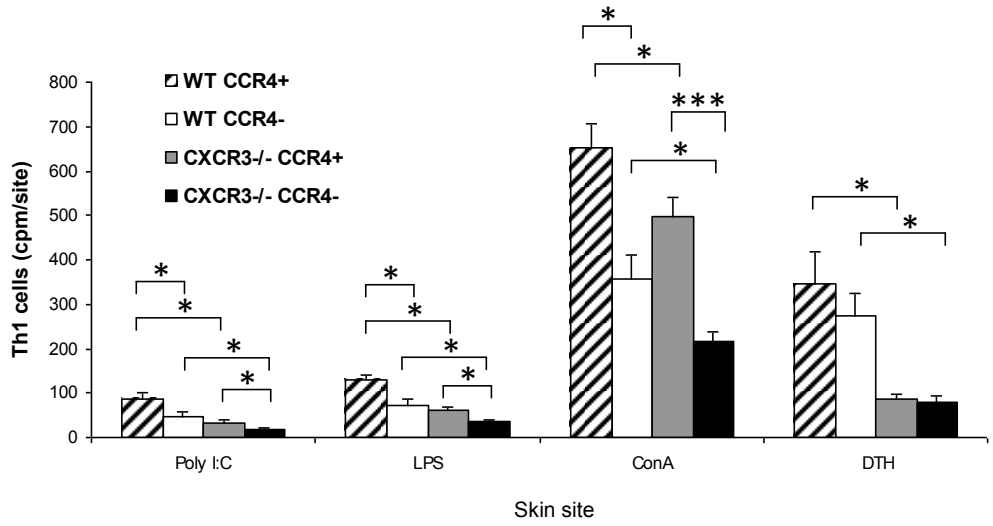

Figure 14. Migration of CCR4$^+$ and CCR4$^-$ Th1 cells from WT and CXCR3$^{-/-}$ mice into sites of dermal inflammation. CCR4$^+$ and CCR4$^-$ were sorted from WT Th1 cells and from CXCR3$^{-/-}$ Th1 cells. They were radiolabeled and injected i.v. into mice that received i.d. injections of inflammatory stimuli. After 20 h, the accumulation of CCR4$^+$ and CCR4$^-$ WT Th1 cells and CCR4$^+$ and CCR4$^-$ CXCR3$^{-/-}$ Th1 cells to dermal sites of inflammation was determined. Bars represent mean increase in cpm ± SEM over control sites; 52 ± 11 for CCR4$^+$, 38 ± 12 for CCR4$^-$ WT Th1 cells, and 24 ± 6 for CCR4$^+$, 18 ± 7 for CCR4$^-$ CXCR3$^{-/-}$ Th1 cells, n=6-12 in 2-4 exps. * $P < 0.05$, *** $P < 0.005$ by Student's t-test

4.6 Summary

The studies described in this chapter demonstrate that CXCR3 mediates part of the migration of CD4 cells, Th1 and Tc1 cells to dermal inflammation. Though $CCR4^+$ Th1 cells are found in greater numbers in most of the inflamed skin sites than $CCR4^-$ cells, I found that CXCR3 mediates part of the migration of both $CCR4^+$ and $CCR4^-$ Th1 cells to inflamed skin. Work presented in the chapter 3 demonstrated that the relationship between the CCR4 expression and the migration of CD4 cells differs between memory and activated cells. Both memory CD4 and activated CD4 cells express CXCR3, whose blockade was shown to reduce the recruitment of T cells to inflamed skin in rats (93). The use of $CXCR3^{-/-}$ mouse T cells enabled the requirement of CXCR3 for migration of T cells to the skin, and the effect of CXCR3 on the recruitment of $CCR4^+$ Th1 cells to be examined.

First, surface expression of CCR4 on mouse T cells was detected using a mAb developed in our laboratory against rat CCR4 (CR4.1). This antibody was found to detect mouse CCR4 on activated WT T cells, while it did not show nonspecific binding on activated $CCR4^{-/-}$ T cells. When CCR4 downregulation was induced, CR4.1 detected reducing levels of CCR4 expression on surface of mouse T cells (Figure 6).

Next, an assay to measure migration of T cells to dermal inflammation induced using TLR agonists (poly I:C, LPS), ConA and DTH was developed. ConA and DTH were shown to induce the recruitment of memory CD4 cells, Th1 and Tc1 cells (Figure 7 and 8). These stimuli recruited more day 6 activated T cells than day 5 activated T cells, an observation that was consistent for both Tc1 cells and Th1 cells (Figure 8). When the migration of day 6 activated T cells to inflamed skin sites was examined, some stimuli such as ConA and DTH were shown to recruit more activated T cells than TLR agonists. This is similar to dermal inflammatory model in rat, where DTH recruited more activated CD4 cells than TLR agonists (Figure 5).

Using CXCR3 deficient T cells, the migration of T cells to dermal inflammation was found to be reduced in the absence of CXCR3. Even though CXCR3 is expressed on most Th1 and Tc1 cells, the requirement of CXCR3 for the recruitment of T cells to inflamed skin sites was more apparent for Th1 cells than for Tc1 cells. In fact, the

recruitment of CXCR3$^{-/-}$ Th1 cells to dermal inflammation was reduced by ~50-60%, but that of CXCR3$^{-/-}$ Tc1 cells was reduced only by ~30% (Figures 12A and 13A). A moderate effect of CXCR3 deficiency on Tc1 cell infiltration compared to Th1 cell infiltration has not been reported before. CXCR3$^{-/-}$ Th1 and Tc1 cells appeared to be normal in that the absence of CXCR3 did not affect their expression of adhesion molecules. However, the proliferation of CXCR3$^{-/-}$ Th1 cells was reduced compared with WT Th1 cells (Figure 9).

My results demonstrate that CXCR3 mediates part of the migration of CD4 T cells to dermal inflammation, whether they were Th1 cells or unstimulated CD4 cells. The expression of CXCR3 is increased after *in vitro* activation of CD4 T cells in type 1 polarizing condition (Figure 9), but the absence of CXCR3 reduced the number of both unstimulated CD4 cells and Th1 cells in the inflamed skin sites by ~60% (Figures 11A and 12A). This suggests that ~60% of the unstimulated or activated CD4 cells that are found in inflamed skin require CXCR3 for their recruitment. Thus, the blockade of CXCR3 would reduce the recruitment of unstimulated and activated CD4 T cells, as previously shown for the memory CD45RC^{-} CD4 cells and *in vivo* activated T cells in rats (93).

The expression of CCR4 was associated with increased accumulation of Th1 cells to inflammatory sites in skin, except for DTH where CCR4^{+} and CCR4^{-} Th1 cells were found at similar levels. A 2-fold increase in the accumulation of CCR4^{+} Th1 cells compared with CCR4^{-} Th1 cells in response to TLR agonists and ConA was observed. I found that the increased accumulation of CCR4^{+} Th1 cells was only partly affected by the CXCR3 deficiency. CXCR3 deficiency reduced the recruitment of CCR4^{+} Th1 cells. However, even amongst CXCR3$^{-/-}$ Th1 cells, there were more CCR4^{+} cells than CCR4^{-} cells in most of the inflamed skin sites (Figure 14). These CXCR3$^{-/-}$ Th1 cells would represent the proportion of Th1 cells that do not require CXCR3 for their recruitment. When comparing the recruitment of CCR4^{+} and CCR4^{-} Th1 cells, 60-70% of the Th1 cells that accumulated in the sites injected with ConA or TLR agonists were of CCR4^{+}; whether they were from WT Th1 cells or CXCR3$^{-/-}$ Th1 cells (Figure 14).

It is of interest to note that CXCR3 mediated part of the recruitment of Th1 cells to inflamed skin sites, whether they were CCR4^{+} or CCR4^{-}. For example, there was ~40-

60% fewer $CCR4^+$ $CXCR3^{-/-}$ Th1 cells in the inflamed skin sites than $CCR4^+$ WT Th1 cells. Similarly, less $CCR4^-$ $CXCR3^{-/-}$ Th1 cells were found in the inflamed skin sites than $CCR4^-$ WT Th1 cells (Figure 14). Therefore, the absence of CXCR3 reduced the recruitment of Th1 cells, and did not appear to have a differential effect on $CCR4^+$ and $CCR4^-$ cells. It is likely that the recruitment of $CCR4^+$ Th1 cells to inflamed skin is mediated in part by CXCR3 and in part by other CKRs including possibly CCR4 itself.

Chapter 5. Effect of CCR4 Deficiency on the *In Vivo* Migration of T Cells to Dermal Inflammation

The association between CCR4 expression on memory and activated T cells and their recruitment into inflammatory sites in different tissues is unclear. The previous studies in the rat suggested that the relationship between CCR4 expression and dermal tropism was complex (Chapter 3). Most of the memory CD4 cells in the inflamed skin were $CCR4^{+}$ (Chapter 3), yet part of the migration of CD4 cells is mediated by CXCR3, as shown by blockade studies in the rat (93), and by deficiency of CXCR3 in mice (Chapter 4). In addition, by utilizing $CXCR3^{-/-}$ Th1 cells, it was shown that migration of Th1 cells was partly dependent on CXCR3, but there was some CXCR3-independent migration by $CCR4^{+}$ and $CCR4^{-}$ Th1 cells (Chapter 4). In order to examine whether the expression of CCR4 on T cells is required for the migration to inflamed skin, CCR4 deficient mice were utilized, and an antibody to detect mouse CCR4 expression was used. Whether CCR4 is required differentially by CD4 or CD8 cells; whether the requirement would vary for different T subsets; unstimulated or memory CD4 cells, Treg cells, activated type 1 or type 2 T cells, were also examined.

5.1 Effect of CCR4 Deficiency on the *In Vivo* Migration of Unstimulated CD4 Cells of WT and CCR4$^{-/-}$ Mice to Sites of Dermal Inflammation

The increased recruitment of CCR4^{+} memory CD4 cells to inflamed skin only demonstrates that CCR4^{+} CD4 cells migrate to inflamed skin (Chapter 3), but does not indicate whether they require CCR4 for this process. In fact, the migration of CD4 cells are only partly mediated by CXCR3, as shown by the reduced migration of CXCR3$^{-/-}$ CD4 cells (Chapter 4). It is therefore necessary to examine whether CCR4 is required for the migration of CD4 cells to inflamed skin.

For this purpose, CD4 splenocytes were isolated from CCR4$^{-/-}$ and WT mice, radiolabeled and injected into mice that received ConA and diluent sites on the back skin. ConA sites recruited ~25 times more WT CD4 cells than control sites. About ~30% more CCR4$^{-/-}$ CD4 cells were found in ConA sites than WT cells ($P < 0.05$) (Figure 15A), and ~2 times more CCR4$^{-/-}$ CD4 cells were found in the LNs than WT cells, but they were found in comparable numbers in the spleen (Figure 15B).

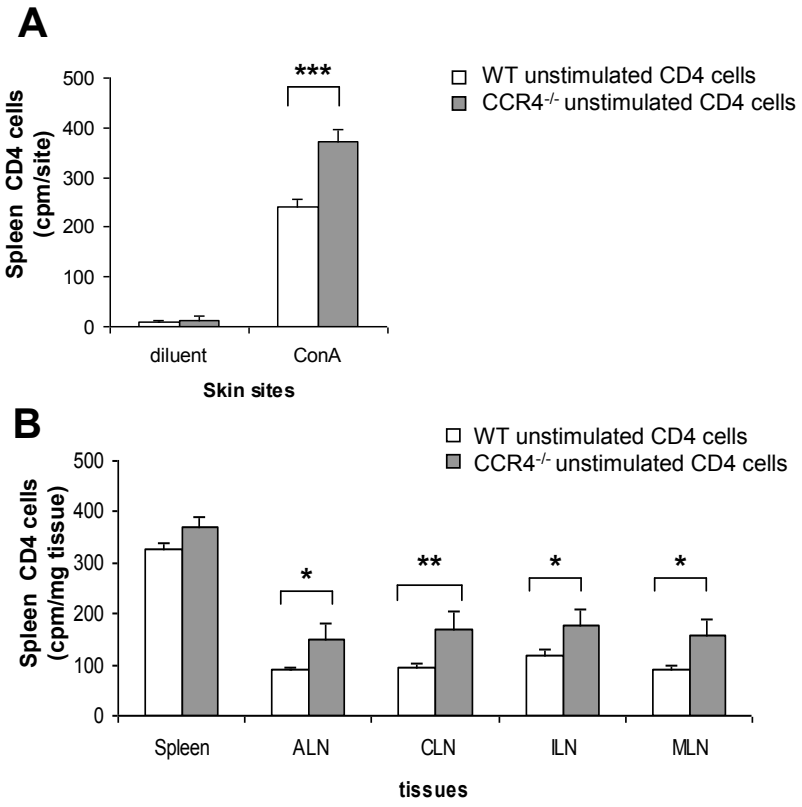

Figure 15. Migration of unstimulated CD4 cells from CCR4$^{-/-}$ and WT mice into sites of dermal inflammation and lymphoid tissues. CD4 cells isolated from spleens of CCR4$^{-/-}$ and WT mice, were radiolabeled and injected i.v. into mice that received i.d. ConA. After 20 h, the radioactivity content of the skin sites (A), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), and mesenteric LN (MLN) (B) was determined as a measure of labeled cell migration. n = 6-17 mice in 4-6 exps. Bars represent mean cpm ± SEM in skin sites for skin homing or mean cpm per mg tissue ± SEM for lymphoid homing. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ by Student's t-test compared to WT CD4 cells.

5.2 Expression of CCR4 and CXCR3 on Memory CD4 Cells from WT and CCR4$^{-/-}$ Mice, and the Effect of CCR4 Deficiency on the Migration of Memory CD4 Cells to Sites of Dermal Inflammation

Unstimulated CD4 cells are ~10% CXCR3^{+} (93) and 2-7% CCR4^{+}, and this is mostly restricted to the memory cells in rats (Figure 4A), which migrate to dermal inflammation significantly better than do naïve CD4 cells (Figure 6D). In order to examine the expression of CXCR3 and CCR4 on memory cells, CD4 splenocytes were stained with antibodies for CD4, CD44, CD45RB and CXCR3 or CCR4. CCR4 was expressed on a small proportion of CD4 cells (2.8 ± 2.0%), while CXCR3 was expressed on 11.2 ± 5.8% of the CD4 cells in the spleen of WT mice. Most of the CKR expression on CD4 cells is present on memory CD4 cells, which are 30 ± 10% CXCR3^{+} and 13.9 ± 7.5% CCR4^{+}. The expression of CXCR3 is normal on CCR4$^{-/-}$ memory CD4 cells, which are 25.8 ± 8.2% CXCR3^{+} (Figure 16A).

It was interesting to observe an increased proportion of memory CD4 cells in the spleen of CCR4$^{-/-}$ mice compared to WT mice. About 18.0 ± 1.1% of the CCR4$^{-/-}$ CD4 cells had a memory phenotype, while 14.1 ± 3.6% of the WT CD4 cells had a memory phenotype. Thus, there was an ~25% increase in the proportion of memory cells amongst the CCR4$^{-/-}$ CD4 cells ($P < 0.05$) (Figure 16A).

Since CCR4$^{-/-}$ splenocytes have a higher proportion of memory cells than WT splenocytes (Figure 16A), the increased recruitment of CCR4$^{-/-}$ CD4 cells may reflect the presence of more memory cells among the CCR4$^{-/-}$ than the WT CD4 cells. To determine whether the increased recruitment was an inherent feature of CCR4$^{-/-}$ memory CD4 cells, memory cells isolated from CCR4$^{-/-}$ and WT splenocytes radiolabeled, and injected into mice that received i.d. injections of inflammatory stimuli. There was ~40% more CCR4$^{-/-}$ memory CD4 cells than WT cells in ConA sites ($P < 0.05$), but they were comparably found in DTH lesions ($P > 0.05$). There was no difference in the accumulation of CCR4$^{-/-}$ and WT memory CD4 cells in lymphoid tissues of these mice (Figure 16B,C). Thus, CCR4 is not required for the migration of memory CD4 cells to dermal inflammation. In contrast, more memory CCR4$^{-/-}$ CD4 cells were found in ConA sites compared to memory WT CD4 cells.

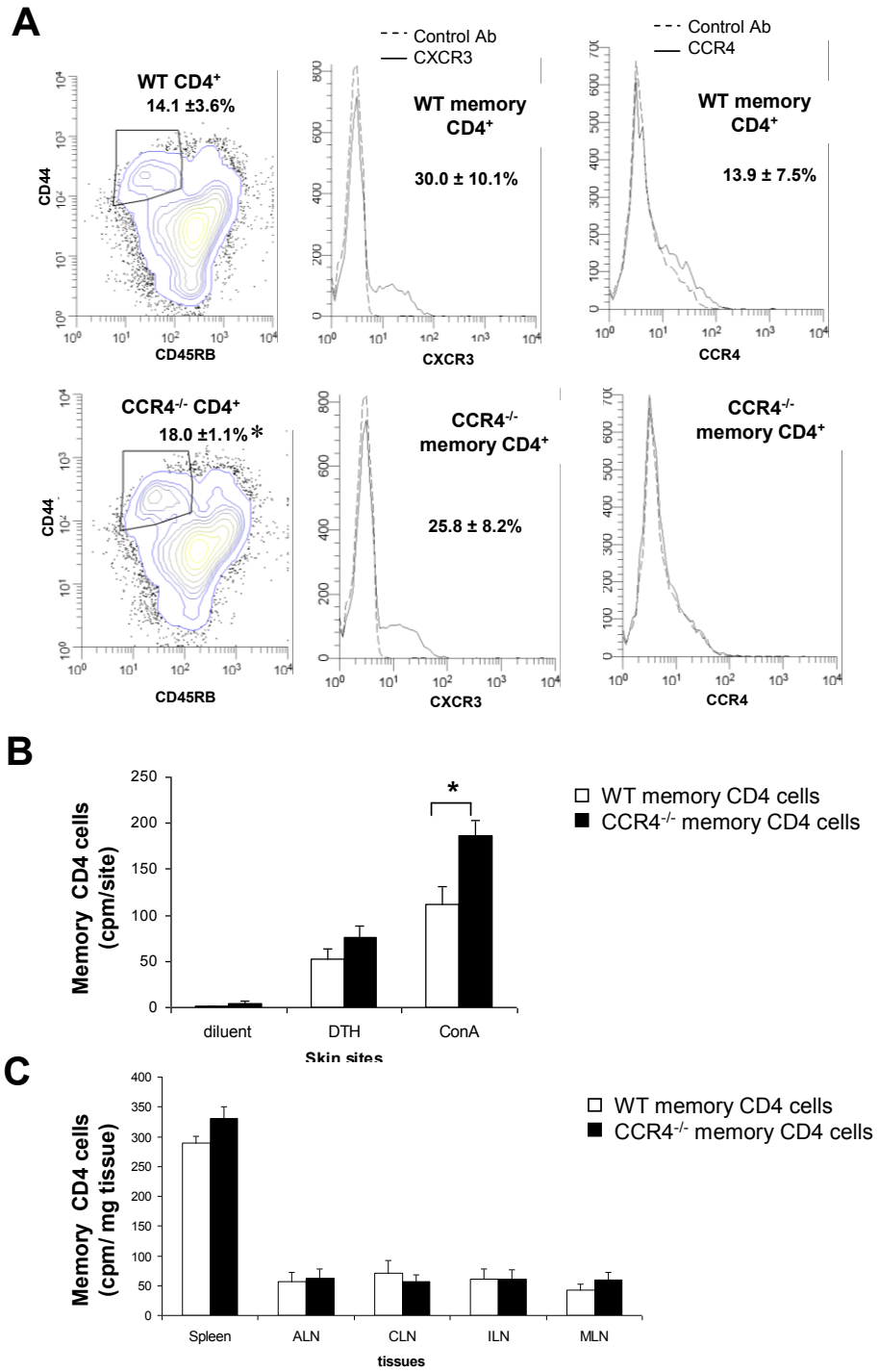

Figure 16. Expression of CCR4 and CXCR3 on memory CD4 cells, and the migration of memory CD4 cells from WT and CCR4$^{-/-}$ mice into sites of dermal inflammation and lymphoid tissues. (A) CCR4$^{-/-}$ and WT CD4 splenocytes were stained to determine proportion of memory cells (CD45RB^{lo} CD44^{hi}) and their CXCR3 and CCR4 expression. n=4-17. (B-C) CCR4$^{-/-}$ and WT memory CD4 cells were radiolabeled and injected i.v. to mice that received i.d. inflammatory stimuli. After 20 h, the radioactivity content of the skin sites (B), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), and mesenteric LN (MLN) (C) was determined as a measure of labeled cell migration. n = 4-12 mice in 1-4 exps. Bars represent mean cpm ± SEM for skin homing or mean cpm per mg tissue ± SEM for lymphoid homing. * $P < 0.05$ by Student's t-test compared to WT CD4 cells.

5.3 Effect of CCR4 Deficiency on the Migration of Treg Cells to Sites of Dermal Inflammation

Whether CCR4 mediates the recruitment of regulatory CD4 $Foxp3^+$ cells to dermal inflammation is not clear. Studies examining this have used normal skin in $RAG2^{-/-}$ mice (156) or OXA-induced CHS (195), but the requirement for CCR4 has not been examined using other inflammatory stimuli in the skin, such as TLR agonists or ConA. For this purpose, CD4 cells were isolated from $CCR4^{-/-}$ and WT mice, and activated in culture conditions to induce and expand Treg cells (> 80% $Foxp3^+$). They were radiolabeled and injected i.v. into mice that received inflammatory stimuli i.d.

As shown in Figure 17, the extent of recruitment of Treg cells varied in response to different inflammatory stimuli; ConA induced a 27-fold recruitment of Treg cells compared to control sites, while TLR agonists induced ~2-3 times more recruitment of Treg cells than the control sites. It should be noted that the $CCR4^{-/-}$ and WT CD4 Treg cells that were differentiated in the presence of α-CD3/α-CD28, IL-2, TGF-β and rapamycin gave similar results to $CCR4^{-/-}$ and WT $CD25^+$ CD4 cells that were expanded in the presence of IL-2. These results were therefore combined (Figure 17). When the migration of $CCR4^{-/-}$ and WT Treg cells was examined, more $CCR4^{-/-}$ Treg cells were found in the inflamed skin more than WT Treg cells; ~40% more $CCR4^{-/-}$ Treg cells in the ConA site, and ~75% times more $CCR4^{-/-}$ Treg cells in poly I:C site ($P < 0.05$), but no difference was noted in LPS site (Figure 17A). The accumulation of $CCR4^{-/-}$ and WT Treg cells in the spleen and LNs was similar (Figure 17B). Therefore, Treg cells were recruited to ConA and poly I:C in the skin, and they were found in increased numbers in the absence of CCR4.

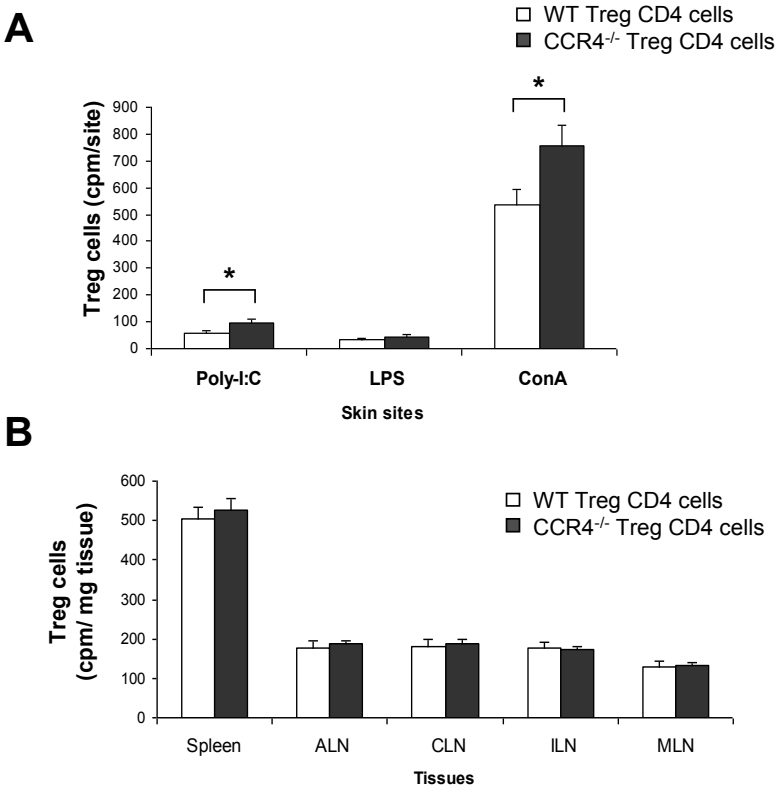

Figure 17. Migration of Treg cells from WT and CCR4$^{-/-}$ mice into sites of dermal inflammation and lymphoid tissues. WT and CCR4$^{-/-}$ Treg cells were radiolabeled and injected i.v. into mice that received i.d. inflammatory stimuli. After 20 h, the radioactivity content of the skin sites (A), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), and mesenteric LN (MLN) (B) was determined as a measure of labeled cell migration. n = 16-20 mice in 4-5 exps. Bars represent mean cpm ± SEM above control sites (20±4 for WT or 32±7 for CCR4$^{-/-}$) for skin homing or mean cpm per mg tissue ± SEM for lymphoid homing. * $P < 0.05$ by Student's t-test compared to WT cells.

5.4 Proliferation and Expression of Adhesion Molecules, and chemokine receptors, CXCR3 and/or CCR4, on Activated T Cells from WT and CCR4$^{-/-}$ Mice

The relationship between the expression of CCR4 on activated T cells and their recruitment into dermal inflammation is not clear. CCR4^{+} Th1 cells are found in greater numbers in most inflamed skin sites. Though part of the migration is mediated by CXCR3, the CXCR3-independent migration of Th1 cells is associated with CCR4 expression, as shown by the increased presence of CCR4^{+} CXCR3$^{-/-}$ Th1 cells in the inflamed skin sites than CCR4^{-} cells (Chapter 4). Therefore, it is important to examine whether CCR4 is required for the *in vivo* migration of activated T cells. CD4 T cells from CCR4$^{-/-}$ and WT mice were activated in type 1 or type 2 polarizing conditions. Cell counts were performed daily during the *in vitro* activation. On day 6, the expression of the activation marker CD25, adhesion molecules (CD44, α_4-integin, L-selectin, ESL and PSL), cytokines (IFN-γ and IL-4), and CKRs (CCR7, CCR4 and/or CXCR3) was determined.

As shown in Figure 18, the proliferation of CCR4$^{-/-}$ Th1 cells was normal. The expression of CD25 on the activated CCR4$^{-/-}$ and WT CD4 cells were similar; ~90% of CD4 cells expressed CD25. These activated cells had a Th1 profile in that 64 ± 7% of the WT CD4 cells and 69 ± 4% of the CCR4$^{-/-}$ CD4 cells expressed IFN-γ but not IL-4 (Table 9). Also, the expression of adhesion molecules on CCR4$^{-/-}$ Th1 cells appeared to be normal. Both WT and CCR4$^{-/-}$ Th1 cells expressed 98% CD44, 55% α_4-integrin, 34% L-selectin, 54% ESL, 40% PSL, 91% CXCR3, and 43% CCR7 (Table 9).

When WT and CCR4$^{-/-}$ CD4 cells were activated in type 2 polarizing conditions, CCR4$^{-/-}$ Th2 cells were found to have reduced proliferation compared to WT Th2 cells ($P < 0.05$) (Figure 18). The expression of CD25 on the activated CCR4$^{-/-}$ and WT CD4 cells were similar; 90% of CD4 cells expressed CD25. These activated cells had a Th2 profile in that they produced IL-4, but there was a significantly lower proportion of IL-4 producing cells on CCR4$^{-/-}$ Th2 cells than on WT cells; 39 ± 8% of the CCR4$^{-/-}$ Th2 cells expressed IL-4 while 60 ± 7% of the WT Th2 cells expressed IL-4 ($P < 0.05$). In addition, ~20% of the CD4 cells produced IFN-γ, but these IFN-γ^{+} cells appeared to be uncommitted in that most of them produced IL-4 or IL-10. There was increased

expression of L-selectin on CCR4$^{-/-}$ Th2 cells (~39%) compared to WT Th2 cells (20%), but the expression of ESL and PSL on CCR4$^{-/-}$ Th2 cells appeared to be normal in that both WT and CCR4$^{-/-}$ Th2 cells expressed 26% ESL and 13% PSL (Table 10).

The expression of CCR4 and CXCR3 on activated CD4 cells differed between those activated in type 1 and type 2 polarizing conditions. The proportion of CD4 cells expressing CCR4 or CXCR3 was increased after *in vitro* activation; with Th1 cells expressing more CCR4 and CXCR3 than did Th2 cells. In fact, CCR4 was expressed on 60% of the WT Th1 cells, but only on ~20% of the Th2 cells (Figure 18 and Tables 9 and 10). Also, 90% of the WT and CCR4$^{-/-}$ Th1 cells expressed CXCR3, while 20% of the WT Th2 cells expressed CXCR3 (Tables 9 and 10). Whereas 85 ± 5% of the CCR4^{+} Th1 co-express CXCR3 (Figure 9), only 22 ± 4% of the CCR4^{+} Th2 cells co-express CXCR3.

Table 9. Expression of adhesion molecules and cytokines by WT and CCR4$^{-/-}$ CD4 cells after 6 days of *in vitro* activation in type 1 polarizing conditions.

	WT Th1 cells			**CCR4$^{-/-}$ Th1 cells**		
Markers	N	% expression mean (±SEM)	MFI mean (±SEM)	N	% expression mean (±SEM)	MFI mean (±SEM)
CD25	14	89.2 (±2.5)	58.9 (±8.1)	13	89.1 (±2.3)	58.6 (±10.8)
Adhesion molecules						
CD44	6	98.0 (±0.6)	371.8 (±144.4)	6	98.1 (±0.5)	433.2 (±191.5)
α_4-integin	8	54.0 (±8.7)	21.6 (±2.8)	8	54.5 (±8.7)	22.5 (±2.3)
L-selectin	14	34.4 (±3.4)	48.5 (±8.3)	13	34.8 (±4.2)	50.1 (±9.1)
ESL	14	54.0 (±5.1)	672.7 (±198.3)	14	52.6 (±5.4)	719.5 (±210.4)
PSL	8	39.6 (±6.6)	941.3 (±443.0)	9	39.8 (±6.6)	952.8(±422.9)
CKRs						
CXCR3	12	91.1 (±2.4)	267.5 (±75.5)	11	92.0(±2.7)	307.4 (±101.2)
CCR4	16	58.4 (±3.8)	30.1 (±3.4)			
CCR7	4	42.8 (±3.6)	18.4 (±3.5)	4	42.7 (±5.1)	21.1 (±5.9)
Cytokines						
IFN-γ	9	64.4 (±7.1)	135.7 (±74.5)	6	69.0 (±4.4)	115.7 (±58.5)
IL-4	3	2.3 (±1.3)	11.1 (±2.5)	2	2.7(±0.6)	13.1(±4.4)

Table 10. Expression of adhesion molecules and cytokines by WT and CCR4$^{-/-}$ CD4 cells after 6 days of *in vitro* activation in type 2 polarizing conditions.

	WT Th2 cells			**CCR4$^{-/-}$ Th2 cells**		
Markers	N	% expression mean(±SEM)	MFI mean(±SEM)	n	% expression mean(±SEM)	MFI mean(±SEM)
CD25	3	90.7 (±0.5)	64.6 (±8.9)	3	88.7 (±2.2)	56.6 (±1.8)
Adhesion molecules						
CD44	1	98.0	56.0	1	94.0	50.0
α4-integin	1	10.7	17.0	1	16.2	18.1
L-selectin	3	19.7 (±4.3)	42.8(±7.4)	3	38.6 (±7.1)*	52.5 (±10.2)
ESL	3	25.5(±10.0)	36.9 (±12.8)	3	27.7 (±5.0)	51.5 (±12.8)
PSL	2	11.1 (±5.6)	24.5 (±8.6)	2	15.6 (±1.9)	26.9 (±2.1)
CKRs						
CXCR3	5	20.1 (±3.4)	10.0 (±0.8)	0	ND	ND
CCR4	11	17.1(±2.5)	14.5 (±2.2)	2	0.1 (±0.1)	11.8 (±1.7)
Cytokines						
IL-4	5	59.8 (±7.3)	24.9 (±7.5)	5	38.5 (±8.3) #	18.6 (±5.8)
IL-10	7	14.7 (±5.9)	11.6 (±1.3)	5	24.2 (±7.2)	14.0(±2.0)
IFN-γ (a)	3	20.4 (±8.8)	73.8 (±43.4)	1	20.3	121.1

# $P < 0.05$ by paired t-test compared to WT cells.

(a) Most of the IFN-γ cells also produced IL-4 or IL-10; ~68.4% (±21.9) of the WT IFN-γ^+ CD4 cells also produced IL-4, and ~43.4% (±14.0) of the IFN-γ^+ CD4 cells also produced IL-10.

* $P < 0.05$ by student's t-test compared to WT cells.

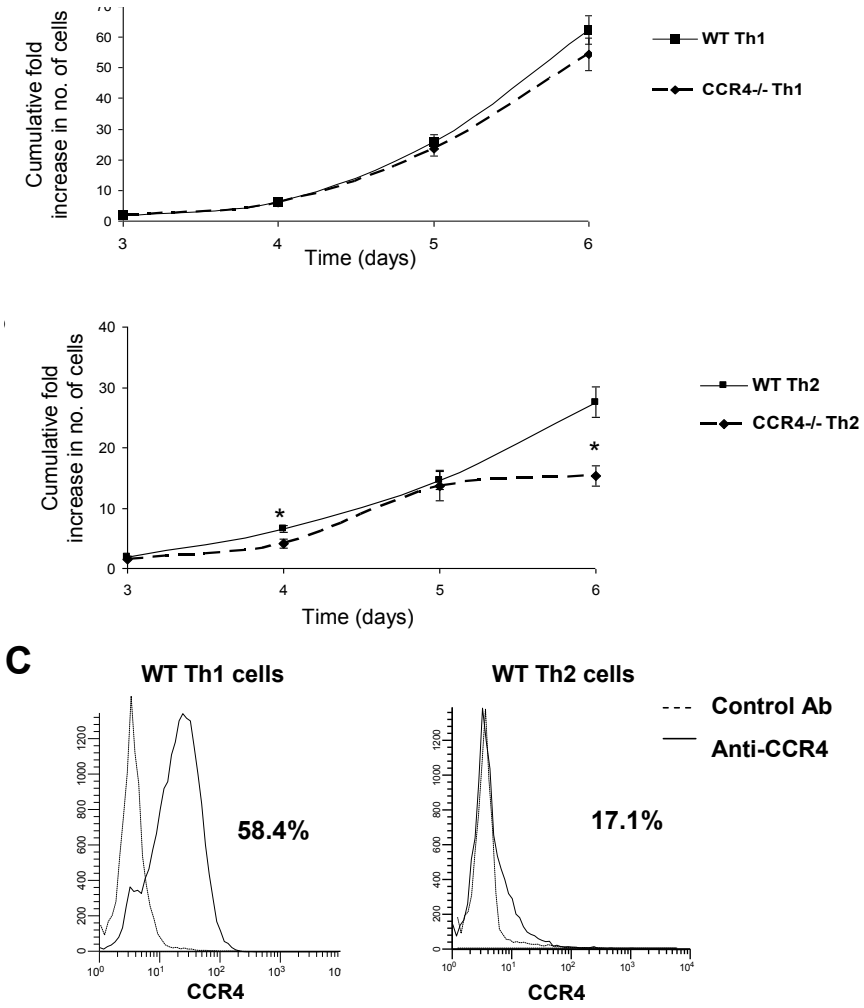

Figure 18. Proliferation and expression of CCR4 on *in vitro* activated CD4 cells from CCR4$^{-/-}$ and WT mice. WT and CCR4$^{-/-}$ CD4 cells were activated in type 1 or type 2 polarizing conditions. (A-B) Cumulative fold increase in the number of cells during *in vitro* proliferation of CCR4$^{-/-}$ and WT Th1 cells (n=36-52) (A), and CCR4$^{-/-}$ and WT Th2 cells (n=10-16) (B) * $P < 0.05$ by Student's t-test compared to WT cells. (C) Expression of CCR4 on activated WT Th1 and Th2 cells (n=11-16). Error bars represent mean ± SEM. Representative histograms with mean of % expression.

CD8 T cells from CCR4$^{-/-}$ and WT mice were also activated in type 1 or type 2 polarizing conditions. Cell counts were performed daily during the *in vitro* activation. After 6 days of activation, the expression of the activation marker CD25, adhesion molecules (CD44, α_4-integin, L-selectin, ESL and PSL), cytokines (IFN-γ and IL-4), and CKRs (CCR7, CCR4 and/or CXCR3) was determined.

As shown in Figure 19, CCR4$^{-/-}$ Tc1 cells proliferated well, but slightly less than WT Tc1 cells. CCR4$^{-/-}$ Tc1 cells proliferated ~100 times, while WT Tc1 cells proliferated ~130% times by day 6 ($P < 0.05$). The expression of CD25 on the activated CCR4$^{-/-}$ and WT CD8 cells was similar; after 5 days of *in vitro* activation: 89 ± 1% of the WT cells and 89 ± 2% of the CCR4$^{-/-}$ cells expressed CD25, and 6 days of *in vitro* activation: 59 ±4% of the WT cells and 55 ± 10% of the CCR4$^{-/-}$ cells expressed CD25. These activated cells had a Tc1 profile in that 79 ± 3% of the WT CD4 cells and 73% of the CCR4$^{-/-}$ CD4 cells expressed IFN-γ but not IL-4 (Table 11). The expression of adhesion molecules on CCR4$^{-/-}$ Tc1 cells appeared to be normal, in that both expressed CD44, α_4-integrin, L-selectin, ESL and PSL on a similar frequency of cells (Table 11).

When WT and CCR4$^{-/-}$ CD8 cells were activated in type 2 polarizing conditions, both WT and CCR4$^{-/-}$ Tc2 cells proliferated equivalently (Figure 19). The expression of CD25 on the activated CCR4$^{-/-}$ and WT CD8 cells were similar; 98% of the WT CD8 cells and 96% of the CCR4$^{-/-}$ CD8 cells expressed CD25 after 5 days of *in vitro* activation, and 80 ± 17% of the WT CD8 cells and 80 ± 18% of the CCR4$^{-/-}$ CD8 cells expressed CD25 after 6 days of *in vitro* activation. These activated cells had a Tc2 profile in that 25 ± 3% of the WT CD8 cells and 18 ± 4% of the CCR4$^{-/-}$ CD8 cells produced IL-4. In addition, 50% of the CD4 cells produced IFN-γ, but some of the IFN-γ^+ cells were uncommitted in that they produced IL-4 or IL-10 (Table 12). *In vitro* generated mouse Tc2 cells are known to secrete both IFN-γ and IL-4 (8, 9).The expression of adhesion molecules on CCR4$^{-/-}$ Tc2 cells appeared to be normal in that both WT and CCR4$^{-/-}$ Tc2 cells expressed L-selectin, ESL and PSL on a similar frequency of cells (Table 12).

The expression of CCR4 and CXCR3 on activated CD8 cells differed between type 1 and type 2 polarizing conditions. The proportion of CD8 cells expressing CCR4 or CXCR3 was increased after *in vitro* activation; with Tc1 cells expressing more CCR4 and

CXCR3 than did Tc2 cells. In fact, CCR4 was expressed on ~20% of the WT Tc1 cells, but only on ~10% of the Tc2 cells (Figure 19 and Tables 11 and12). Also, ~96% of the WT Tc1 cells expressed CXCR3, while ~20% of the WT Tc2 cells expressed CXCR3 (Tables 11 and 12).

Table 11. Expression of adhesion molecules and cytokines by WT and CCR4$^{-/-}$ CD8 cells after 6 days of *in vitro* activation in type 1 polarizing conditions.

	WT Tc1 cells			**CCR4$^{-/-}$ Tc1 cells**		
Markers	N	% expression mean (±SEM)	MFI mean (±SEM)	N	% expression mean (±SEM)	MFI mean (±SEM)
CD25	3	59.2 (±4.2)	24.5 (±7.0)	3	55.4 (±10.5)	25.1 (±6.4)
Adhesion molecules						
CD44	2	97.8 (±1.6)	80.2 (±20.1)	2	95.9 (±3.7)	77.2 (±23.2)
α_4-integin	4	62.6 (±6.9)	26.8 (±2.1)	3	67.1 (±10.5)	25.8 (±1.4)
L-selectin	3	22.8 (±1.4)	58.1 (±17.4)	3	23.7 (±1.7)	61.3(±19.6)
ESL	4	74.8 (±6.1)	931.9 (±436.6)	3	72.8 (±5.3)	988.2 (±165.4)
PSL	4	53.4(±10.1)	355.6 (±89.8)	3	54.6 (±16.0)	248.4 (±25.2)
CKRs						
CXCR3	3	96.8 (±1.3)	60.8 (±4.3)		ND	ND
CCR4	6	22.4 (±5.9)	46.5 (±16.4)	3	2.3 (±1.0)	18.8 (±3.5)
Cytokines						
IFN-γ	2	79.6 (±3.2)	386.1 (±58.5)	1	72.8	478.8
IL-4	3	1.0 (±0.8)	11.1 (±3.2)	2	0.1(±0.1)	12.1(±4.4)

ND= not determined

Table 12. Expression of adhesion molecules and cytokines by WT and CCR4$^{-/-}$ CD8 cells after 6 days of *in vitro* activation in type 2 polarizing conditions.

	WT Tc2 cells			**CCR4$^{-/-}$ Tc2 cells**		
Markers	N	% expression mean (±SEM)	MFI mean (±SEM)	N	% expression mean (±SEM)	MFI mean (±SEM)
CD25	2	80.2 (±17.2)	75.1 (±27.9)	2	79.9 (±18.1)	87.0 (±25.1)
Adhesion molecules						
CD44	1	98.0	46.0	1	95.0	64.0
L-selectin	1	34.1	31.0	1	31.9	29.0
ESL	2	52.6 (±8.3)	368.2 (±73.4)	2	51.3(±8.2)	342.3 (±37.3)
PSL	2	8.9 (±5.3)	110.7 (±42.4)	2	8.7 (±5.1)	105.9 (±43.2)
CKRs						
CXCR3	3	17.4 (±20.5)	11.2 (±0.9)	0	ND	ND
CCR4	6	11.2(±2.9)	33.3 (±9.1)			
Cytokines						
IL-4	5	24.5 (±3.1)	13.4 (±2.5)	3	17.9 (±4.0)	14.9 (±5.0)
IL-10	6	16.5 (±5.9)	12.6 (±1.1)	3	21.0(±10.5)	14.7(±3.2)
IFN-γ [(a)]	3	50.7 (±11.2)	156.3 (±106.3)	1	76.3	432.2

ND= not determined

[(a)] Most of the IFN-γ cells also produced IL-4 or IL-10; ~42.3% (±10.7) of the WT IFN-γ^+ CD8 cells also produced IL-4, and ~47.9% (±6.9) of the IFN-γ^+ CD8 cells also produced IL-10.

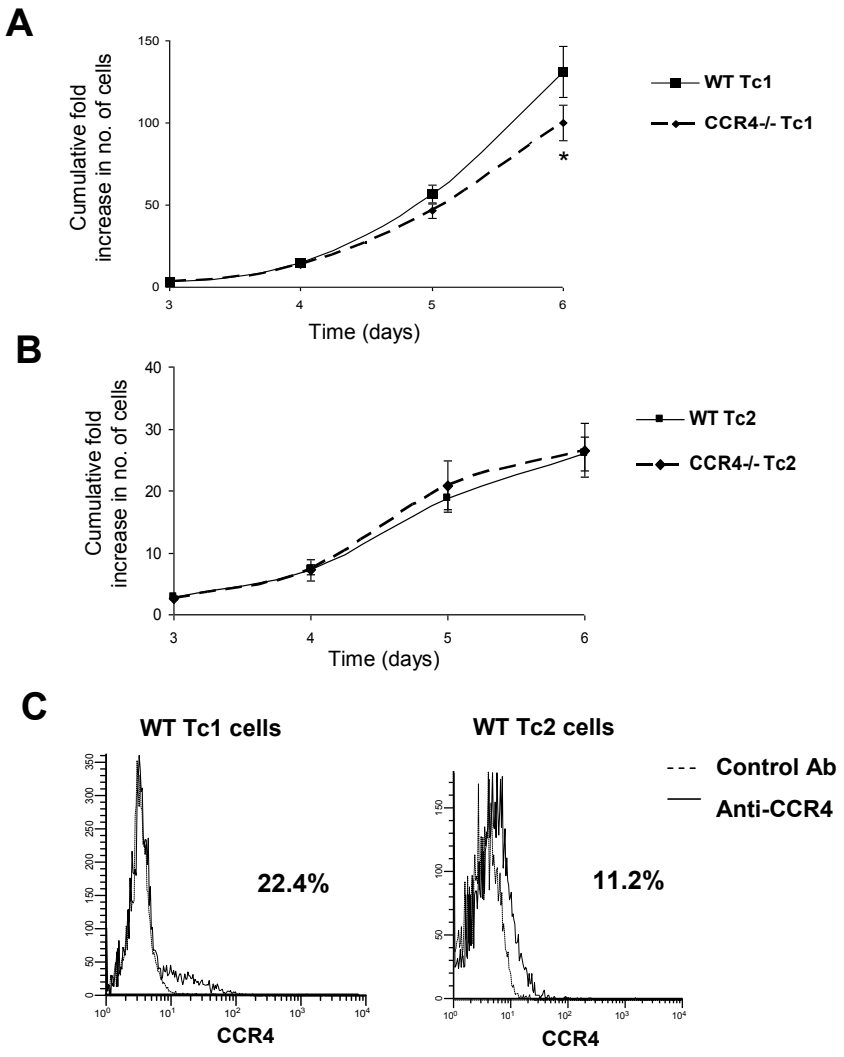

Figure 19. Proliferation and expression of CCR4 on *in vitro* activated CD8 cells from CCR4$^{-/-}$ and WT mice. WT and CCR4$^{-/-}$ CD8 cells were activated in type 1 or type 2 polarizing conditions. (A-B) Cumulative fold increase in the number of cells during *in vitro* proliferation of CCR4$^{-/-}$ and WT Tc1 cells (n=16-20) (A), and CCR4$^{-/-}$ and WT Tc2 cells (n=9-15) (B) * $P < 0.05$ by Student's t-test compared to WT cells. (C) Expression of CCR4 on activated WT Tc1 and Tc2 cells (n=5-6). Error bars represent mean ± SEM. Representative histograms with mean of % expression.

5.5 Effect of CCR4 Deficiency on the Migration of Activated T Cells to Sites of Dermal Inflammation

The contribution of CCR4 to the infiltration of activated T cells into inflammatory sites in the skin is unclear. CCR4 expression might be related to the migration of activated T cells, such as Th1 cells, since $CCR4^{+}$ Th1 cells are present in the inflamed skin (Chapter 4). However, the recruitment of activated Th1 cells in dermal inflammation is partly dependent on CXCR3 (Chapter 4), but it is unclear whether CCR4 is required for the CXCR3-independent migration of Th1 cells. It is also unclear whether type 1 or type 2 T cells differ in their requirement for CCR4 in their migration to dermal inflammation. For this purpose, activated T cells from $CCR4^{-/-}$ mice were used to examine whether CCR4 expression is required for migration to inflamed skin.

CCR4 is expressed on ~60% of the Th1 cells and ~20% of Th2 cells (Figure 18), but whether CCR4 mediates the migration of Th1 and Th2 cells to dermal inflammation induced by different stimuli has not been determined. For this purpose, CD4 cells were isolated from $CCR4^{-/-}$ and WT mice, activated in type 1 or type 2 polarizing conditions, radiolabeled and injected i.v. into mice that received inflammatory stimuli. The extent of recruitment of activated Th1 or Th2 cells varied to the different stimuli (Figure 20). As shown in Figure 20, the recruitment of $CCR4^{-/-}$ Th1 cells to inflamed skin sites, spleen and LNs was similar to that of WT cells (Figure 20A, B). In contrast, there were ~30% fewer $CCR4^{-/-}$ Th2 cells in ConA sites than the WT Th2 cells ($P < 0.05$), but they accumulated similarly in poly-I:C and DTH sites ($P > 0.05$). The recruitment of $CCR4^{-/-}$ Th2 cells was reduced by ~20% in the spleen, but was normal in the LNs of recipient mice (Figure 20C, D). Therefore, the migration of Th1 cells to dermal inflammation does not appear to be mediated by CCR4, even though it is expressed on ~60% of the cells. While CCR4 is not required for the recruitment of Th2 cells to poly-I:C or to DTH sites in the skin, it mediates part of the migration of Th2 cells to ConA-induced dermal inflammation. This is different from Th1 cells which had normal recruitment to inflamed skin, including ConA, in the absence of CCR4.

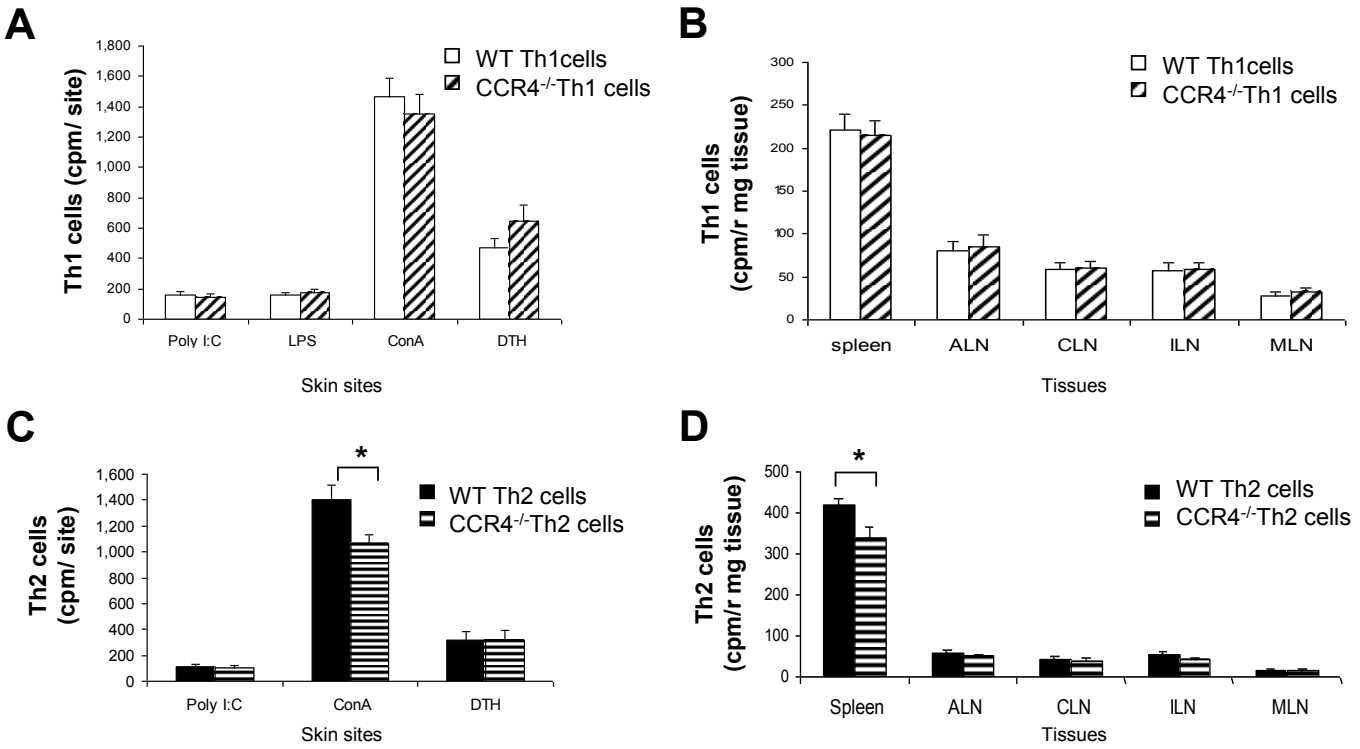

Figure 20. Migration of Th1 and Th2 cells from CCR4$^{-/-}$ and WT mice into sites of dermal inflammation and lymphoid tissues. CD4 cells of CCR4$^{-/-}$ and WT mice were activated in type 1 or type 2 conditions, radiolabeled and injected i.v. to mice that had received i.d. injections for inflammatory stimuli. Migration of CCR4$^{-/-}$ and WT Th1 cells to inflamed skin sites above that of control sites (33±6 for WT or 42±6 for CCR4$^{-/-}$) (A), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), and mesenteric LN (MLN) (B). n = 10-16 mice in 5-7 exps (for A and B). Migration of CCR4$^{-/-}$ and WT Th2 cells to inflamed skin sites above that of control sites (34±13 for WT or 42±5 for CCR4$^{-/-}$) (C), spleen, ALN, CLN, ILN, and MLN (D). n = 4-15 mice in 2-6 exps (for C and D). Bars represent mean cpm ± SEM above control sites for skin homing or mean cpm per mg tissue ± SEM for lymphoid homing. * $P < 0.05$ by Student's t-test compared to WT cells.

The relationship between CCR4 expression on activated CD8 T cells and their migration to inflamed skin has not been examined *in vivo*. For this purpose, CD8 cells were isolated from $CCR4^{-/-}$ and WT mice and activated in type 1 or type 2 polarizing conditions. Cells were radiolabeled and injected i.v. into mice that received i.d. inflammatory stimuli. Like activated CD4 cells, the extent of recruitment of activated CD8 T cells varied with different stimuli (Figure 21). The recruitment of $CCR4^{-/-}$ Tc1 cells to inflamed skin sites, spleen and LNs was not different from WT cells (Figure 21A, B). In contrast, the recruitment of $CCR4^{-/-}$ Tc2 cells was reduced to ConA by ~35% ($P < 0.05$), but not to poly I:C site ($P > 0.05$). The accumulation of $CCR4^{-/-}$ Tc2 cells to spleen was also normal, but there were ~30-40% fewer $CCR4^{-/-}$ Tc2 cells in some of the LNs of these mice compared to WT cells (Figure 21C, D). Thus, CCR4 does not mediate the migration of Tc1 cells to inflamed skin, however the recruitment of Tc2 cells to ConA sites, but not to poly-I:C, was partially mediated by CCR4.

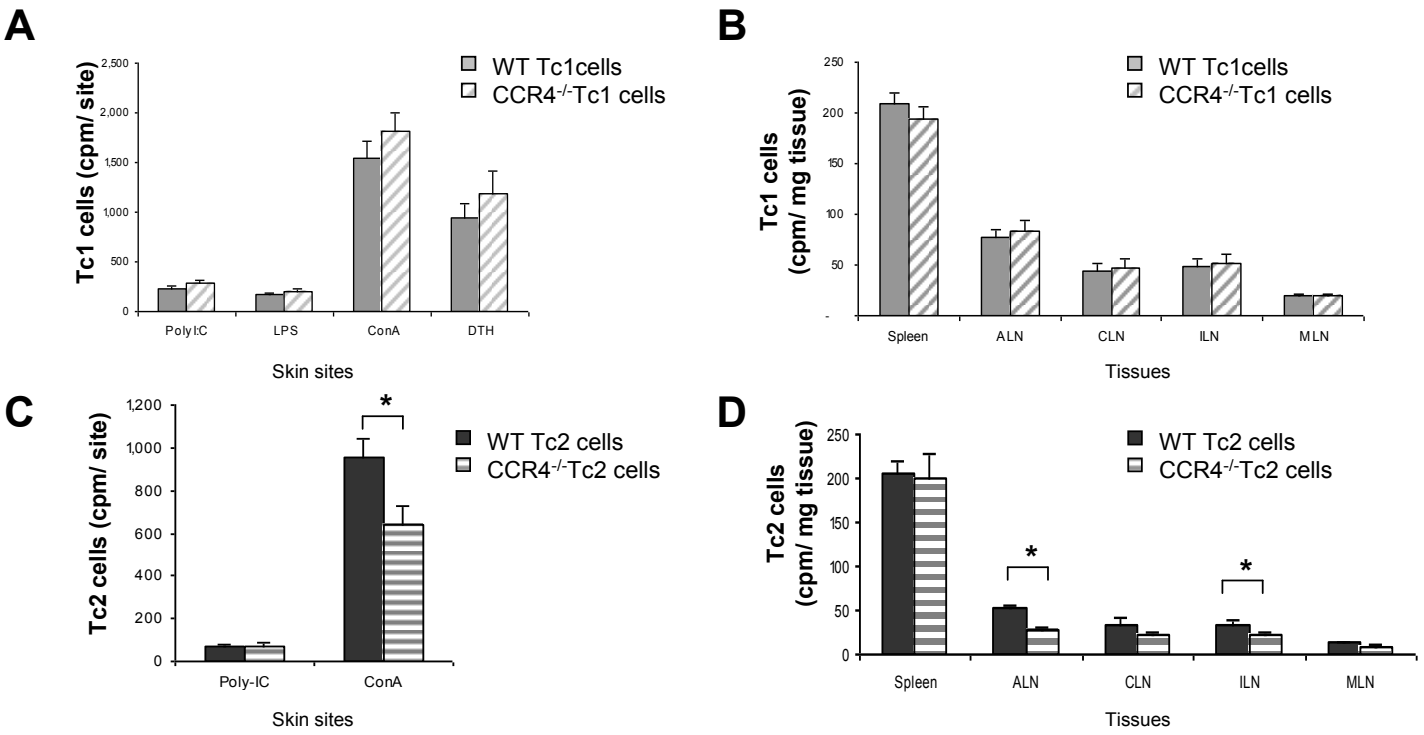

Figure 21. Migration of Tc1 and Tc2 cells from CCR4$^{-/-}$ and WT mice into sites of dermal inflammation and lymphoid tissues. CD8 cells of CCR4$^{-/-}$ and WT mice were activated in type 1 or type 2 conditions, radiolabeled and injected i.v. to mice that had received i.d. injections of inflammatory stimuli. Migration of CCR4$^{-/-}$ and WT Tc1 cells to inflamed skin sites above that of control sites (27±3 for WT or 44±7 for CCR4$^{-/-}$) (A), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), and mesenteric LN (MLN) (B). n = 7-13 mice in 5 exps (for A and B). Migration of CCR4$^{-/-}$ and WT Tc2 cells to inflamed skin sites above that of control sites (45±10 for WT or 48±10 for CCR4$^{-/-}$) (C), spleen, ALN, CLN, ILN, and MLN (D). n = 5-9 mice in 3-4 exps (for C and D). Bars represent mean cpm ± SEM above control sites for skin homing or mean cpm per mg tissue ± SEM in lymphoid homing.
* $P < 0.05$ by Student's t-test compared to WT cells.

5.6 Summary

The use of CCR4 deficient T cells is a valuable tool to determine whether CCR4 is required for the migration of T cells to dermal inflammation. CCR4 expression is associated with the migration of memory CD4 cells in rats (Chapter 3) and of Th1 cells in mice (Chapter 4), but this does not definitely prove that CCR4 is required for skin homing. Both memory CD4 cells and Th1 polarized cells express CXCR3, which mediates part of the migration of CD4 cells, Th1 and Tc1 cells to inflamed skin (Chapter 4). CCR4 is known to be expressed on memory CD4, $Foxp3^+$ CD4 cells (154), activated CD4 and CD8 cells, but whether CCR4 is required for the migration of those T cells to inflammatory sites in the skin is not known. T cells from $CCR4^{-/-}$ mice were used in determining whether the increased expression of CCR4 on activated T cells mediates T cell recruitment into dermal inflammation.

First, when the recruitment of CD4 cells to ConA was examined, the migration of unstimulated CD4 cells was found to be unhindered when they were deficient in CCR4. In fact, more $CCR4^{-/-}$ CD4 cells were found in the ConA induced inflammation than WT cells (Figure 16A). Since there was an increased proportion of memory cells in $CCR4^{-/-}$ splenocytes than in WT splenocytes (Figure 16A), it was necessary to examine the recruitment of equivalent numbers of memory $CCR4^{-/-}$ and WT CD4 cells. When memory CD4 cells were injected, the recruitment of CD4 cells to ConA was still increased in the absence of CCR4. Therefore, memory CD4 cells do not require CCR4 for their recruitment to dermal inflammation (Figure 16B), even though most of the memory CD4 cells present in dermal inflammation in rats were $CCR4^+$ (Figure 4).

Whether the expression of CCR4 by regulatory $Foxp3^+$ CD4 (Treg) cells is necessary for their infiltration into dermal inflammation is not clear. The deficiency of CCR4 on Treg cells may reduce their accumulation to skin during homeostasis (156) or may increase their accumulation in the CHS skin in $CCR4^{-/-}$ mice (195). In order to examine the requirement of CCR4 for the migration of Treg cells to inflamed skin induced by TLR agonists and ConA, CD4 cells were cultured *in vitro* to allow for the induction of Foxp3 expression, the activation and expansion of Treg cells (48-50). Dermal inflammation induced by poly-I:C and LPS recruited 2-3 fold more WT Treg

cells than control sites, while ConA recruited 27 fold more WT Treg cells than control sites. When the effect of CCR4 deficiency on the recruitment of Treg cells was determined, I found an increased accumulation of *in vitro* expanded $CCR4^{-/-}$ $Foxp3^{+}$ CD4 cells in ConA and poly I:C sites compared to WT Treg cells, but no difference was observed in LPS site (Figure 17A). This suggests that CCR4 does not mediate the migration of Treg cells to inflamed skin, which may be mediated by other CKRs that are known to be expressed by Treg CD4 cells (149). In fact, CCR4 deficiency lead to an increase in the numbers of Treg cells found in the inflamed skin sites. I found this with dermal inflammation induced not only in poly I:C and ConA (Figure 17A), and others observed this in OXA-induced CHS as well (195).

The expression of CCR4 on different T cell subsets was found to differ with the activation status and the type 1 / type 2 polarization of the T cells. The expression of CCR4 has been shown to increase during *in vitro* activation of CD4 cells in rats (Figure 5A), and in mice; where CCR4 is present on ~ 60% of the Th1 cells, but on less than 5% of the unstimulated CD4 cells. In addition, more Th1 cells expressed CCR4 than did Th2 cells; ~60% of the Th1 cells were $CCR4^{+}$, while only ~20% of Th2 cells were $CCR4^{+}$ (Figure 18). This was also observed for CD8 cells; ~20% of the Tc1 cells were $CCR4^{+}$, while only ~10% of the Tc2 cells were $CCR4^{+}$ (Figure 19). This is in contrast with other reports, where CCR4 was found to be expressed on human Th2 and Tc2 cell lines rather than on Th1 and Tc1 cell lines (137, 142, 144) or mouse BALB/c Th2 cells, but not on Th1 cells (141). It is possible that strain differences would account for the biased expression of CCR4 on Th1 cells of C57Bl/6 mice versus Th2 cells of BALB/c mice.

The relationship between the expression of CCR4 on Th1 and Th2 cells and a requirement of CCR4 for skin homing is unclear. My studies demonstrated the increased infiltration of $CCR4^{+}$ Th1 cells compared to $CCR4^{-}$ Th1 cells in the inflamed skin (Chapter 4), but this does not establish a requirement for CCR4. The ability of $CCR4^{+}$ human Th2 cells to infiltrate skin in SCID mice treated with CCL22 was demonstrated (245), but whether CCR4 mediates the infiltration in response to inflammatory stimuli is not clear. Interestingly, I found that recruitment of $CCR4^{-/-}$ Th2 cells was reduced by ~30% in ConA, but was unaffected in responses to TLR agonists and DTH. In contrast, the migration of $CCR4^{-/-}$ Th1 cells to inflamed skin was normal (Figure 20). Though

$CCR4^+$ Th1 cells are present in the inflamed skin, they may possibly utilize other CKRs that are co-expressed with CCR4, such as CXCR3 which mediates ~60-70% of the recruitment to inflamed skin (Chapter 4), or CCR10; the inhibition of which reduces the accumulation of *in vivo* activated $CCR4^{-/-}$ lymph node cells to CHS (237).

Like activated CD4 cells, the migration of Tc1 cells to the inflamed skin was found to be CCR4-independent. The migration of Tc2 cells to ConA was partially mediated by CCR4 while recruitment to other sites did not need CCR4 (Figure 21). It should be noted that the recruitment of Tc1 cells to ConA and DTH sites in the skin was reduced only by ~30% in the absence of CXCR3 (Chapter 4). Hence, ~70% of the recruitment of Tc1 cells to dermal inflammation is independent of CXCR3, and their migration was CCR4-independent. Interestingly, the deficiency of CCR4 did not affect the migration of Tc2 cells to TLR agonists, but reduced the migration in ConA by ~35%; similar to the effect of CCR4 deficiency on Th2 cells. This is unexpected, as CCR4 is present only on ~20% of Th2 cells and ~10% of Tc2 cells. Thus, like Th2 cells, CCR4 mediates part of the recruitment of Tc2 cells to ConA, but it is not required for their migration to other inflamed skin sites.

In summary, studies presented in this chapter demonstrate that 1) CCR4 is not required for the skin homing of unstimulated CD4 cells or total memory CD4 cells, but that increased accumulation of $CCR4^{-/-}$ memory CD4 cells are found in ConA. 2) CCR4 is not required for the migration of *in vitro* cultured $Foxp3^+$ CD4 cells to inflamed skin; but more $CCR4^{-/-}$ $Foxp3^+$ CD4 cells were found in ConA and poly I:C sites than WT cells. 3) Th1 cells, which are ~60% $CCR4^+$, do not require CCR4 for their migration to inflammatory sites in skin. In contrast, a deficiency in CCR4 reduced the migration of Th2 cells to ConA, while recruitment to other sites was unaffected. 4) Similarly, CCR4 does not mediate the migration of Tc1 cells to inflamed skin, but it mediates part of the migration of Tc2 cells to ConA.

Chapter 6. Effect of CXCR3 and CCR4 Deficiency on the *In Vivo* Migration of Activated T Cells to Joint Inflammation, and the Effect of CCR4 Deficiency on the Development of Collagen-Induced Arthritis

Both CCR4 and CXCR3 are expressed on T cells in synovial fluid (SF) of patients with arthritis (89, 135, 305-307, 311), but their requirement for the migration of activated T cells to joint inflammation is unclear. The contribution of CXCR3 is suggested by the inhibitory effect of CXCR3 blockade on ~50% of the recruitment of T cell lymphoblasts to inflamed joints in a rat model of adjuvant arthritis (310). Still, CXCR3 blockade may have indirectly affected the recruitment of T cells, since it reduced the severity of the arthritis and reduced the recruitment of PMNs to inflamed joints (310). The presence of CCR4 on CD4 cells in the SF or PB of patients with joint inflammation (305, 309, 312, 313) does not necessarily relate to a requirement for their *in vivo* migration. It is therefore necessary to develop an assay to measure the migration of activated T cells to inflamed paws of mice with collagen-induced arthritis (CIA), and to use $CXCR3^{-/-}$ or $CCR4^{-/-}$ T cells to determine whether these CKRs mediate the migration of activated T cells to inflamed paws. In addition, I examined the development of CIA in $CCR4^{-/-}$ mice and the ability of T cells to migrate to inflamed paws of $CCR4^{-/-}$ mice, since CCR4 is present on different immune cells in the synovium.

6.1 Development of an Assay to Measure *In Vivo* Migration of Th1 Cells to Paws of Mice With Collagen-Induced Arthritis

In order to measure the migration of Th1 cells to joint inflammation, arthritis was induced in mice using CIA, in which mice were s.c. immunized with collagen II and *M. tuberculosis* in CFA and boosted on day 21. This resulted in the development of paw inflammation in ~50% of the mice, with ~50% of the maximum possible severity score on day 30 (Figure 26 A, B). WT Th1 cells were radiolabeled and injected i.v. to immunized mice, and their accumulation into the paws was assessed after 5h. Inflamed paws of mice with CIA were found to recruit ~4 times more Th1 cells than paws of normal mice or uninflamed paws of immunized mice (Figure 22A).

The *in vivo* migration of WT Th1 cells into inflamed paws of immunized mice for different durations was compared. As shown in Figure 22B, more WT Th1 cells were recruited to inflamed paws than to uninflamed paws at 5 h or 18 h post cell injection. There was no significant difference between the recruitment of Th1 cells to inflamed paws at 5 h and 18h ($P > 0.05$). Therefore, the assay was established so that *in vivo* migration of activated T cells was measured after 5 h of cell injection into immunized mice. Recruitment of T cells to normal paws of immunized mice was comparable to that of normal mice, and can therefore serve as a control for the recruitment of T cells to inflamed paws.

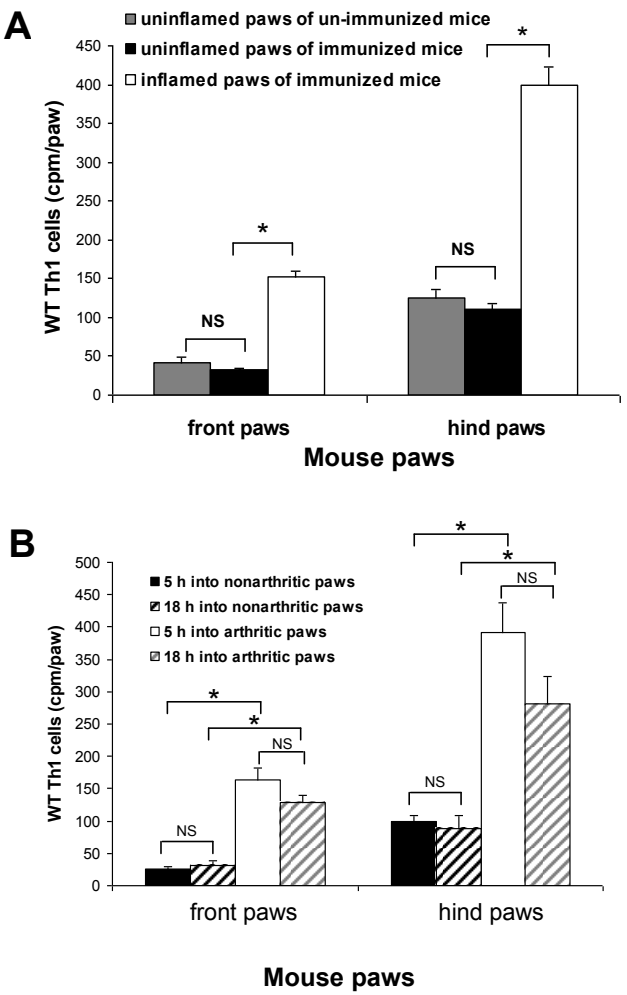

Figure 22. Migration of WT Th1 cells to normal paws and paws of mice with collagen-induced arthritis. Mice were immunized with collagen II / CFA on days 0 and 21. WT Th1 cells were radiolabeled and injected i.v. to immunized mice with inflamed or uninflamed paws. The radioactivity content of the paws was determined as a measure of labeled cell migration. (A) Accumulation of WT Th1 cells in paws of un-immunized or immunized mice after 5 h. n=16-54 paws in 8-32 mice. (B) Accumulation of WT Th1 cells in normal or inflamed paws of immunized mice 5 h or 18 h after injection. n=6-17 paws in 6-11 mice. Bars represent mean ± SEM.
* $P < 0.05$ by ANOVA.

6.2 Effect of CXCR3 Deficiency on the *In Vivo* Migration of Th1 Cells to Joint Inflammation

The expression of CXCR3 on CD4 T cells in synovium of RA patients has been reported (135, 306), and the blockade of CXCR3 has been shown to reduce recruitment of *in vivo* activated LN T cells and PMNs to joint inflammation in rats (310). However, this does not definitely prove a requirement for CXCR3 in the recruitment of Th1 cells to joint inflammation. For this purpose, $CXCR3^{-/-}$ and WT CD4 cells were activated *in vitro* in type 1 polarizing conditions, and the Th1 cells were radiolabeled and injected i.v. into immunized mice that had inflamed paws, After 5 hours, the radioactivity content of the paws and tissues was measured to assess T cell recruitment.

Our results demonstrate that CXCR3 deficiency is not required for recruitment of Th1 cells into arthritic paws. Inflamed paws recruited more Th1 cells than did uninflamed paws, but the accumulation of $CXCR3^{-/-}$ Th1 cells into inflamed paws was equivalent to WT Th1 cells (Figure 23A). $CXCR3^{-/-}$ and WT Th1 cells were found comparably in the spleen and in the LNs draining the inflamed paw (i.e. popliteal LN). However, there was reduced accumulation of $CXCR3^{-/-}$ Th1 cells in the LNs draining the site of immunization (i.e. Inguinal LN), but not in other LNs (e.g. PLN, CLN) (Figure 23B). Therefore, CXCR3 is not required for the recruitment of Th1 cells to inflamed paws, even though it affected their accumulation in the LN draining the site of immunization.

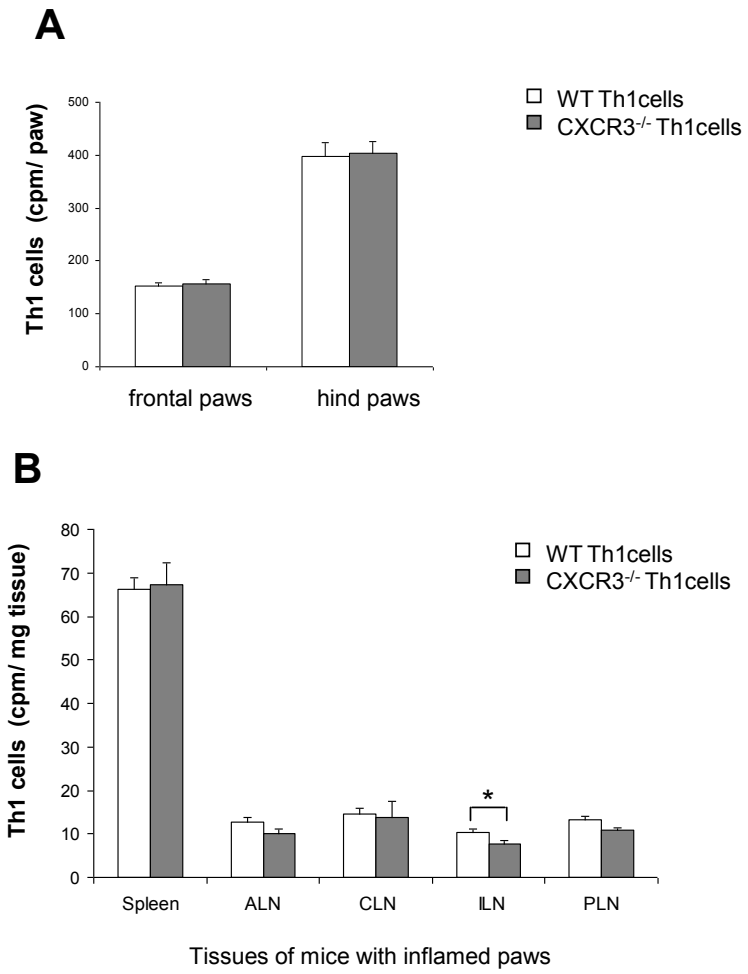

Figure 23. Migration of CXCR3$^{-/-}$ and WT Th1 cells to inflamed paws and lymphoid tissues of mice with collagen-induced arthritis. Mice were immunized with collagen II / CFA on days 0 and 21. CXCR3$^{-/-}$ and WT Th1 cells were radiolabeled and injected i.v. to immunized mice that had inflamed paws. The radioactivity content of the paws (A) and), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), and popliteal LN (PLN) (B) were determined as a measure of labeled cell migration after 5 h. n=30-54 paws of 22-32 mice in 3-7 exps. Bars represent mean cpm ± SEM per paw for homing to joint inflammation, or per mg tissue or lymphoid homing. * $P < 0.05$ by Student's t-test compared to WT Th1 cells.

6.3 Effect of CCR4 Deficiency on the Migration of T Cells to Joint Inflammation

CCR4 is expressed on CD4 cells in the synovial fluid (SF) (89, 305, 311) and circulation of patients with joint inflammation (309, 312, 313), but the relationship between the expression of CCR4 and the recruitment of activated T cells to joint inflammation is not known. For this purpose, the *in vivo* migration of activated T cells from $CCR4^{-/-}$ mice was compared to cells from WT mice. CD4 and CD8 T cells from $CCR4^{-/-}$ and WT mice were activated in type 1 polarizing conditions, radiolabeled and injected i.v. into collagen II and CFA- immunized mice which had inflamed paws. As shown in Figure 24, inflamed paws recruited more Th1 cells than uninflamed paws of immunized mice. This was also true for Tc1 cells (Table 13 and Figure 25).

When the migration of $CCR4^{-/-}$ and WT Th1 cells into inflamed paws was compared, the recruitment of $CCR4^{-/-}$ type 1 cells and WT cells was equivalent (Figure 24A). The recruitment of $CCR4^{-/-}$ and WT Th1 cells has also comparable in the spleen, the LNs draining the inflamed paws (i.e. ALN and PLN), and LNs draining the site of immunization (ILN), and in nondraining LNs (CLN and MLN) (Figure 24B). Thus, the recruitment of Th1 cells to inflamed paws did not depend on CCR4.

Moreover, the *in vivo* migration of $CCR4^{-/-}$ and WT Tc1 cells into inflamed paws was similar (Figure 25A). The number of $CCR4^{-/-}$ and WT Tc1 cells were also comparable in the spleen, the LNs draining the inflamed paws (i.e. ALN and PLN), and LNs draining the site of immunization (ILN), and in nondraining LNs (CLN and MLN) (Figure 25B). My results therefore demonstrate that CCR4 is not required for the recruitment of type 1 cells to inflamed paws.

Table 13. Accumulation of radiolabeled WT or $CCR4^{-/-}$ T cells to normal paws of immunized mice with collagen induced arthritis.

T cell type	WT or $CCR4^{-/-}$ T cell	No. of paws	No. of mice	No. of exps	Front paws (mean ± SEM cpm/paw)	Hind paws (mean ± SEM cpm/paw)
Th1	**WT**	38	19	9	37 ± 3	117 ± 6
	$CCR4^{-/-}$	30	15	8	43 ± 2	125 ± 7
Tc1	**WT**	18	9	6	43 ± 4	148 ± 8
	$CCR4^{-/-}$	18	9	6	50 ± 5	141 ± 8

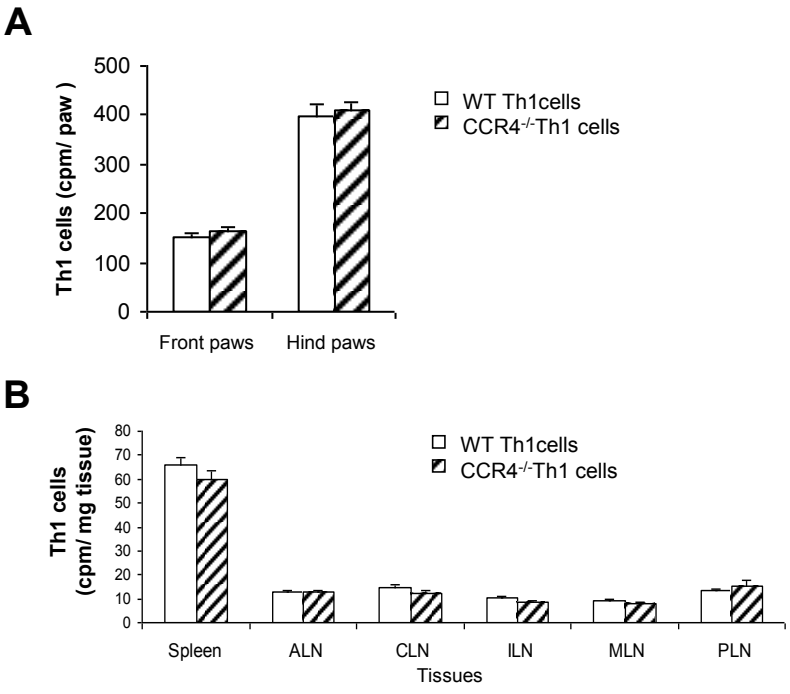

Figure 24. Migration of CCR4$^{-/-}$ and WT Th1 cells to inflamed paws and lymphoid tissues of mice with collagen-induced arthritis. Mice were immunized with collagen II / CFA on days 0 and 21. CCR4$^{-/-}$ and WT Th1 cells were radiolabeled and injected i.v. into immunized mice that had inflamed paws. After 5h, the radioactivity content of the paws (A), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), mesenteric LN (MLN), and popliteal LN (PLN) (B) was determined as a measure of labeled cell migration. n=31-54 paws of 20-31 mice in 6-7 exps. Bars represent mean cpm ± SEM.

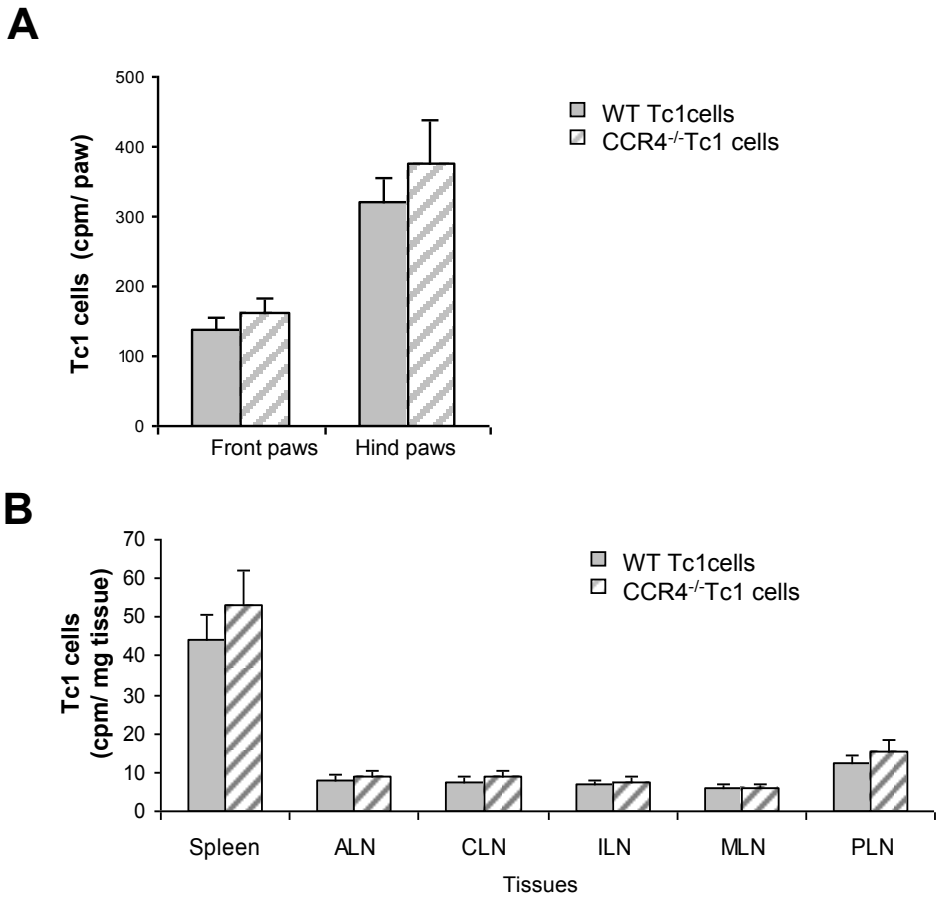

Figure 25. Migration of CCR4$^{-/-}$ and WT Tc1 cells to inflamed paws and lymphoid tissues of mice with collagen-induced arthritis. Mice were immunized with collagen II / CFA on days 0 and 21. CCR4$^{-/-}$ and WT Tc1 cells were radiolabeled and injected i.v. into immunized mice that had inflamed paws. After 5h, the radioactivity content of the paws (A), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), mesenteric LN (MLN), and popliteal LN (PLN) (B) was determined as a measure of labeled cell migration. n =4-21 paws of 8-15 mice in 6-8 exps. Bars represent mean cpm ± SEM.

6.4 Development of Collagen-Induced Arthritis in CCR4$^{-/-}$ Mice

Given that CXCR3 and CCR4 are present on T cells in the SF of RA and JRA patients (89, 135, 305-307, 311), it was unexpected to find that neither CXCR3 nor CCR4 mediated the migration of Th1 cells to joint inflammation induced by CIA in mice (Figures 23 and 24). CXCR3 blockade was previously shown to reduce the severity of arthritis in rats (310), but it is not known whether CCR4 deficiency would affect the development of CIA in mice.

In order to examine this, CIA was induced in both CCR4 deficient and WT mice. CCR4$^{-/-}$ and WT mice were immunized and boosted after 21 days with an emulsion that contained collagen II and CFA adjuvant. Mice were weighed and their paws were scored when redness and/or swelling developed. Symptoms of arthritis started to develop on day 25 and increased gradually till day 29 post-immunization. Both CCR4$^{-/-}$ and WT mice developed arthritis, however the incidence of arthritis was reduced in CCR4$^{-/-}$ mice; in that CCR4$^{-/-}$ mice had delayed onset of CIA (Figure 26A).

The severity of the poly-arthritis was assessed and scored on alternate days. On day 29 post-immunization, 2 or 3 paws affected in each mouse with peak severity scores. The severity scores of the inflamed paws of CCR4$^{-/-}$ and WT mice was found to be comparable (Figure 26B). The severity was also assessed by determining the change in weight and the difference in the weight of inflamed paws over that of normal paws, with no difference between CCR4$^{-/-}$ and WT mice. Immunized mice did not lose any body weight until after day 27; both CCR4$^{-/-}$ and WT arthritic mice lost ~5% of their body weight relative to that on day 21, while nonarthritic mice gained 1-2% of their body weight (Figure 26C). The weight of inflamed paws were found to increase above normal paws by 30-40%; both for CCR4$^{-/-}$ and WT mice (Figure 26D).

In order to further assess the severity of collagen-induced arthritis, the accumulation of intravenously injected ^{125}I-albumin into the inflamed paws was used as a measure of vascular permeability. As shown as Figure 26E, inflamed paws of WT mice accumulated 9-11 μl more labeled albumin than uninflamed paws. The increase of accumulation of labeled albumin in inflamed paws was similar in CCR4$^{-/-}$ and WT mice, suggesting similar vascular permeability in the inflamed paws of CCR4$^{-/-}$ and WT mice.

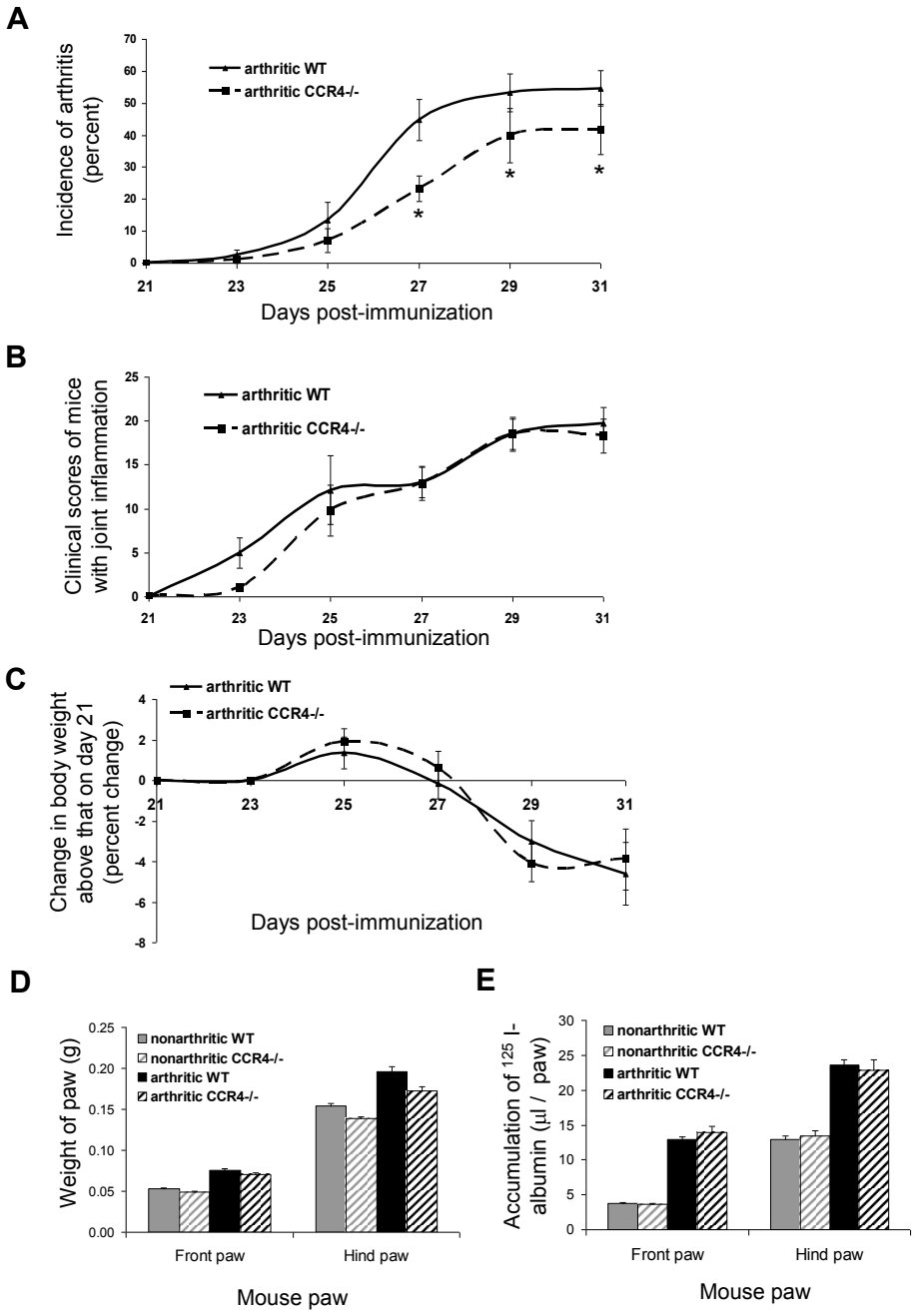

Figure 26. Incidence and severity of collagen-induced arthritis developed in CCR4$^{-/-}$ and WT mice. CCR4$^{-/-}$ and WT mice were immunized with collagen II and adjuvant, and boosted 21 days later. (A) The incidence of development of arthritis as a percentage of mice with joint inflammation (total n= 82 WT and 86 CCR4$^{-/-}$ immunized mice in 11 exps). The clinical score of the mice with joint inflammation out of maximum score of 42 (B), and the change of the body weight of mice with joint inflammation (C) in 42 WT mice and 34 CCR4$^{-/-}$ mice. The weight of paws (D), and the accumulation of ^{125}I- albumin in paws of arthritic and nonarthritic mice (E). n = 31-88 mice. Error bars represent mean ± SEM. * $P < 0.05$ by Mann Whitney test.

6.5 Expression of Adhesion Molecules and CXCR3 in Lymph Nodes of Collagen II - Immunized CCR4$^{-/-}$ and WT Mice

In order to examine the effect of CCR4 deficiency on the number CD4 and CD8 cells present in the LN draining the site of inflammation during the later phase of arthritis, CCR4$^{-/-}$ and WT mice were immunized with collagen II and adjuvant and booster on day 21. Then, on day ~45, the LNs draining the site of immunization (inguinal LN; ILN) and nondraining LNs (cervical LN; CLN) were collected and the number of CD4 and CD8 cells was determined. As shown in Figure 27A, there were more cells isolated from the draining LNs than the nondraining LNs. The increase in the size of draining LN was affected by the deficiency of CCR4, and was reflected by the presence of ~20-30% fewer CD4 and CD8 cells in the draining LNs of CCR4$^{-/-}$ mice compared to WT mice.

The CD4 cells in the LNs were stained with antibodies for the activation marker CD25, adhesion molecules (ESL, PSL), CXCR3 and IFN-γ to determine whether deficiency in CCR4 affected the number of T cell subpopulations in draining LNs. As shown in Figure 27B, there were ~4 times more CD4 cells in the draining LN (ILN) than nondraining LN in WT mice. In contrast, the number of CD4 cells was only increased ~2 fold in draining versus nondraining LNs of CCR4$^{-/-}$ mice. Immunization induced similar the recruitment and/or upregulation of the CD4 cells expressing CXCR3, PSL, ESL, and IFN-γ that were in ILN. The number of CD4 cells expressing CD25 was also affected; with fewer CD25^{+} CD4 cells in CCR4$^{-/-}$ mice (Figure 27B). Therefore, my results indicate a reduced accumulation of CD4 cells and CD25^{+} CD4 cells in the draining LNs in collagen-immunized mice that are deficient in CCR4.

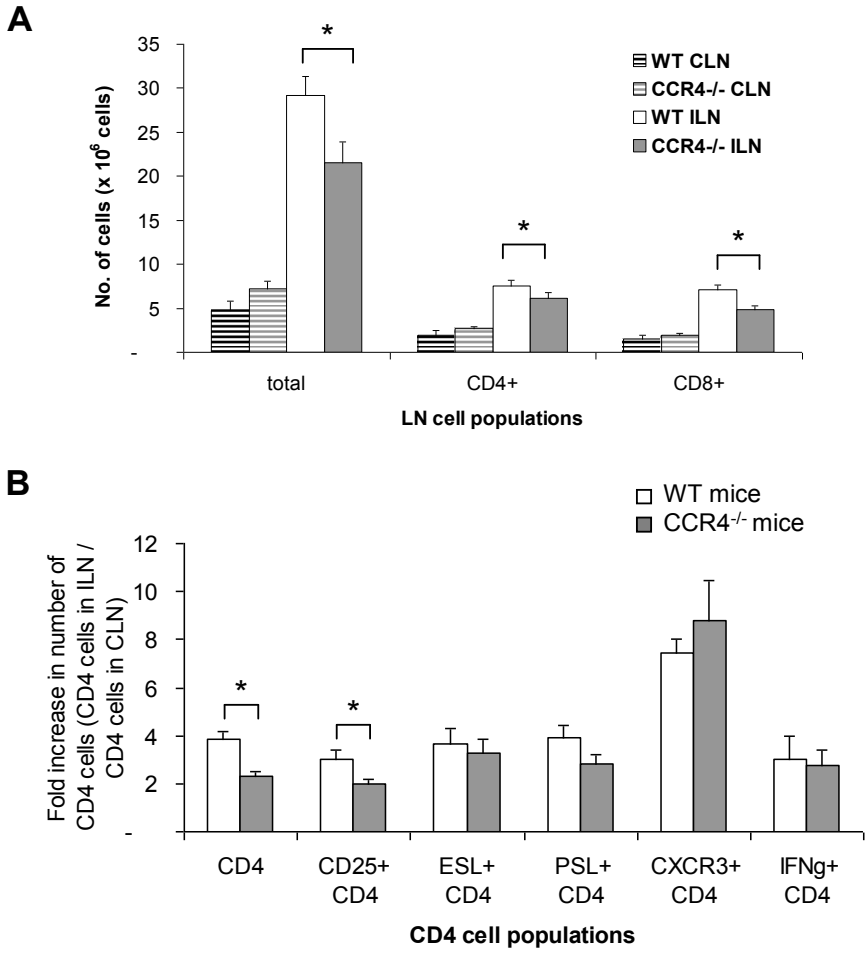

Figure 27. Number of CD4 and CD8 cells, and the increase in the number of CD4 cells expressing adhesion molecules, CXCR3 and CD25 on CD4 cells in LNs of collagen-immunized CCR4$^{-/-}$ and WT mice. CCR4$^{-/-}$ and WT mice were immunized with collagen II and adjuvant. The number of CD4 and CD8 cells in the LN draining the site of immunization (ILN) and nondraining LN (CLN) of immunized mice was assessed after ~45 days (A). LN cells were stained for CD25, ESL, PSL, CXCR3, and intracellular IFN-γ to assess the fold increase in the number of these CD4 cell populations in ILN as a ratio of their number in CLN (B) in WT and CCR4$^{-/-}$ mice. n = 3-12 mice in 1-4 exps. Error bars represent mean ± SEM. * $P < 0.05$ by Student's t-test.

6.6 *In Vivo* Migration of Activated T Cells in Inflamed Paws of CCR4$^{-/-}$ Mice

When the recruitment of WT Th1 cells into paws of CCR4$^{-/-}$ recipients was examined, ~20-30% fewer WT Th1 cells were found in the inflamed paws of CCR4$^{-/-}$ mice compared to WT mice. Accumulation into normal paws of CCR4$^{-/-}$ mice was not affected (Figure 28A). The accumulation of WT Th1 cells in lymphoid tissues of mice with inflamed paws was unaffected by the deficiency of CCR4 (Figure 28B). Thus, the extrinsic deficiency of CCR4 affected the recruitment of Th1 cells into inflamed paws, suggesting a role for CCR4 on non-T cells in determining Th1 cell recruitment, rather than a direct role for CCR4 on Th1 cells in this process.

Migration of CCR4$^{-/-}$ Th1 cells to inflamed paws in CCR4$^{-/-}$ mice was assessed. Similar to what was observed for WT Th1 cells, there was reduced recruitment of CCR4$^{-/-}$ Th1 cells to inflamed paws of CCR4$^{-/-}$ recipients compared to WT recipients (Figure 28C). The accumulation of CCR4$^{-/-}$Th1 cells in lymphoid tissues of mice with inflamed paws was comparable in WT and CCR4$^{-/-}$ recipients (Figure 28D). Therefore, CCR4 deficient mice recruit fewer Th1 cells to inflamed paws, whether the Th1 cells were derived from WT or CCR4$^{-/-}$ mice.

Inflamed paws in CCR4 deficient mice recruited fewer Th1 cells, but whether CCR4 deficiency affected the recruitment of other activated T cells, such as Tc1 cells, to joint inflammation is not known. As shown in Figure 29A, radiolabeled WT Tc1 cells were recruited to inflamed paws of CCR4$^{-/-}$ and WT mice in equivalent numbers. Accumulation in lymphoid tissues was also unaffected by the deficiency of CCR4 (Figure 29). This is in contrast to the reduced recruitment of Th1 cells to the inflamed paws of CCR4$^{-/-}$ mice (Figure 28).

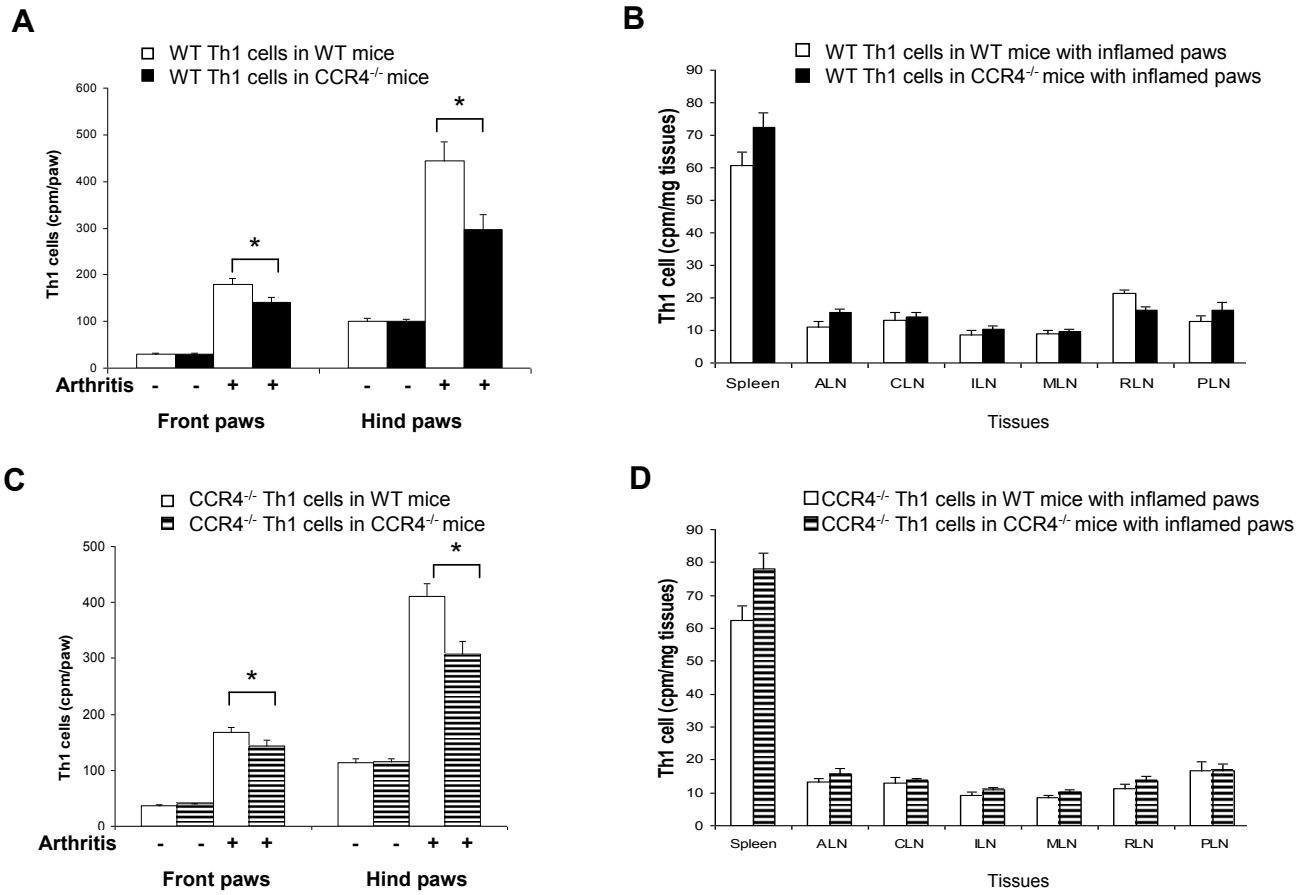

Figure 28. Migration of Th1 cells to joint inflammation and lymphoid tissues of CCR4$^{-/-}$ and WT mice. WT and CCR4$^{-/-}$ CD4 cells were activated in type 1 polarizing conditions, radiolabeled, and injected i.v. into immunized WT and CCR4$^{-/-}$ mice that had normal paws (of nonarthritic mice) or inflamed paws (of arthritic mice). *In vivo* migration of WT Th1 cells to paws of WT and CCR4$^{-/-}$ mice (A), spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), mesenteric LN (MLN), retroperitoneal LN (RLN), and popliteal LN (PLN) of mice with inflamed paws (B). n=9-12 mice in 3-7 exps (for A and B). *In vivo* migration of CCR4$^{-/-}$ Th1 cells to paws of WT and CCR4$^{-/-}$ mice (C), spleen, ALN, CLN, ILN, MLN, RLN, and PLN of mice with inflamed paws (D). n=11-16 mice in 3-7 exps (for C and D). Error bars represent mean ± SEM. * $P < 0.05$ by Student's t-test compared to WT recipient mice.

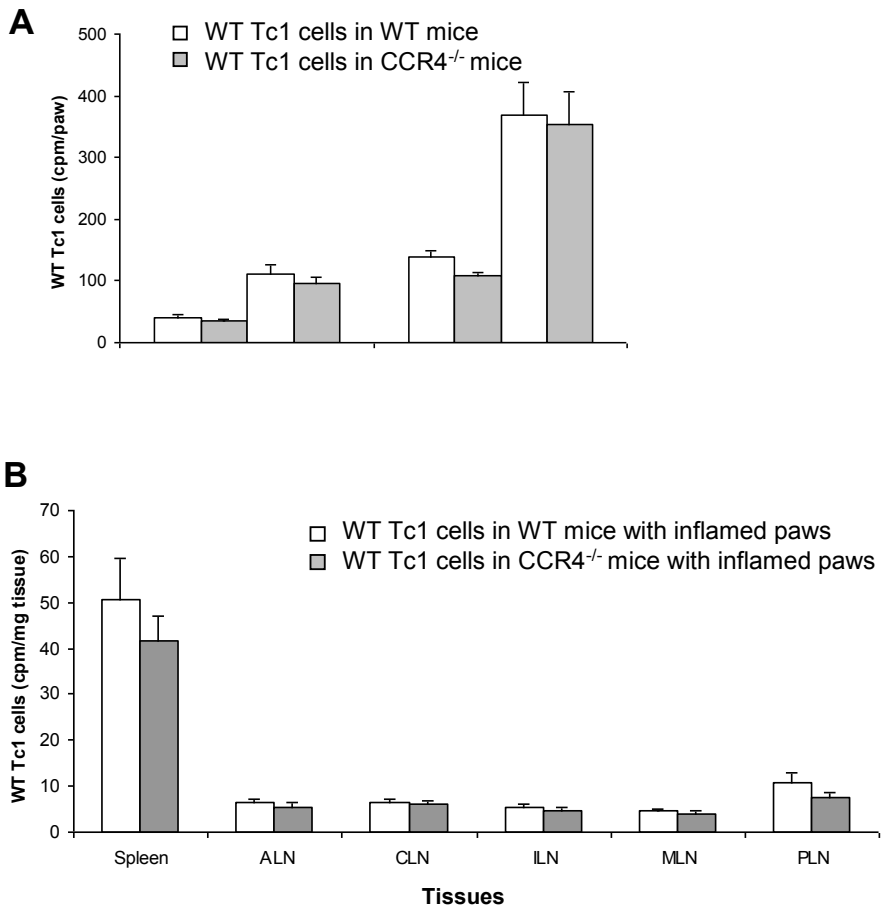

Figure 29. Migration of WT Tc1 cells to joint inflammation and lymphoid tissues of CCR4$^{-/-}$ and WT mice. CD8 cells were activated in type 1 polarizing conditions, radiolabeled, and injected i.v. into immunized CCR4$^{-/-}$ and WT mice that had normal paws (of nonarthritic mice) or inflamed paws (of arthritic mice). After 5 hours, the content of radioactivity in the normal or inflamed paws (A) and the spleen, axillary LN (ALN), cervical LN (CLN), inguinal LN (ILN), mesenteric LN (MLN), and popliteal LN (PLN) of mice with inflamed paws (B) was assessed as a measure of labeled cell migration. n= 5-9 mice in 3-6 exps. Error bars represent mean ± SEM.

6.7 Summary

In this chapter, I have examined the contribution of CXCR3 and CCR4 to the *in vivo* migration of Th1 cells to joint inflammation. Also, the effect of CCR4 on the development of collagen-induced arthritis was assessed by examining the incidence and severity of CIA in $CCR4^{-/-}$ mice, the accumulation of CD4 cells in the draining LNs, and the *in vivo* migration of activated Th1 and Tc1 cells to inflamed paws of $CCR4^{-/-}$ mice.

First, an assay to measure the migration of activated T cells to inflamed paws in mice with collagen induced arthritis was developed. More Th1 cells were recruited to inflamed paws compared to uninflamed paws of arthritic mice or uninflamed paws of un-immunized mice (Figure 22A). The increased recruitment of Th1 cells to inflamed paws was observed when the assay continued for 5 h or 18 h (Figure 22B).

Though CXCR3 are expressed on T cells in the SF of RA patients (135, 306), the normal migration of $CXCR3^{-/-}$ Th1 cells to inflamed paws and their draining LNs (popliteal LN) (Figure 23) suggests that the migration of Th1 cells to joint inflammation does not depend on CXCR3. In earlier studies, CXCR3 blockade reduced the recruitment of *in vivo* activated T cells to inflamed joints in rats (310), which may have been an indirect effect resulting from the reduced development of arthritis and reduced recruitment of PMNs to inflamed joints (310), or an effect on other T cells that can be induced in arthritis, such as IL-17 producing T cells, rather than on IFNγ-producing Th1 cells which migrate to inflamed paws in a CXCR3-independent manner.

Interestingly, my work also shows that the migration of Th1 and Tc1 cells to inflamed paws does not depend on CCR4. The recruitment of $CCR4^{-/-}$ Th1 cells to inflamed paws was normal (Figure 24), and recruitment of $CCR4^{-/-}$ Tc1 cells to inflamed paws was also normal (Figure 25). It is likely that the recruitment of type 1 T cells to inflamed paws depends on other CKRs, like CCR5, which is known to be expressed on CD4 cells in the synovium of RA patients (307).

Furthermore, the development of collagen-induced arthritis was assessed in $CCR4^{-/-}$ mice to determine to determine the effect of CCR4 deficiency. $CCR4^{-/-}$ mice developed CIA at reduced incidence, but with normal severity, as assessed by the severity scores, weight of inflamed paws, and vascular permeability, which was evaluated using the

accumulation of ^{125}I-albumin in the paws. They also have similar changes in their body weight (Figure 26). Yet, the LNs draining the site of immunization during a later phase (~ day 45 post-immunization) had fewer CD4 cells in CCR4$^{-/-}$ mice. The increase in the ratio of CD4 CD25^{+} cells in the ILN *versus* nondraining LN (CLN) was lower in CCR4 deficient mice than in WT mice (Figure 27). Thus, a deficiency of CCR4 did not affect the severity of CIA, but there were fewer CD4 CD25^{+} cells in the ILN.

Moreover, CCR4 expressed on T cells is not required for their migration to joint inflammation (Figure 24), but it is possible that the presence of CCR4 on other cells affects the extent with which Th1 cells are recruited to the joint inflammation. the recruitment of Th1 cells to joint inflammation was found to be reduced in CCR4$^{-/-}$ recipient mice. This was demonstrated with both CCR4$^{-/-}$ Th1 and WT Th1 cells (Figure 28A,C). It is interesting to note that, in contrast to Th1 cells, the inflamed paws of CCR4$^{-/-}$ and WT mice recruited Tc1 cells equivalently (Figure 29).

Chapter 7. Effect of Deficiency of CXCR3 and CCR4 on the Accumulation of Th1 Cells in Lymph Nodes Draining Sites of Immunization

The association between CXCR3 and CCR4 expression on activated T cells and the accumulation in LNs draining a site of immunization is unclear. Reactive LNs are known to have properties of inflammatory sites, such as the upregulation of ligands for E- and P-selectin (361), increased blood flow, and increased expression of CKs related to recruitment of CXCR3 expressing tumor cells or T cells (338, 339), in addition to $CCR7^+$ T cells (344). The migration of activated T cells to draining LNs is also of interest, as it may assist in the understanding of whether CCR4 or CXCR3 are needed for activated T cells to migrate to tertiary lymphoid tissues, such as arthritis (346, 347), which contain CK-producing stromal cells, B-cells, DCs (353), and CKR^+ T cells (354). Also, LNs draining a site of immunization can be used as a inflammatory site from which the migrating T cells can be collected, and the effect of CXCR3 or CCR4 deficiency on their accumulation can be assessed. It was necessary to develop an assay to measure the accumulation of T cells in draining LNs, in order to determine the effect of deficiency of CXCR3 or CCR4 on the accumulation of Th1 cells to the draining LNs. Also, the expression of CKRs and adhesion molecules on these T cells can be characterized, in order to determine whether the deficiency of CCR4 affects the recruitment of T cell populations differently.

7.1 Development of an Assay to Measure Accumulation of Th1 Cells to Lymph Nodes Draining Sites of Immunization

The development of an assay to measure the migration of Th1 cells to draining LNs involved: 1) A comparison between the activation of LNs draining a site of immunization with OVA-CFA and OVA-Alum to determine the optimal immunization protocol. 2) The measurement of the expression and kinetics of CD25, CXCR3 and ESL on CD4 cells in the draining LNs at multiple days post-immunization (p.i.). 3) The accumulation of labeled Th1 cells in the LNs at different time points in immunized mice, to determine the kinetics of accumulation of Th1 cells in the draining LNs.

First, to compare the effect of Alum- or CFA-immunization on the activation of CD4 cells in draining LNs, mice were immunized subcutaneously with an emulsion that contained OVA-CFA or OVA-Alum. Draining LNs (inguinal LNs) were collected and stained to determine the expression of CD25, CXCR3, ESL and IFN-γ on CD4 cells. As shown in Figure 30, LNs draining the site of Alum immunization had fewer CD4 cells compared to CFA immunization. The number of CD4 cells expressing CD25, CXCR3 or ESL in LNs draining sites of Alum immunization was found to be lower than in LNs draining CFA immunization (Figure 30). The number of CD4 cells expressing CD25, CXCR3, or ESL in LNs draining sites of CFA immunization appears to increase at day 3 p.i., and remained elevated on day 6 (Figure 31). On day 6 p.i., CD4 cells in the draining LNs were ~3% $CCR4^+$; with $0.14x10^6$ $CCR4^+$ CD4 cells following OVA/Alum immunization, and $0.37x10^6$ $CCR4^+$ CD4 cells following OVA/CFA immunization (n=3).

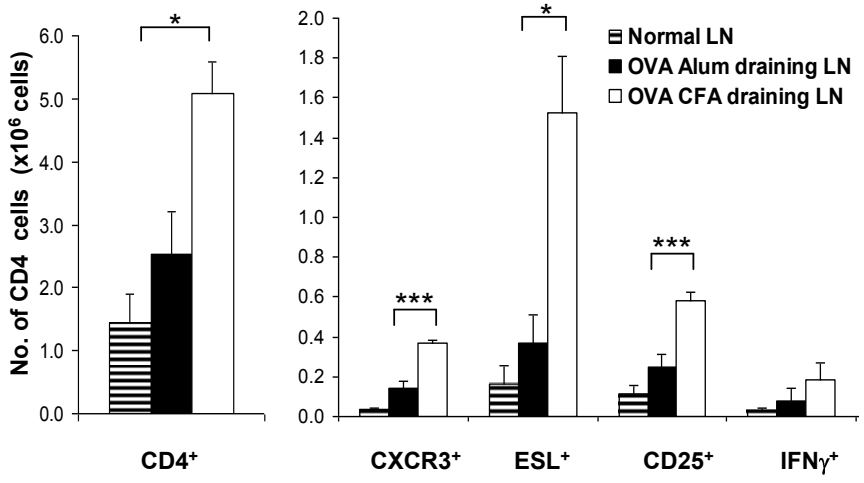

Figure 30. Expression of CXCR3, ESL, CD25 and IFN-γ on CD4 cells from the draining LN after immunization with OVA-Alum or OVA-CFA. Mice were immunized subcutaneously with emulsion that contains ovalbumin in Alum or ovalbumin in CFA, and the draining LN (inguinal LN) was collected after 6 days. The number of CD4 cells expressing CD25, CXCR3, ESL or IFN-γ in the draining LN and normal LN are shown. n=3-4 mice per group. Bars represent mean ± SEM. * $P < 0.05$, ** $P < 0.005$ by Student's t-test.

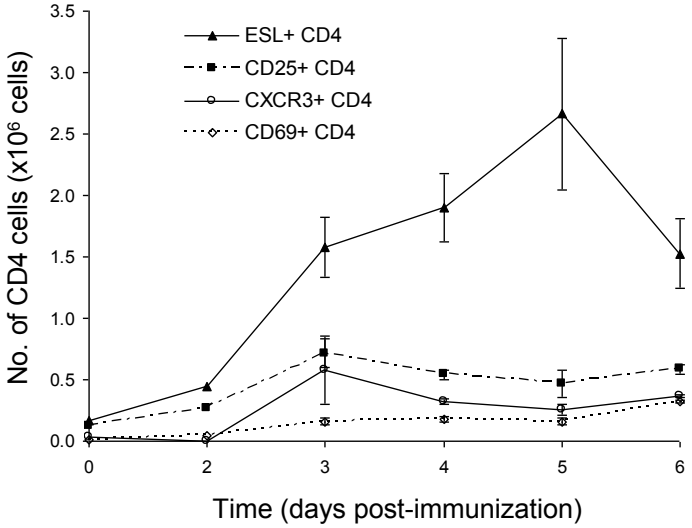

Figure 31. Time course of CXCR3, ESL and CD25 expression on CD4 cells from the draining LNs after immunization with OVA-CFA at different time points. Mice were immunized subcutaneously with emulsion containing ovalbumin in CFA. The number of CD4 cells expressing CD25, CD69, CXCR3 or ESL in the draining LN are shown, n=2-4 mice per time point. Bars represent mean ± SEM.

It was necessary to optimize the assay to measure the accumulation of intravenously injected Th1 cells in LNs draining sites of immunization. WT Th1 cells were fluorochrome-labeled and injected i.v. into mice that were immunized subcutaneously with OVA-CFA at the base of the tail ~ 3 days earlier. At different time points, draining LN (inguinal LN), nondraining LN (axillary and cervical LN), and spleen were collected and stained with anti-CD4 mAb in order to assess the number of labeled Th1 cells in these tissues. So, the number of labeled Th1 cells recovered from the nondraining LNs, draining LNs and spleen after i.v. injection was estimated.

As shown in Figure 32, increasing numbers of Th1 cells were found in the LNs up to 72 h post cell injection. The number of Th1 cells recovered from draining LNs at 72 h was 2-3 fold higher than at 24 h, even after normalizing the number of CD4 cells recovered to 10^6 CD4 cells in the tissue (Figure 32A). In this assay, labeled Th1 cells are injected intravenously, and are present in the circulation. Therefore, Th1 cells were found in the spleen, but their number did not significantly change in different time points (1200-1700 CD4 cells per 10^5 cells injected / 10^6 CD4 cells in the spleen) (Figure 32B).

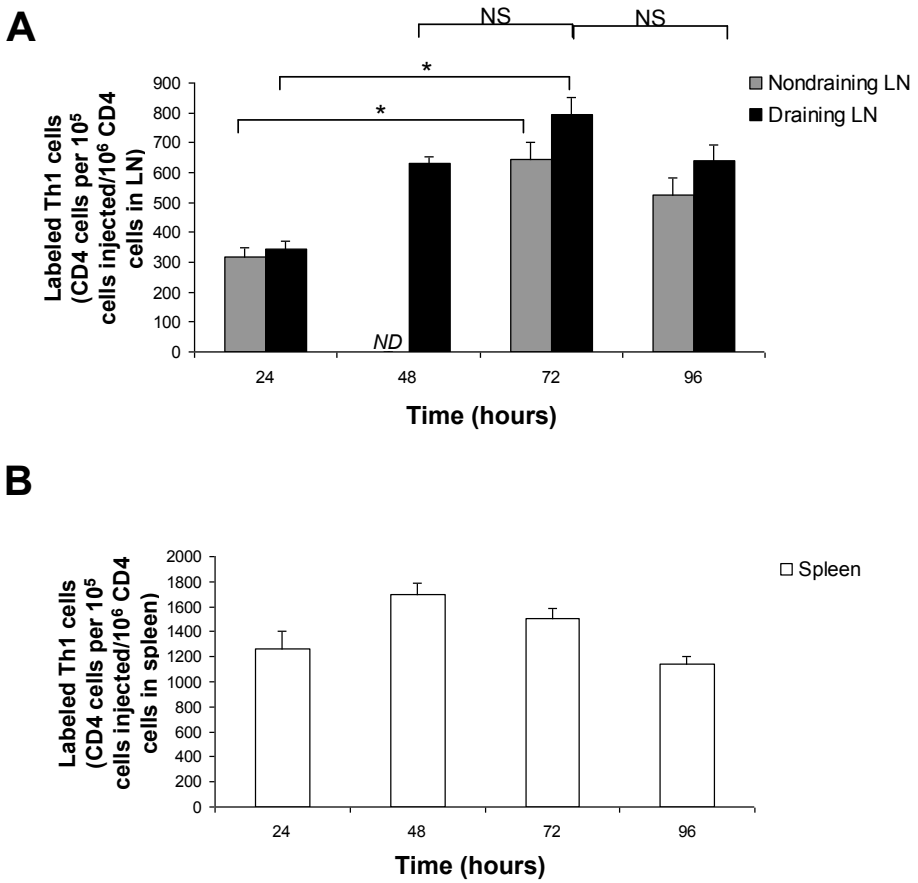

Figure 32. Accumulation of Th1 cells in the draining LN, nondraining LN and spleen of OVA-CFA- immunized mice. Th1 cells were fluorochrome- labeled and injected i.v. into mice that were immunized with CFA 3 days earlier. The number of labeled Th1 cells recovered from nondraining LN (axillary and cervical LNs), and draining LN (inguinal LN) (A) and spleen (B) at 24 – 96 h after i.v. injection. n=4-17 mice in 1-6 exps. Bars represent mean ± SEM of number of labeled Th1 cells per 10^6 cells in the tissue for 10^5 Th1 cells injected. * $P < 0.05$ by ANOVA. *ND*: not determined

7.2 Effect of CXCR3 Deficiency on the Migration of Th1 Cells to Lymph Nodes Draining Sites of Immunization

It is not known whether CXCR3 expression on Th1 cells is required for their accumulation in reactive LNs. In order to study the relationship between the expression of CXCR3 on Th1 cells and their accumulation in LNs draining site of immunization, $CXCR3^{-/-}$ and WT Th1 cells were fluorochrome-labeled and injected i.v. into mice that were immunized with OVA-CFA 3 days earlier. Then, the number of labeled $CXCR3^{-/-}$ and WT Th1 cells present in the spleen and LNs draining site of immunization (inguinal LN), and nondraining LNs (axillary and cervical LN) were found using flow cytometry. The number of labeled CD4 cells recovered from the tissues was estimated, and divided by 10^5 cells injected to normalize for the number of cells injected, then divided by 10^6 CD4 T cells in the tissue to normalize for the size of the tissue. Increasing numbers of WT Th1 cells were found in the draining LNs up to 72 h post cell injection. The increase of number of Th1 cells recovered from draining LNs above that of nondraining LNs was calculated and shown in Figure 33A.

Interestingly, CXCR3 was required for a significant proportion of the accumulation of Th1 cells in the draining LNs. Though the accumulation of $CXCR3^{-/-}$ Th1 cells to draining LN was normal at 24 h post-injection, the requirement for CXCR3 was most apparent at later time points, when the number of $CXCR3^{-/-}$ Th1 cells recovered from the draining LNs was significantly lower than that of WT Th1 cells ($P < 0.05$) (Figure 33A). This suggests that most of the increased accumulation of Th1 cells in draining LNs is related to CXCR3 expression. The number of $CXCR3^{-/-}$ Th1 cells recovered from the spleen of these mice was equivalent at different time points (Figure 33B).

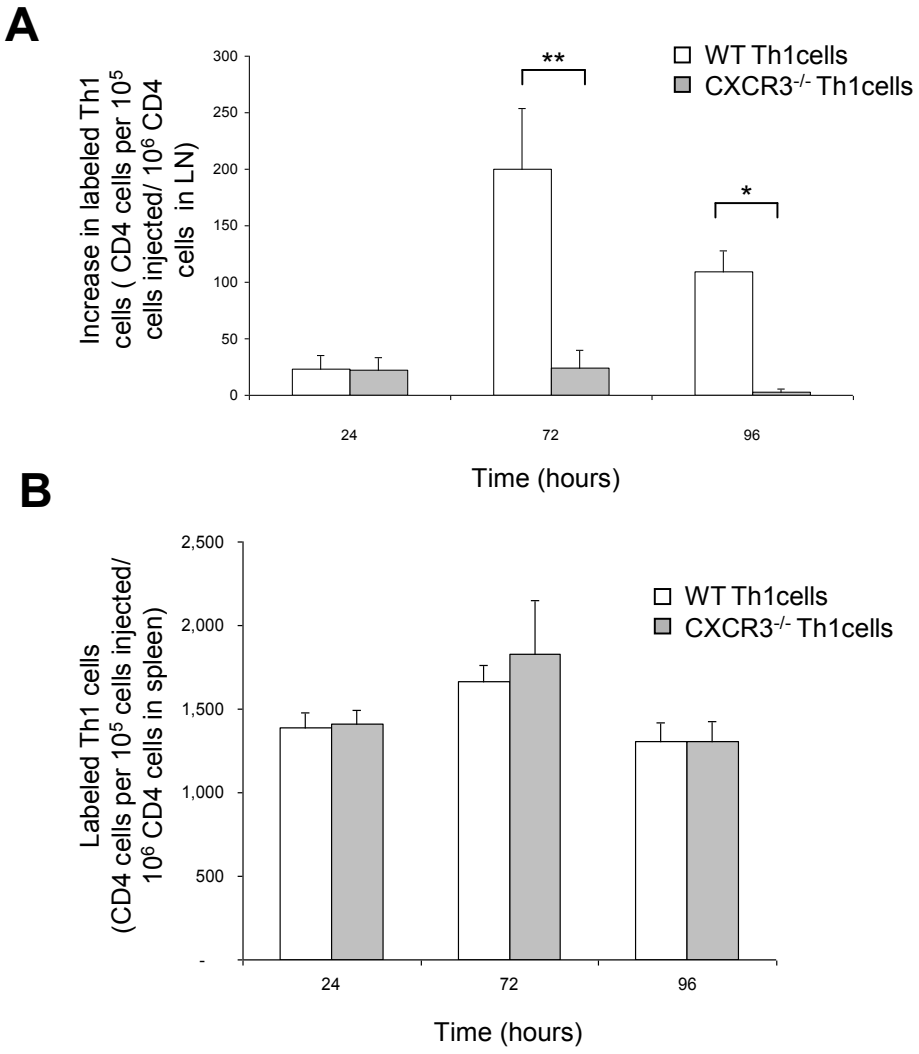

Figure 33. Number of CXCR3$^{-/-}$ and WT Th1 cells recovered from spleen and LNs of immunized mice at 24-96h post-i.v. cell injection. CXCR3$^{-/-}$ and WT Th1 cells were fluorochrome-labeled and injected i.v. into mice that were immunized with CFA 3 days earlier. The increase in the number of labeled Th1 cells recovered from draining LNs (inguinal LN) over nondraining LNs (axillary and cervical LN) (A), and the number of labeled Th1 cells recovered from the spleen (B). n = 3-8 mice in 1-3 exps. Bars represent mean ± SEM of number of labeled Th1 cells / 10^6 CD4 cells in tissue per 10^5 CD4 cells injected * $P < 0.05$, ** $P < 0.01$ by Student's t-test compared to WT cells.

7.3 Effect of CCR4 Deficiency on the Accumulation of Th1 Cells After Intravenous Injection to Lymph Nodes Draining Sites of Immunization

The effect of CCR4 deficiency on the accumulation of Th1 cells in the lymphoid tissues has not been examined. $CCR4^{-/-}$ mice were shown to have increased numbers of CD4 cells in the LNs draining skin CHS sites (195), but the phenotype of these CD4 cells was not examined. The accumulation of $CCR4^{-/-}$ and WT Th1 cells in LNs and spleen was compared to demonstrate whether CCR4 influences the recruitment of Th1 cells to lymphoid tissues. $CCR4^{-/-}$ and WT Th1 cells were fluorochrome-labeled and injected i.v. into CFA-immunized mice. At different time points, the spleen, draining LN (inguinal LN), and nondraining LNs (axillary and cervical LNs) were collected and the number of labeled CD4 cells was examined by flow cytometry. The number of labeled CD4 cells recovered from the tissues was estimated, and divided by 10^5 cells injected to normalize for the number of cells injected, then divided by 10^6 CD4 T cells in the tissue to normalize for the size of the tissue. Then, the increase of number of Th1 cells recovered from draining LNs above that of nondraining LNs was calculated and shown in Figure 34A.

In contrast to what was observed in the absence of CXCR3, the deficiency in CCR4 resulted in an increased accumulation of Th1 cells in the draining LNs. Compared to WT Th1 cells, $CCR4^{-/-}$ Th1 cells were found in increased numbers in the draining LN at 72 h post injection ($P < 0.05$) (Figure 34A). The number of $CCR4^{-/-}$ Th1 and WT Th1 cells in the spleen was similar on day 1, but there were ~20% more $CCR4^{-/-}$ Th1 cells than WT Th1 cells in the spleen on day 3 post-i.v. (Figure 34B). Therefore, more Th1 cells accumulate in the draining LNs and spleen when CCR4 is deficient.

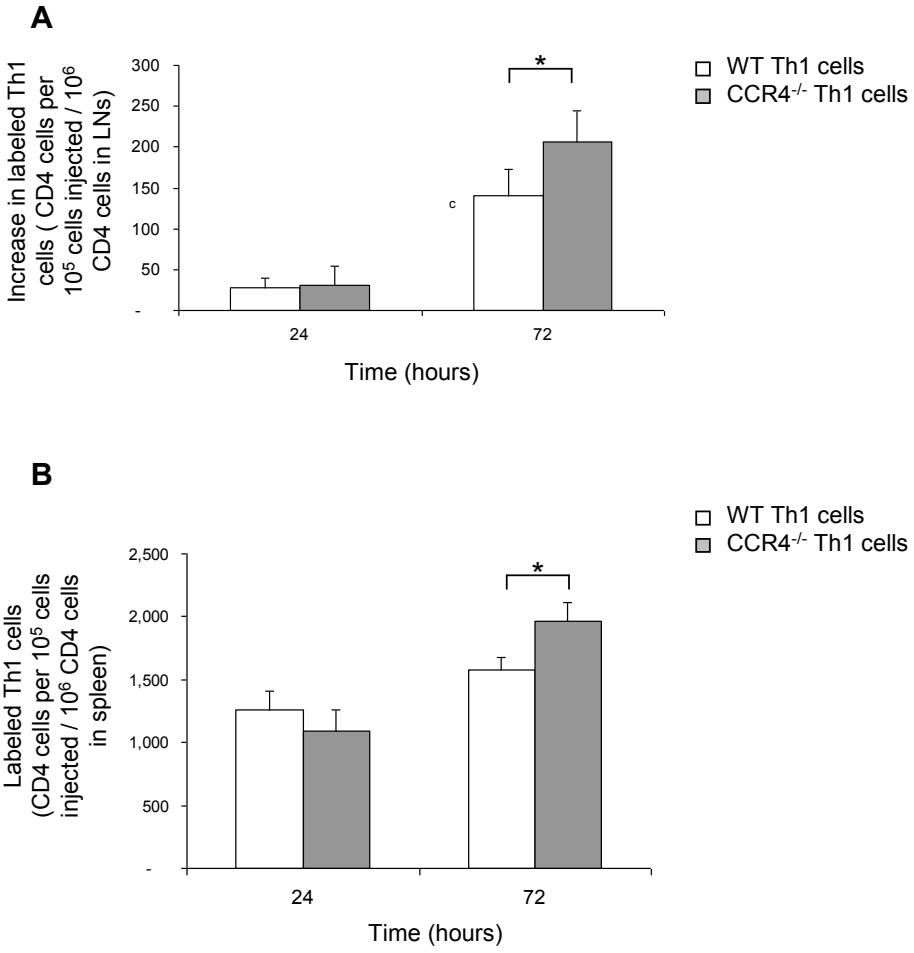

Figure 34. Number of CCR4$^{-/-}$ and WT Th1 cells recovered from spleen and LNs of immunized mice at 24-96h post-i.v. cell injection. CCR4$^{-/-}$ and WT Th1 cells were fluorochrome-labeled and injected i.v. into mice that were immunized with CFA 3 days earlier. The increase in the number of labeled Th1 cells recovered from draining LNs (inguinal LN) over nondraining LNs (axillary and cervical LN) (A), and the number of labeled Th1 cells recovered from the spleen (B). Bars represent mean ± SEM of number of labeled Th1 cells / 10^6 CD4 cells in tissue per 10^5 CD4 cells injected, n (24 h): 2 exps with 4 mice, n (72 h): 6 exps with 20 mice. * $P < 0.05$ by Paired t-test of 6 exps at the 72 h time point.

7.4 Effect of CCR4 Deficiency on the Phenotype of Th1 Cells That Accumulate in Lymph Nodes Draining Sites of Immunization

The deficiency in CCR4 increased the accumulation of Th1 cells in draining LN at 72 h after intravenous injection (Figure 34), but whether this differentially affects the accumulation of ESL^+, PSL^+ $CXCR3^+$, L-selectin$^+$ or α_4-integrin$^+$ Th1 cells has not been examined. For this purpose, labeled Th1 cells were injected i.v. into immunized mice. The spleen, draining and nondraining LNs were collected and stained to determine the number of WT or $CCR4^{-/-}$ CD4 cells that express CD25, CXCR3, PSL, ESL, L-selectin and α_4-integrin that accumulated. As shown in Figure 35, there was a significant increase in the number of $CXCR3^+$ $CCR4^{-/-}$ Th1 cells in the draining LNs above that of nondraining LNs ($P < 0.05$) (Figure 35A). The accumulation of $CXCR3^+$ cells in the spleen was unaffected by the deficiency of CCR4 (Figure 35B). The number of ESL^+ $CCR4^{-/-}$ CD4 cells in draining LN over nondraining LN exceeded that of WT cells by ~2 fold ($P < 0.05$), while their accumulation was comparable in the spleen. Also, there were ~50% more PSL^+ $CCR4^{-/-}$ CD4 cells in the draining LN over nondraining LNs than WT cells ($P < 0.05$), but they were found in similar numbers in the spleen. As shown in Table 14, there was no difference in the accumulation of $CCR4^{-/-}$ and WT CD4 cells that express α_4 integrin or L-selectin. Therefore, these results indicate the influence of CCR4 deficiency on $CXCR3^+$, ESL^+ or PSL^+ Th1 cells, but not α_4 integrin$^+$ or L-selectin$^+$ cells.

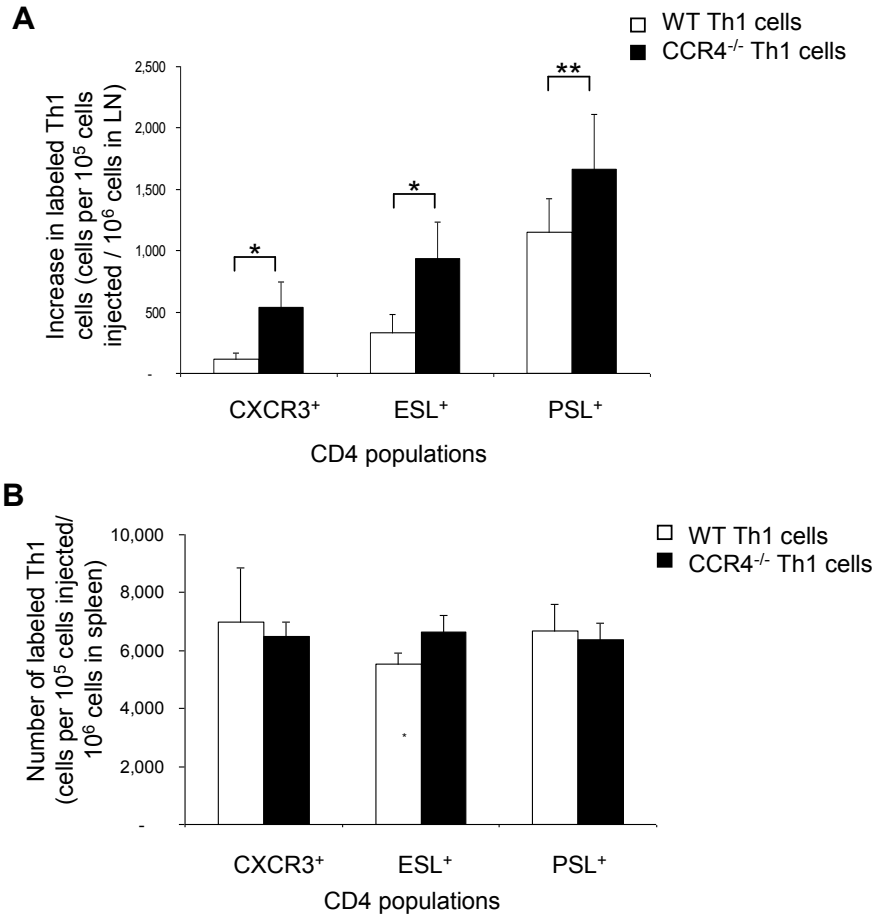

Figure 35. Number of CCR4$^{-/-}$ and WT Th1 cells expressing CXCR3, ESL and PSL that were recovered from spleen and LNs in immunized mice at 72 h post-i.v. injection. CCR4$^{-/-}$ and WT Th1 cells were fluorochrome-labeled and injected i.v. into mice that were immunized with CFA 3 days earlier. LNs and spleen were recovered at 72 h and were stained to estimate the number of labeled Th1 cells that express CXCR3, ESL or PSL. (A) The increase in the number of labeled Th1 cells recovered from draining LN. (B) The number of labeled Th1 cells recovered from the spleen. Error bars represent mean ± SEM. * $P < 0.05$, ** $P < 0.01$ Paired t-test comparing CCR4$^{-/-}$ and WT Th1 cells in 2 exps (CXCR3), 4 exps (PSL) or 5 exps (ESL) that include total of 6-19 mice.

Table 14. Number of CD4 cells that express CXCR3, ESL, PSL, α_4-integrin or L-selectin in draining LN and spleen at 72 h post-i.v. injection [(a)]

Th1 cells	**WT Th1** (Mean number of CD4 of cells per 10^5 cells injected per 10^6 cells in tissue (±SEM))	**CCR4$^{-/-}$ Th1** (Mean number of CD4 of cells per 10^5 cells injected per 10^6 cells in tissue (±SEM))
DRAINING LN		
CXCR3^{+} [(a)]	2,456 (± 760)	4,656 (± 691)
ESL^{+} [(a)]	3,159 (± 402)	4,396 (± 373)
PSL^{+} [(a)]	4,245 (± 1430)	5,950 (± 1158)
α4 integrin^{+} [(b)]	2,783 (± 615)	2,413 (± 502)
L-selectin^{+} [(b)]	2,801 (± 375)	2,889 (± 317)
SPLEEN		
CXCR3^{+} [(a)]	6,996 (± 1871)	6,474 (± 523)
ESL^{+} [(a)]	6,060 (± 776)	6,622 (± 579)
PSL^{+} [(a)]	6,689 (± 902)	6,362 (± 590)
α4 integrin^{+} [(b)]	4,599 (± 764)	5,488 (± 1016)
L-selectin^{+} [(b)]	2,837 (± 683)	3,182 (± 574)

(a) N = 6-19 mice 3-5 experiments
(b) N = 3-5 mice in 3 experiments

7.5 Surface Expression of CCR4 and CXCR3 on WT Th1 Cells Recovered from the Lymphoid Tissues of Immunized Mice

Even though ~50% of the Th1 cells were $CCR4^+$ when injected into immunized mice, most of the Th1 cells recovered from the lymphoid tissues were $CCR4^-$; only 4-5% of the labeled Th1 cells recovered from the spleen and LNs at 72 h post-injection were $CCR4^+$ (Figure 36). In comparison, Th1 cells were mostly $CXCR3^+$ at the time of injection and half of the WT Th1 cells recovered from the lymphoid tissues expressed CXCR3 (Figure 36). Therefore, WT Th1 cells appear to downregulate the surface expression of CXCR3, but this does not occur to the same extent as observed with CCR4. Since half of the WT Th1 cells recovered from the spleen and LNs, which are mostly $CCR4^-$, express CXCR3, it is likely that CXCR3 rather than CCR4 mediate part of the accumulation of Th1 cells in the draining LNs.

It should be noted that while WT Th1 cells downregulated the expression of CXCR3 to 47± 8%, a higher proportion of $CCR4^{-/-}$ Th1 cells were $CXCR3^+$ (71± 6%) at the time of recovery. $CCR4^{-/-}$ Th1 cells do not downregulate CXCR3 as WT cells do. Also, most of labeled $CCR4^{-/-}$ and WT CD4 cells found in lymphoid tissues downregulated CD25 after they were injected *in vivo*. CD25 is expressed on 3.0 ± 0.8% of the labeled CD4 cells in the draining LN after 72 h post-i.v, and on 4.8 ±0.1% of CD4 cells in the draining LN at 24 h post-i.v.

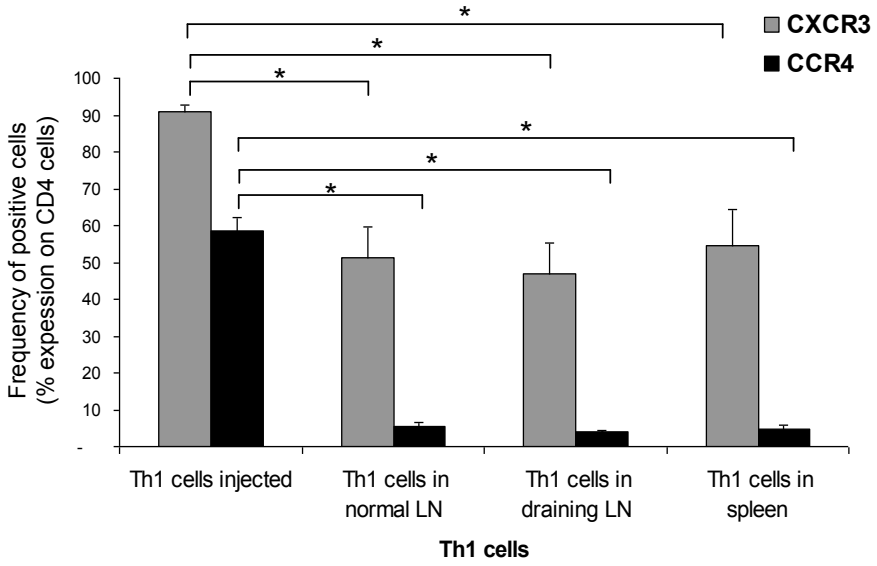

Figure 36. Expression of CXCR3 and CCR4 on WT Th1 cells recovered from the lymphoid tissues of immunized mice at 72 h post-i.v. injection. Flurochrome labeled WT Th1 cells were injected into immunized mice. Nondraining (normal) LN, draining LN, and spleen were collected after 72 hours. Expression of CXCR3 and CCR4 on labeled Th1 cells prior to injection and after recovery from LNs and spleen is shown. n = 5-20 mice in 3-6 exps. Error bars represent mean ± SEM. * $P < 0.05$ by ANOVA.

7.6 Effect of CCR4 Deficiency on the Accumulation of Th1 Cells After Subcutaneous Injection to Lymph Nodes Draining Sites of Immunization

The deficiency of CCR4 resulted in an increased accumulation of Th1 cells in the draining LNs. In order to establish whether this effect is related to the route of administration or whether it relates to the retention of Th1 cells in the draining LNs, cell accumulation in the draining LNs was examined after subcutaneous injection. Mice were immunized subcutaneously with *M. butyricum* at the hock and after 3 days later, $CCR4^{-/-}$ and WT Th1 cells were fluorochrome-labeled and injected subcutaneously at the hock. At different time points, draining LNs (popliteal and inguinal LNs) and spleen were collected and stained with anti-CD4 mAb in order to assess the number of labeled Th1 cells in these tissues.

As shown in Figure 37, increasing numbers of Th1 cells were found in the LNs up to 48 h post-s.c. cell injection. Accumulation of $CCR4^{-/-}$ Th1 cells in the draining LNs, it was found to exceed that of WT Th1 cells 2-3 fold at 24-48 h after s.c. injection ($P < 0.05$). However, at 72 h post-s.c. injection, the number of $CCR4^{-/-}$ or WT Th1 cells in the draining LNs at was comparable..

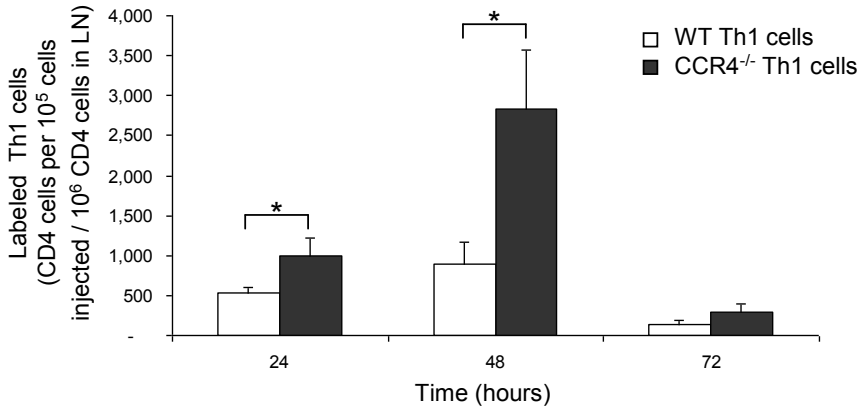

Figure 37. Number of $CCR4^{-/-}$ and WT Th1 cells recovered from draining LNs in immunized mice after s.c. injection. Mice were immunized with *M. butyricum* subcutaneously at the hock and 3 days later they received s.c. injection of flurochrome labeled $CCR4^{-/-}$ and WT Th1 cells. Number of labeled Th1 cells in LN draining site of immunization (popliteal and inguinal LNs) is shown at different time points. n = 4-9 mice in 1-3 exps. Error bars represent mean ± SEM. * $P < 0.05$ by Student's t-test compared to WT cells.

7.7 Effect of CCR4 Deficiency on the Phenotype of Th1 Cells That Accumulate in Lymph Nodes from Subcutaneous Sites

Labeled $CCR4^{-/-}$ and WT Th1 cells were injected subcutaneously into immunized mice. After 48 h, the spleen and draining LNs (inguinal and popliteal LNs) were collected and stained. The accumulation of $CCR4^{-/-}$ and WT Th1 cells expressing CXCR3, ESL, PSL or L-selectin was examined. About 3 fold more $CXCR3^+$ $CCR4^{-/-}$ Th1 cells were found in the draining LNs compared to WT Th1 cells at 48 h post-s.c. ($P < 0.05$). These changes in the accumulation of $CXCR3^+$ cells do not occur as extensively in the spleen ($P > 0.05$) (Table 15). In addition, there was no significant difference between the accumulation of ESL^+, PSL^+, or L-$selectin^+$ of WT and $CCR4^{-/-}$ Th1 cells in the draining LNs or the spleen ($P > 0.05$) (Table 15). Therefore, these results indicate the effect of CCR4 deficiency on the increased accumulation of $CXCR3^+$ Th1 cells, which was also observed when the Th1 cells were injected via the i.v. route.

Table 15. Number of Th1 cells that express CXCR3, ESL, PSL or L-selectin in draining LNs and spleen at 48 h post-s.c. injection [(a)]

	No. of Th1 cells in draining LN Mean (±SEM)		**Normalized number of cells in spleen**	
Th1 cells	**WT Th1**	**$CCR4^{-/-}$ Th1**	**WT Th1**	**$CCR4^{-/-}$ Th1**
$CXCR3^+$	5,616 (± 2186)	19,394 (± 5137)*	946 (±302)	1,305 (± 433)
ESL^+	11,609(± 3530)	25,884 (± 7181)	3,313(± 947)	4,928 (± 1641)
PSL^+	9,048 (± 5341)	17,627(± 2103)	2,845(± 43)	2,419 (± 222)
L-$selectin^+$	898 (± 217)	3,931 (± 1368)	736 (± 346)	1,131 (± 314)

[(a)] N = 3-6 mice in 1-2 experiments.
* $P < 0.05$ Student's t-test

7.8 Apoptosis of CCR4$^{-/-}$ and WT Th1 Cells Induced after IL-2 Deprivation *In Vitro*

More CCR4$^{-/-}$ Th1 cells were recovered from the draining LNs than WT Th1 cells, and the reason behind this observation is unclear. In order to determine if the increase in the number of CCR4$^{-/-}$ cells is related to ability of these cells to resist apoptosis, *in vitro* deprivation of IL-2 cells was used to examine apoptosis of CCR4$^{-/-}$ and WT Th1 cells. CD4 cells were activated in type 1 polarizing conditions which included IL-2. After 6 days, Th1 cells were cultured in the absence of IL-2 to induce apoptosis, or in the presence of IL-2 as controls. The induction of apoptosis and reduction of viability of the IL-2 deprived Th1 cells was compared to control Th1 cells using trypan blue exclusion assay and Annexin V / 7AAD staining.

CCR4$^{-/-}$ and WT Th1 cells that were cultured in the absence of IL-2 for 24 hours did not change in the number, however after 48-72 hours of IL-2 deprivation, the number of cells was reduced by ~50% (Figure 38A). The cumulative change in the number of IL-2 deprived cells was similar for CCR4$^{-/-}$ and WT Th1 cells. Also, Th1 cells have reduced viability when cultured in the absence of IL-2 as assessed by trypan blue exclusion assay. About ~40% of the Th1 cells excluded trypan blue after 48 hours of IL-2 deprivation, whereas the cells that were cultured in the presence of IL-2 were ~85% viable (Figure 38B). The reduction in the viability of IL-2 deprived CCR4$^{-/-}$ and WT Th1 cells were similar.

The induction of apoptosis by IL-2 deprived CCR4$^{-/-}$ and WT Th1 cells was compared to assess whether the deficiency of CCR4 affects the induction of apoptosis. As shown in Figure 38C, ~24% of the Th1 cells were found to be apoptotic, as assessed by the positivity of Annexin staining. This was similar for CCR4$^{-/-}$ Th1 cells. Most of the apoptotic cells excluded 7AAD, hence they were estimated to be undergoing early apoptosis. Only 6-7% of the Th1 cells were found to be Annexin^{+} 7AAD^{+}, suggesting that they were undergoing late stage apoptosis. The induction of apoptosis, the viability of the cells and the number of cells was found to be similar for CCR4$^{-/-}$ and WT Th1 cells after IL-2 deprivation *in vitro*. Therefore, the increase in the number of CCR4$^{-/-}$ Th1 cells recovered in the draining LN compared to that of WT Th1 cells does not appear to be related to differences in the induction of apoptosis, as shown by the lack of IL-2 *in vitro*.

It should be noted that trypan blue exclusion appeared to be more sensitive than the Annexin staining; ~70% Th1 cells that were deprived of IL-2 for 48 hours were found to viable by Annexin staining (Figure 38C), but only ~40% of those Th1 cells excluded trypan blue.

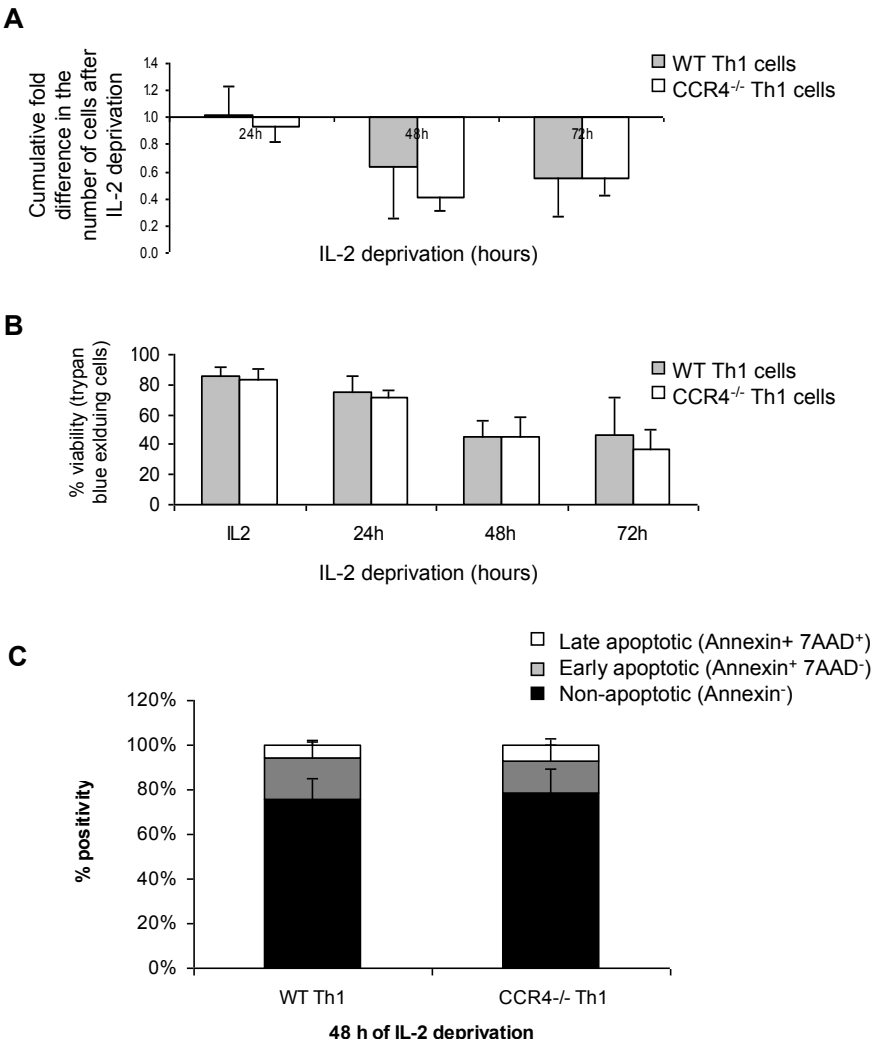

Figure 38. Apoptosis induced by IL-2 deprivation of CCR4$^{-/-}$ and WT Th1 cells. WT and CCR4$^{-/-}$ CD4 cells were cultured in type 1 polarizing conditions. After 6 days, cells were deprived of IL-2, or control cells were cultured with IL-2. (A) Fold difference in the number of cells compared to day 6 cells. (B) Cell viability was assessed by trypan blue exclusion in the presence or absence of IL-2. The cells that were stained blue (dead) and those that excluded the stain (viable) were counted per field to assess the percent cell viability. (C) Th1 cells deprived of IL-2 for 48 hours were stained with Annexin V and 7AAD to assess proportion of cells that were non-apoptotic (Annexin^{-}), early apoptotic (Annexin^{+}7AAD^{-}), and late apoptotic (Annexin^{+}7AAD^{-}). n = 2-4. Error bars represent mean ± SEM.

7.9 Summary

The requirement for CXCR3 and CCR4 in the accumulation of Th1 cells in draining LNs has not been examined. Draining LNs can act as an inflammatory site from which T cells can be collected and characterized. First, it was necessary to develop and assay to measure the accumulation of Th1 cells in LNs draining the site of immunization. A comparison was made between the expression of CD25, ESL and CXCR3 on CD4 cells following an immunization with OVA-CFA or OVA-Alum (Figure 30). Since there was an increased number of $CD25^{+}$, ESL^{+} and $CXCR3^{+}$ CD4 cells in the LNs draining sites of CFA immunization, this was selected for further experiments. It should be noted that this was the same immunization strategy used to sensitize mice for DTH in the skin (Chapters 4 and 5). The kinetics of expression of CD25, CD69, ESL and CXCR3 on CD4 cells were examined, and the number of CD4 cells expressing these markers appears to increase by day 3 p.i. (Figure 31).

When labeled Th1 cells are injected i.v. into immunized mice, the accumulation of Th1 cells was noted in the LNs. As shown in Figure 34, the number of labeled Th1 cells recovered in the draining LN after i.v. injection were ~2 times higher at 72 h compared to 24 h. Other studies reported the recovery of increasing numbers of adoptively transferred *in vivo* activated T cells from draining LNs (237). It was therefore necessary to examine the accumulation of Th1 cells in the draining at 72 h post-i.v. injection in mice immunized 3 days earlier with an emulsion that contained CFA.

CXCR3 was found to mediate part of the increased accumulation of Th1 cells to LNs draining site of immunization. At 72 h and 96 h post injection, the number of $CXCR3^{-/-}$ Th1 cells in draining LNs was significantly lower than WT Th1 cells (Figure 33). The absence of CXCR3 did not affect the circulation of Th1 cells, as shown by the normal numbers of $CXCR3^{-/-}$ Th1 cells present in the spleen.

The contribution of CCR4 to the accumulation of Th1 cells in the draining LNs has not been examined. LNs draining the CHS in $CCR4^{-/-}$ mice were shown to contain increased numbers of CD4 cells, but the phenotype of these cells was not examined (195). Increasing numbers of Th1 cells were recovered from the LN draining the site of immunization after i.v. injection with a peak after 72 h. The deficiency of CCR4

increased cell accumulation in the draining LNs by ~ 40% and in the spleen by ~ 20% after 72 h (Figure 34). Even when the cells were injected s.c., the accumulation of $CCR4^{-/-}$ Th1 cells was increased by ~ 3 times after 48 h (Figure 37). Therefore, the deficiency of CCR4 resulted in an increased accumulation of Th1 cells in the draining LNs whether they cells were introduced via bloodstream after i.v. injection or via the lymphatic system after s.c. injection.

$CCR4^{-/-}$ and WT Th1 cells in the draining LN and the spleen were stained to determine the expression of adhesion molecules. There were more PSL^+ and ESL^+ cells among the $CCR4^{-/-}$ Th1 cells in the draining LNs than WT Th1 cells (Figure 35), whereas the accumulation of α_4 $integrin^+$ or $L\text{-}selectin^+$ CD4 cells was not affected by the deficiency of CCR4 (Table 14). The increased accumulation of the ESL^+ and PSL^+ $CCR4^{-/-}$ Th1 cells in the draining LNs appear to result from the inflammatory response in the draining LN, rather than the mere increase in the size of draining LN, as the number of labeled Th1 cells is normalized to the number of CD4 cells in the LN. The deficiency of CCR4 increased the accumulation of ESL^+ and PSL^+ CD4 cells in the draining LNs after i.v. injection (Figure 35), and this also appears to be the case with cells injected s.c. (Table 15). My results show that Th1 cells can accumulate into the draining LNs in the absence of CCR4, and that the deficiency of CCR4 increased the accumulation of ESL^+ and PSL^+ CD4 cells in the draining LN. It is interesting to note that $L\text{-}selectin^+$ cells, which presumably utilize L-selectin to accumulate in the LNs, are not affected by the deficiency of CCR4.

Like WT Th1 cells, $CCR4^{-/-}$ Th1 cells are ~90% $CXCR3^+$. Since $CXCR3^{-/-}$ Th1 cells have reduced accumulation in the draining LNs (Figure 33), CXCR3 may mediate part of the accumulation of the $CCR4^{-/-}$ Th1 cells. There were more $CXCR3^+$ cells among $CCR4^{-/-}$ Th1 cells in the draining LN than WT Th1 cells, and this was observed both after i.v. (Figure 35) and s.c. injection (Table 15). This may provide an alternative explanation for the increased accumulation of ESL^+ and PSL^+ $CCR4^{-/-}$ CD4 cells. The deficiency of CCR4 caused an increased accumulation of $CXCR3^+$ cells, thus it is likely that the presence of CXCR3, rather than the deficiency of CCR4, might have mediated the recruitment and/or accumulation of the ESL^+ and PSL^+ cells.

It is interesting that WT Th1 cells maintained ~50% of their expression of CXCR3 after they were injected, while most of the expression of CCR4 was downregulated *in vivo* (Figure 36). CCR4 can be downregulated if the ligands for CCR4 were present in the lymphoid tissues. Th1 cells also downregulated CD25 *in vivo*, even at 24 h post-i.v. injection (Chapter 7.4). This suggests that the increased accumulation of $CCR4^{-/-}$ Th1 cells in the draining LNs of immunized mice may not be a direct result of the absence of CCR4, but may result indirectly if the deficiency of CCR4 influenced other pathways that mediate the retention of CD4 cells in the draining LNs.

Furthermore, I examined the possibility that $CCR4^{-/-}$ Th1 cells may resist apoptosis and have improved survival ability in the draining LNs. Apoptosis was induced by the deprivation of IL-2 *in vitro* in order to assess the ability of $CCR4^{-/-}$ and WT Th1 cells to undergo apoptosis (Figure 38). The reduction in cell viability and induction of apoptosis appeared to be similar in $CCR4^{-/-}$ and WT Th1 cells after IL-2 deprivation *in vitro*, suggesting that this might not be the reason for the increased accumulation of $CCR4^{-/-}$ Th1 in the draining LNs.

Results from this chapter demonstrate that 1) the accumulation of Th1 cells in the draining LNs was decreased by the deficiency of CXCR3, but increased by deficiency of CCR4. The increased accumulation into draining LN of $CCR4^{-/-}$ Th1 cells was observed whether the cells were introduced via the bloodstream or subcutaneous route. 2) The deficiency of CCR4 increased the accumulation of $CXCR3^{+}$, ESL^{+} and PSL^{+} Th1 cells, but not L-selectin^{+} Th1 cells in the draining LNs. 3) Th1 cells downregulate CD25 and CCR4 *in vivo* after i.v. injection, but they maintain ~50% of their expression of CXCR3. 4) When $CCR4^{-/-}$ and WT Th1 cells were deprived from IL-2 *in vitro*, the reduction in cell viability and induction of apoptosis were comparable, indicating that the $CCR4^{-/-}$ Th1 cells do not inherently resist the induction of apoptosis.

Chapter 8. Discussion

The relationship between the expression of CCR4 and CXCR3 on T cells and their migration to inflammatory sites in the skin, joints and LNs is not known. This is the first study to examine the effect of CCR4 and CXCR3 deficiency on the migration of different T cell subsets to the inflamed skin, arthritic paws, and LNs draining a site of CFA-immunization. In addition, the effect of CCR4 deficiency on the development of CIA has been explored.

In the following chapter, I will discuss my studies examining the contribution of CXCR3 and CCR4 in the migration of T cells to inflammatory sites in the skin, inflamed paws and LNs, in relation to the related literature. Aspects of T cell migration that influence the interpretation of the related literature will be highlighted. Also, a model will be presented and future studies will be suggested to overcome the limitations of the current studies and to expand the knowledge base on the role of CCR4 and CXCR3 in the migration of T cells.

8.1 Contribution of CCR4 and CXCR3 in the Recruitment of T Cells to Sites of Dermal Inflammation

The chemokine receptors, CCR4 and CXCR3, are expressed by several T cell subsets, but the relationship between their expression and the recruitment of T cells to inflamed skin is not clear. The findings of my studies demonstrate that the presence of $CXCR3^+$ or $CCR4^+$ Th1 cells in inflamed skin does not necessarily reflect the requirement of these CKRs for Th1 cell recruitment. This is the first time that the migration of $CCR4^+$ and $CCR4^-$ Th1 cells to dermal inflammation has been compared *in vivo*. This is also the first study to examine the migration of $CXCR3^{-/-}$ and $CCR4^{-/-}$ T cells to stimuli, such as TLR agonists, ConA and DTH, associated with different types of inflammatory responses.

A contribution for CXCR3 in the CD4 cell recruitment to inflamed skin has been suggested by histological findings showing $CXCR3^+$ CD4 cells in the chronic lesions of patients with AD or psoriasis (104, 229). CXCR3 ligands present in the lesions of allergic contact dermatitis and psoriasis (230), may recruit CD4 cells, since CXCL10 injections into skin grafts in mice lead to the infiltration of CD4 cells (238). Previous studies in our laboratory demonstrated that most CD4 cells migrating into dermal inflammatory sites have $CXCR3^+$ memory phenotype, though CXCR3 is only expressed on ~10% of the memory CD4 cells in rats (93). CXCR3 blockade resulted in a reduced infiltration of memory CD4 cells into dermal inflammatory sites (93). In addition, the blockade of CXCR3 inhibited most the recruitment of *in vivo* activated T cell lymphoblasts to inflamed skin (93). In other studies, chronic *Leishmania major* lesions in the skin of $CXCR3^{-/-}$ mice were reported to have fewer infiltrating IFN-γ^+ CD4 cells (241).

CXCR3 is not only expressed on activated and memory CD4 cells (93, 138, 141, 142, 149, 371, 372), but also by CD8 cells activated *in vitro* and *in vivo* (142, 146). Therefore, CXCR3 was found on CLA^+ CD8 T cells in psoratic lesions (104). It was suggested to contribute to the recruitment of CD8 T cells to inflammatory sites in the skin, based on *in vivo* recruitment of CD8 memory cells to inflammatory sites in the skin (93) and the number of CD8 T cells in chronic *Leishmania major* – induced dermal lesions (241). However, these studies did not examine the relationship between the

expression of CXCR3 on different T cell subsets and their recruitment to dermal inflammation induced by LPS, poly-I:C, ConA or DTH.

I found that CXCR3 deficiency reduced the recruitment of CD4 cells to ConA (Figure 11), which confirms the inhibitory effect of CXCR3 blockade on the recruitment of memory CD4 cells to dermal inflammatory sites in rats (93). The proportion of CD4 splenocytes with a memory phenotype was not affected the deficiency of CXCR3 (Section 4.3). However, I did not determine if CXCR3 deficiency influenced the proportion of central memory (T_{CM}) and effector memory CD4 cells (T_{EM}), neither did I examine the effect of CXCR3 deficiency on the homing of these different memory CD4 cell subsets. Both of T_{CM} and T_{EM} cells are known to express CXCR3 (371, 372), but they preferentially home to different tissues; T_{CM} cells preferentially home to lymphoid tissues, while T_{EM} is more likely to be found in nonlymphoid tissues (61). It is not clear if CXCR3 deficiency influences the homing of T_{CM} and T_{EM} differentially.

Also, it is possible that CXCR3 deficiency affects the expression of other CKRs on memory cells. For instance, if the expression of CCR7 on $CXCR3^{-/-}$ memory CD4 cells is increased, then it may explain the increased homing of $CXCR3^{-/-}$ CD4 cells to LNs, that I observed (Figure 11). Nevertheless, ~50 % of the recruitment of CD4 cells to ConA site was CXCR3-independent (Figure 11), like what was observed for memory CD4 cells infiltrating dermal inflammatory sites in rats (93). This may be possibly mediated by CCR5, which is found on skin-infiltrating memory CD4 cells (89), or CCR4 that is co-expressed on half of the $CXCR3^{+}$CD4 cells (138) and on proportion of $CXCR3^{+}$ T_{CM} and T_{EM} cells (371, 372). However, the recruitment of CCR4 deficient memory cells to dermal inflammation was unhindered (Figure 16).

In addition, I found that a deficiency of CXCR3 differentially reduced the recruitment of T cell subsets to inflammatory sites in the skin; it reduced the recruitment of Tc1 cells to sites of dermal inflammation by ~30% only (Figure 13), while it reduced the recruitment of Th1 cells to these sites by 50-60% (Figure 12). Though CXCR3 blockade inhibits the infiltration of *in vivo* activated T cells to dermal inflammatory sites in rats (93), the differential effect of CXCR3 deficiency on the skin homing of activated CD4 and CD8 T cells was not demonstrated prior to this study. In other words, though most Th1 and Tc1 cells express CXCR3 (Figures 9B and 10B), CXCR3 mediates the

recruitment of different proportions of Th1 cells (50-60%) and Tc1 cells (~30%) (Figures 12A and 13A). Still, a proportion of Th1 cells (~50%) and Tc1 cells (~70%) were recruited in the absence of CXCR3. This may be associated with CCR4 expressing cells, since CCR4 is expressed on half of the $CXCR3^+$ Th1 cells (Figure 9B).

CCR4 is regarded as a marker for the skin homing potential of CD4 T cells (136). This is primarily based on the presence of $CCR4^+$ CD4 cells in the lesions of patients with skin inflammation (90, 110, 228, 229, 233, 235). The presence of CCR4 ligands in the inflamed skin of patients (110, 233, 234) and mice (196), and its production by keratinocytes (109) and DCs (112, 234) was suggested to recruit $CCR4^+$ CD4 cells into inflamed skin, as shown by *in vitro* studies (107) and the co-localization with DCs in skin biopsies (234). *In vivo* studies showed the ability to intradermally administered CCR4 ligands to recruit CD4 cells into skin grafts in mice (145, 245). These studies suggested a possible relationship between the expression of CCR4 and the infiltration of memory CD4 cells into inflamed skin. However, the contribution of CCR4 has not been related to the *in vivo* recruitment of specific T cell subsets, so the relationship between the expression of CCR4 on T cell subsets and their recruitment to dermal inflammation is not clear. Whether a deficiency of CCR4 would affect the migration to inflammation induced by stimuli, such as the cytokines IFN-γ and TNF, TLR agonists, ConA and DTH, has not been previously determined.

My studies demonstrate the increased infiltration of $CCR4^+$ CD4 cells than $CCR4^-$ CD4 cells to sites of dermal inflammation; $CCR4^+$ memory CD4 cells were recruited in the inflamed skin ~5-7 fold more than $CCR4^-$ cells (Figure 4B). They represented most of the infiltrating memory CD4 cells in the inflamed skin, though CCR4 is present on only 5-20% of CD4 cells in the LNs and spleen of rats (Figure 4A). Likewise, CCR4 is present on most of the skin-infiltrating memory CD4 T cells (89), though it is expressed on ~20% of circulating memory CD4 cells of humans (136, 137). It should be noted that this effect was more apparent on memory CD4 cells than on activated CD4 cells in rats (Figure 4,5) or even on Th1 cells in mice (Figure 14). In fact, there was an equivalent number of $CCR4^+$ and $CCR4^-$ activated CD4 cells in certain dermal inflammatory sites, namely cytokine-injected skin sites in rats (Figure 5), and DTH in mice (Figure 14), which was not observed with $CCR4^+$ and $CCR4^-$ memory CD4 cells (Figure 4), thus reflecting the

complexity of the relationship between CCR4 expression and the skin homing of CD4 T cells.

Nevertheless, I found that CCR4 is not required for the recruitment of memory CD4 cells or Th1 cells to sites of dermal inflammation. $CCR4^{-/-}$ Th1 cell recruitment to inflamed skin was normal (Figure 20 A), though the expression of CCR4 was associated with the recruitment of Th1 cells to most sites of dermal inflammation (Figure 14). Similarly, $CCR4^{-/-}$ memory CD4 cells infiltrated normally to DTH (Figure 16), though $CCR4^{+}$ memory CD4 cells preferentially infiltrated to DTH (Figure 4B). My work demonstrates that neither memory CD4 cells nor Th1 cells depend on CCR4 for their recruitment to TLR agonists, ConA and DTH induced skin inflammation, which complements studies reporting normal recruitment of *in vivo* activated $CCR4^{-/-}$ CD4 cells to CHS (237). Though I have not specifically examined the localization of these cells in the inflamed skin sites, it is unlikely that CCR4 deficiency would alter this aspect of skin homing, as shown by the equivalent infiltration of $CCR4^{-/-}$ memory CD4 cells between the dermal and epidermal layers of CHS sites (247).

Interestingly, I found that the dermal recruitment of memory $CCR4^{-/-}$ CD4 cells in response to ConA was increased (Figure 16B). Although there was a higher proportion of memory cells among the $CCR4^{-/-}$ CD4 splenocytes than WT cells (Figure 16A), the increased recruitment of $CCR4^{-/-}$ CD4 cells to ConA (Figure 15A) was re-capitulated when homing was examined, using equal numbers of $CCR4^{-/-}$ and WT memory CD4 cells (Figure 16B). The increased infiltration of $CCR4^{-/-}$ CD4 cells may not be limited to ConA, as it was also reported in OXA-induced CHS of $CCR4^{-/-}$ mice (195). This is in contrast to studies that examined accumulation of CD4 cells to antigen-injected sites over 6-10 days; fewer $CCR4^{-/-}$ CD4 cells were found in these sites (246, 247). The production of CCR4 ligands by DCs *in vitro* (112, 113) and in atopic skin lesions (112) explains their co-localization with $CCR4^{+}$ CD4 T cells in inflamed skin (234). Antigen-specific CD4 cells were shown to proliferate in the skin injected with antigen plus adjuvant (246, 247). So, the formation of CD4 T cells-DC clusters and the subsequent activation / proliferation of CD4 cells would possibly lead to their retention. I speculate that $CCR4^{+}$ CD4 cells interact with antigen-bound DCs and are retained longer in the skin than $CCR4^{-}$ CD4 cells, even though the initial recruitment of CD4 cells into the skin is CCR4-

independent. This explains why CCR4 deficiency reduced the number of antigen-specific CD4 cells after 6 or 10 days (246, 247), but did not hinder their infiltration during the first 20 h in my studies, in which the antigen-specific interaction between total memory CD4 cells and CCL22 producing DCs in the skin sites is not likely to occur. In my opinion, it is important to distinguish the CKRs that affect T cell retention *versus* those involved in tissue infiltration. CD4 T cells present in skin lesions with chronic or repeated acute inflammation (90, 110, 197, 201, 229, 234) may represent a combination of T cells that have newly arrived and those that are retained, and the CKRs involved in these two processes are not well examined.

My studies also demonstrate similarity between the effect of CCR4 deficiency on Treg cells and memory CD4 cell infiltration; in that the recruitment of $CCR4^{-/-}$ Treg cells to TLR agonists or ConA was ~50% greater than that of the control Treg cells (Figure 17). It should be noted that the results observed using $CD25^{+}$ CD4 cells that were cultured in the presence of IL-2, were similar to those obtained using CD4 cells that were cultured in the presence of IL-2, TGF-β, and rapamycin. The increased recruitment of $CCR4^{-/-}$ Treg cells to inflamed skin sites (Figure 17) mimics the increased number of $Foxp3^{+}$ CD4 cells found in OXA-induced CHS of $CCR4^{-/-}$ mice (195). This predicts that the expression of CCR4 by CLA^{+} Treg cells (150), by Treg cells in the in *Paracoccidioides brasiliensis* induced lesions in mice (242) or by induced Treg cells (154, 155) may not relate to their recruitment to dermal inflammation. In contrast, the expression of CCR4 on Treg cells in normal skin (243) may relate to their skin homing during homeostasis. In fact, the reduced infiltration of $CCR4^{-/-}$ Treg cells in the normal skin of $RAG^{-/-}$ mice (156) suggests that the requirement for a certain CKR may be related to the inflammatory microenvironment in the skin. In the absence of an ongoing immune response in the skin, CCR4 may be required for homeostatic skin homing of Treg cells (156), while during an inflammatory response in skin sites, such as ConA, CCR4 is dispensable for the infiltration of Treg cells. This does not exclude the role of CCR4 in mediating the infiltration of Treg cells to other inflamed tissues, as in islet allografts (154) and tumors (183, 185).

In addition, I examined the recruitment of Tc1 cells to sites of dermal inflammation, and found it to be CCR4-independent (Figure 21A). Even though CD8 T

cells are recruited to CCL22-injected skin grafts (238), $CCR4^{-/-}$ Tc1 cells can recruit to sites of dermal inflammation normally. I observed this for TLR agonists, ConA and DTH sites (Figure 21A), and may also apply to CHS, as shown by the normal number of CD8 T cells in CHS in $CCR4^{-/-}$ mice (195). The production of CCR4 ligands by IFN-γ or TNF stimulated keratinocytes (109, 110), or during PPD induced granulomatous responses (171) suggests a role for CCR4 on type 1 responses. A deficiency of CCR4 was shown to induce less IFN-γ in response to secondary challenge with PPD (171).Yet, CCR4 deficiency did not affect the skin homing of Tc1 cells and Th1 cells (Figures 20A and 21A) or their expression of IFN-γ (Tables 9 and 11). Instead, the recruitment of these cells to sites of dermal inflammation was found to be partly mediated by CXCR3 (Figures 12 and 13). So, the role of CCR4 on type 1 T cells remains to be determined.

In contrast to Th1 and Tc1 cells, I found that CCR4 deficiency reduced the recruitment of Th2 and Tc2 cells to ConA injection sites by ~35%. Yet, their infiltration to TLR agonists was not affected by the deficiency of CCR4. Studies which examined the recruitment of $CCR4^{+}$ Th2 cells to the CCL22-injected skin sites may have limited the recruitment to $CCR4^{+}$ Th2 cells, and did not allow for the recruitment of $CCR4^{-}$ Th2 cells to be studied (145, 245). My studies demonstrate a partial contribution of CCR4 in the recruitment of ~35% of the Th2 and Tc2 cells to ConA induced skin inflammation. ConA is known to induce the recruitment and the activation of eosinophils, mast cells and monocyte/macrophages (203, 216, 218, 219), and to induce the production of different cytokines (219, 220). Activated mast cells (117), DCs (112), IL-4 or IL-13 stimulated monocytes/macrophages (112, 115), IL-4 and TNF stimulated eosinophils (111) can produce CCR4 ligands, so it may possibly lead to the infiltration of $CCR4^{+}$ Th2 and Tc2 cells. I have not determined the levels of different CKs or the infiltration of cells such as eosinophils in ConA sites. This information may assist in relating CK production to eosinophil recruitment. The requirement of CCR4 in Th2 cell recruitment might be genuinely “skin” specific, or might be related to the prior infiltration of eosinophils in the tissue. The correlation of $CCR4^{+}$ CD4 cells in the circulation with eosinophilia in atopic patients (110, 174, 229, 232), and the reduced migration of $CCR4^{-/-}$ Th2 cells in eosinophil-recruiting lesions, namely allergic pulmonary inflammation (170) suggests the latter.

In addition, I found that the relationship between CCR4 and Th2 cells is not as profound as suggested in the literature. Reports of the production of CCR4 ligands by IL-4 stimulated APCs (112, 113, 115), and the production of IL-4 by $CCR4^{+}$ $CD45RO^{+}$ CD4 cells (137, 138, 372) suggest an association between CCR4 expression and IL-4 production. I find this to be reasonable, since $CCR4^{-/-}$ Th2 cells produced less IL-4 (Table 10). Also, CCR4 expression is not necessarily required for the skin homing of activated CD4 cells. More CCR4 was expressed on Th1 cells than Th2 cells (Figure 18C), yet it only mediated part of the recruitment of Th2 cells to ConA sites, but did not play a role in the recruitment of Th1 cells (Figures 20A andC). The discrepancy between CCR4 expression on Th1 cells and their *in vivo* recruitment to CCR4 ligands was also shown by others; CCL17 mediates the migration of both Th1 and Th2 cells *in vitro*, but recruits only Th2 cells *in vivo* (145). The reason behind this observation is still unclear. I speculate that the correlation of CCR4 ligands with the severity of AD may link CCR4 to the progression of the inflammatory disease, rather than a specific role in tissue migration, especially since these studies report the presence of CCR4 ligands in the circulation, but do not examine their presence in the atopic lesions (110, 229, 232).

All in all, my studies demonstrate differential roles for CXCR3 and CCR4 deficiency on the recruitment of T cells to sites of dermal inflammation. The recruitment of memory CD4 cells is associated with CCR4 expression, but is not dependent on CCR4, and instead was partly mediated by CXCR3. This was similar for Th1 cells, in which CXCR3 mediated part of the infiltration of both $CCR4^{+}$ and $CCR4^{-}$ cells. Interestingly, the increased infiltration of $CCR4^{+}$ Th1 cells was partly CXCR3-independent as shown by $CCR4^{+}$ and $CCR4^{-}$ $CXCR3^{-/-}$ Th1 cells (Figure 14). I also found that the contribution of CKRs to T cell recruitment was not necessarily linked to their expression; the dependence on CXCR3 for the recruitment of Th1 and Tc1 cells differed, though CXCR3 was equivalently expressed on these cells. Also, the effect of CCR4 deficiency on the recruitment of T cells varied for different T cell subsets; no effect for Th1 and Tc1 cell recruitment, increased infiltration of $CCR4^{-/-}$ memory CD4 and Treg cells especially to ConA, and reduced recruitment of $CCR4^{-/-}$ Th2 and Tc2 cells to ConA. Though CXCR3 mediated part of the recruitment of memory CD4 cells, Th1, and Tc1

cells, and CCR4 mediated part of the recruitment of Th2 and Tc2 cells to sites of dermal inflammation, 50-70% of the T cell infiltration was independent of these CKRs.

It is challenging to interpret the literature in relation to the different T cells subsets, because of the generalizations that have been made in studies that examined the contribution of CKRs to the migration of T cells. Correlations between disease severity and CKR expression on T cells or CK levels are often generated using blood (110, 229, 232), so they may not reflect the actual players in tissue homing. Similarly, the co-expression of CKRs with CLA^+ T cells (90, 104, 239) as a signature for skin-infiltrating T cells (373) does not necessitate their requirement for migration. Neither do correlations between transcript levels of CKR or CKs with numbers of T cells in the skin (144, 196), which are identified with only a few markers (229, 234). In some studies, CCR4 expressing CD4 cells are identified by memory phenotype (89, 104, 229) or as Treg cells (156, 195), but in others, it is difficult to relate other studies to Th1 or Th2 cell migration, due to lack of sufficient characterization of CD4 cells (195, 237). Some studies examine the migration of CD4 cells that are not well-defined, such as those activated *in vivo* (237) or generated after bone marrow reconstitution (156, 247). Finally, most *in vivo* studies rely on CHS models in mice (194, 195, 237, 246), though CHS may recruit CD4 and CD8 T cells at different phases (207). Other studies depend on histological examination of atopic and psoriatic lesions (104, 229), or use the ligands of CXCR3 and CCR4 as stimuli of skin homing *in vivo* (145, 238, 245). Thus, evidence relating CCR4 or CXCR3 to the migration of different T cell subsets to dermal inflammation is not well established.

The contribution of CKRs to T cell recruitment into sites of dermal inflammation needs to be carefully examined in the future, in order to overcome the limitations of this study and further our understanding. First, only part of the migration of memory CD4, type 1 activated T cells (Th1 and Tc1 cells) is mediated by CXCR3 and only part of the migration of type 2 activated T cells (Th2 and Tc2 cells) is mediated by CCR4. The CKRs responsible for the CXCR3- and CCR4- independent infiltration are not known. There is a significant deficit in the knowledge base of CKRs required for recruitment of T cells to sites of dermal inflammation. In addition, the reason why the recruitment of $CCR4^{-/-}$ Treg cells and $CCR4^{-/-}$ memory CD4 cells to ConA site in the skin was increased (Figure 16, 17) is unclear, and my studies did not address the CKRs responsible for the

infiltration of those cells to inflamed skin sites. The expression of different CKRs on $CCR4^{-/-}$ memory CD4 and $CCR4^{-/-}$ Treg cells is not available from my study or other studies (156, 195, 246, 247). It would be of interest to determine if a deficiency of CCR4 is overcome by an increased expression of other CKRs which may possibly explain the increased recruitment of $CCR4^{-/-}$ Treg cells and $CCR4^{-/-}$ memory CD4 cells in sites of dermal inflammation (Figures 16 and17). This aspect cannot be simply deduced using the increases in the transcript levels of chemokines in the skin, as reported for CHS sites of $CCR4^{-/-}$ mice (195). The implications of this observation need to be closely examined, as it might raise concerns on the efficacy of CCR4 antagonism in the control of dermal inflammation.

In this regard, several CKRs, other than CCR4 and CXCR3, may contribute to the recruitment of T cells to dermal inflammation. For instance, $CCR10^{+}$ CD4 cells are present in lesions of patients with skin inflammation (198, 228), so CCR10 may mediate CD4 cell migration to inflamed skin where CCL27 is produced (198, 199, 248). The co-expression of CCR4 and CXCR3 on $CCR10^{+}$ CD4 cells (90, 134, 248) may explain the increased infiltration of $CCR4^{+}$ memory CD4 or Th1 cells to inflamed skin that I have observed (Figures 4, 5 and 14). It is not clear if blocking CCR10 would reduce the recruitment of $CCR4^{+}$ and/or $CCR4^{-}$ CD4 cells. Interestingly, one study reported the efficacy of CCR10 blockade in inhibiting the recruitment of *in vivo* activated T cells to CHS (198). In another study, this effect was limited to *in vivo* activated $CCR4^{-/-}$ CD4 cells but not WT cells (237) suggesting possible redundancy between CCR4 and CCR10 for the recruitment of CD4 cells to CHS. My studies found the recruitment of CD4 cells (e.g. memory CD4 cells or Th1 cells) was unhindered by CCR4 deficiency, but I cannot exclude the contribution of CCR10 in the recruitment of $CCR4^{-/-}$ CD4 T cell subsets.

Also, CCR5 is expressed on Th1 cells (120), Tc1 cells (142, 146) and was suggested to contribute to homing of T cells to inflamed skin. CCR5 is found on skin-infiltrating CD4 cells, namely memory CD4 cells (89) and Treg cells (242). The increased transcript of ligands of CCR5 (i.e. CCL3, 4 and 5) in CHS lesions of $CCR4^{-/-}$ mice (195) may reflect the contribution of CCR5 to the recruitment of T cells, and some redundancy between CCR4 and CCR5 in the skin homing of T cells. In fact, $CCR4^{+}$ cells were found to be recruited to both CCL5 and CCL22 injected skin grafts (238), which

highlights the ability of CCL5 to recruit CD4 and CD8 T cells to the skin (238) and the relevance of CCR5 expression on $CCR4^+$ cells for their skin homing.

Likewise, the expression of CCR8 on Th2 cell clones (143) may be associated to their recruitment to CCR8 ligand, that is present in atopic skin lesions (162). The relevance of CCR8 in Th2 cell recruitment was also suggested by the reduced recruitment of *in vivo* activated OTII $CCR8^{-/-}$ Th2 cells to OVA injected skin sites (374).It may be related to the recruitment of other CCR8 expressing T cells, such as Tc1 cells (162) and Treg cells (32, 148, 149).

Moreover, the presence of several T cell subsets in the inflamed skin that express the same CKR needs to be considered when interpreting the measurement of transcript levels of CKs or CKRs. For instance, several T cell subsets appear to infiltrate simultaneously during CHS. The number of $Foxp3^+$ CD4 Treg cells and CD4 cells in the skin and draining LNs during CHS appear to follow the same kinetics (195). The presence of both IFN-γ^+ $CD8^+$ and IL-4^+ $CD4^+$ cells in the LNs draining sites of CHS was also demonstrated (375). My work demonstrates the requirement for different CKRs may vary between T cell subsets, even if they are migrate to the same lesion; CXCR3 mediates part of the Th1 cell migration to ConA, while the CCR4 mediates part of the Th2 cell recruitment (Figures 12 and 20C).

In addition, the functionality of CKR expression on hematopoietic and structural cells that are present in the inflamed skin should be examined. For instance, the role of CCR4 on eosinophils (158-160), NK cells (163), monocytes (161), dermal endothelium (165) and keratinocytes (164) is unclear. They might respond to the CKs produced in the tissue during inflammation or they may be indirectly activating and / or recruiting CD4 cells from the draining LNs if they are involved in the transfer CKs from the skin to draining LNs, as shown by intradermally injected CCR7 ligands (332) or CCL27 induced in DNFB-treated skin (95, 199).

Furthermore, the sequential infiltration of T cells and other leukocytes should be considered. It is possible that the first of wave of leukocytes infiltrating the skin influences the subset of T cells that infiltrate later. For instance, eosinophils can be recruited by ConA (203) or by possibly skin infiltrating Tc2 cells (10, 21), and can produce CCR4 ligands (111). This may explain why CCR4 mediates part of the

recruitment of type 2 T cells to ConA (Figures 20C and 21C). One can use eosinophil recruiting stimuli in skin, such as parasitic infections, or tape-strip skin models (376), or employ strategies to enhance or block the recruitment of eosinophils, such as the IL-4 activation of endothelium (376), the adoptive transfer eosinophils or the use of anti-eotaxin. This would be useful in assessing the relationship between eosinophil and $CCR4^{+}$ Th2 cell recruitment. I did not examine the overlapping infiltration of different cells into inflamed skin, but this may contribute to the knowledge on CKRs required for T cell migration. It is unclear if this scenario would apply to different tissue infiltrating T cells, I would speculate that the production of CKs by activated T cells after their infiltration into the tissue may influence the CKRs required for the subsequent wave of infiltrating T cells. The production of CKs by activated T cells is reported (119, 120) and might possibly be a factor in influencing the sequential entry of CD4 T cells after CD8 T cells as seen in CHS (200, 207). However, the significance of this is unclear, since the production of CKs by APCs is more pronounced than that by T cells (112, 114).

Results of this study and others suggest a model in which the requirement for CXCR3, CCR4 and other CKRs for the migration of T cells into the inflamed skin varies according to the T cell subset and the phase of accumulation (Figure 39) (93, 156, 195, 198, 199, 237, 238, 246-248, 374). During the early stage of T cell accumulation, the infiltration of memory CD4 cells requires CXCR3 (93) and other CKRs that are co-expressed with CCR4, like CCR5 (89, 138, 238) or CCR10 (90, 134, 248). Memory CD4 cells present in inflamed skin express CCR4, but do not require it for skin infiltration. The recruitment of Treg CD4 cells is also CCR4-independent (195), but may rely on other CKRs expressed by Treg cells, such as CCR5 (242) or CCR8 (32, 148, 149). I speculate that the increased expression of other CKRs on $CCR4^{-/-}$ memory CD4 cells and $CCR4^{-/-}$ Treg CD4 cells may explain their increased infiltration to lesions, like ConA. The recruitment of type 1 T cells is not mediated through CCR4. Though Th1 cells in inflamed skin express CCR4, their migration is partly dependent on CXCR3. Also, they may depend on other CKRs, such as CCR5 or CCR10, as suggested by their expression on $CCR4^{+}$ CD4 cells, the presence of their ligands in skin sites or their effect on the recruitment of *in vivo* activated CD4 T cells (195, 198, 199, 203, 219, 237, 238). Type 2 T cells requires CCR4 for part of their migration to ConA and possibly to other lesions

that recruit and activate eosinophils and/or mast cells (218, 248). However, type 2 T cells migrate to other inflammatory stimuli in a CCR4-independent manner, possibly using other CKRs, such as CCR8 (374).

In contrast, during later phases of accumulation, in the presence of antigen in the skin, antigen presenting cells (e.g. DCs) may interact with antigen-specific CD4 (memory) cells via CCR4 (234). After the activation and/or proliferation of antigen-specific CD4 cells in the antigen injected sites, $CCR4^{+}$ CD4 cells are retained in the inflamed skin sites (246, 247). In addition, $CD103^{hi}$ Treg cells may require CCR4 for migration during skin homeostasis (156) or possibly for their retention in inflamed skin.

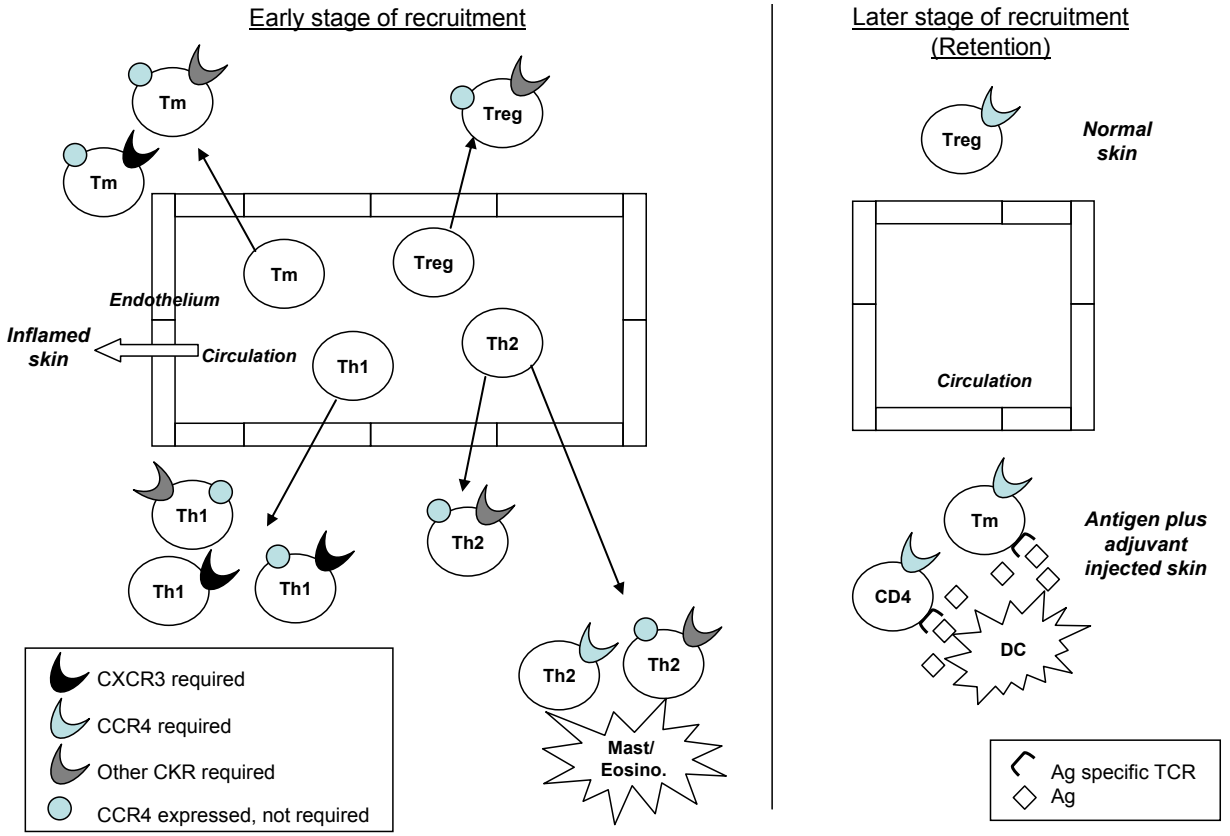

Figure 39. Model for the contribution of CCR4 and CXCR3 in the recruitment of T cells to sites of dermal inflammation. The initial stage of recruitment may involve CCR4, CXCR3 and other CKRs for different T cell subsets. The infiltration of memory CD4 cells requires CXCR3 and other CKRs that are co-expressed with CCR4, e.g. CCR5 or CCR10, but does not depend on CCR4. Also Treg CD4 cell recruitment does not require CCR4 but may rely on other CKRs, e.g. CCR5 or CCR8. The recruitment of Th1 cells is associated with CCR4, but depends on CXCR3 and other CKRs, e.g. CCR5 or CCR10. However, the infiltration of Th2 cells requires CCR4 for part of their migration to ConA and possibly other eosinophils and/or mast cell recruiting lesions, but not to other sites like TLR agonist sites. Other CKRs, like CCR8, is also involved in Th2 cell recruitment. In contrast, in presence of antigen plus adjuvant, antigen presenting cells (e.g. DCs) interact with antigen-specific CD4 (memory) cells via CCR4, so that $CCR4^{+}$ CD4 cells are retained in the inflamed skin during this later phase. Also, CCR4 may mediate Treg cell recruitment to normal skin.

8.2 Contribution of CCR4 and CXCR3 in the Recruitment of T Cells to Joint Inflammation

It is known that Th1 and Tc1 cells are present in the SF of RA patients (17, 295), and that activated CD4 and CD8 T cells migrate across monolayers of fibroblast-like synoviocytes (323). Th1 cell clones were also shown to migrate *in vitro* to supernatants of ST from RA patients (296). However, CKRs that are responsible for the recruitment of activated CD4 and CD8 T cells to the inflamed synovium are not well characterized.

The presence of CCR4 ligands in the SF of patients with arthritis (311, 313), and the increased production of CCR4 ligands by DCs from those patients (314) makes it possible that CCR4 ligands lead to $CCR4^+$ CD4 cell accumulation in the inflamed joints. If so, this would explain the increased proportion of $CCR4^+$ CD4 $CD45RO^+$cells in the SF of RA and JRA patients (309, 311, 313) and the increased CCR4 transcripts in ankles of rats with AA (308). However, it is difficult to deduce the requirement for CCR4 for activated T cell migration, due to the lack of sufficient characterization of these CD4 T cells, as well as the expression of CCR4 on other cells in the tissue, like Treg cells (151), monocytes (161) and endothelial cells (263, 313). So, it is unclear whether CCR4 mediates Th1 or Tc1 cell recruitment to inflamed joints.

In addition, CXCR3 is present on a high proportion of CD4 cells in the SF of RA patients (80-90%) (135, 306). They may be attracted to CXCR3 ligands that are also present in the SF of patients with arthritis (306). The blockade of CXCR3 was shown to reduce the severity of arthritis and to reduce the migration of *in vivo* activated T cells to joint inflammation in rats (310). Still, whether CXCR3 on Th1 cells is required for their migration to joint inflammation is not known.

Some studies examining the contribution of CKRs to T cell recruitment in patients with arthritis have examined CKR expression of T cells in the circulation (309, 312, 313), which may reflect the circulating T cells, rather than those in the tissue. Even when the expression of CXCR3 and CCR4 on T cells in the SF or ST of patients was examined (135, 151, 306, 307, 309, 311), the characterization was not sufficient so as to associate the expression of CKRs with Th1 or Tc1 cells. Given that T cells in the synovium express other CKRs, such as CCR5 (306, 315, 316), the evidence for the requirement of CCR4 or CXCR3 in the *in vivo* migration of type 1 T cells is lacking.

In order to examine the contribution of CXCR3 and CCR4 in the migration of type 1 T cells to joint inflammation, an assay was developed using collagen induced arthritis (CIA) (256, 257). I found that the infiltration of Th1 cells was 3-4 fold higher in the inflamed paws of arthritic mice (Figure 22). However, the accumulation of $CXCR3^{-/-}$ and $CCR4^{-/-}$ Th1 cells in inflamed paws and lymphoid tissues was normal (Figures 23 and 24). Therefore, Th1 cell recruitment to inflamed paws is independent of CCR4 and CXCR3, though they are co-expressed on Th1 cells (Figure 9). This is the first study to examine the requirement of CCR4 or CXCR3 in the recruitment of activated T cells to inflamed joints. Though the ability of *in vivo* activated T cells to transfer disease was blocked in rats using anti-CXCR3 mAb (310), CXCR3 blockade may have inhibited CD8 T cells, which were likely present among the *in vivo* activated T cells, rather than CD4 T cells, which do not require CXCR3 for their recruitment to joint inflammation. Alternatively, CXCR3 blockade may have influenced the lymphoblast migration indirectly by affecting the $CXCR3^{+}$ lining cells of ST (263) or by reducing the migration of neutrophils to inflamed joints (310), rather than directly affecting lymphocytes, which may not express CXCR3 during the inflammatory phase of AA in rats (263).

The recruitment of Th1 cells to joint inflammation may depend on other CKRs, such as CXCR4 and CCR5. This has been suggested by the efficacy of blocking CXCL12, CCL2 or CCL5 in reducing the *in vitro* migration of Th1 cell clones to supernatants derived from ST of RA patients, which was more effective than blocking CXCL10 (296). Also, the reduced migration of $CXCR4^{-/-}$ *in vivo* activated T cells to joints of mice (325) suggested that the expression of CXCR4 on CD4 T cells in SF or ST of patients (323) or mice with arthritis (325) may be associated with their migration to inflamed joints. Also, it is likely that CCR5 mediates the recruitment of Th1 cells, given the presence of CCR5 ligands in the SF of RA patients (306, 315) and the expression of CCR5 on 70-85% of the CD4 cells in the ST or SF of RA patients (306, 315, 316). Indeed, CCR5 is expressed by most $CXCR3^{+}$ CD4 cells in the SF (135, 305) or the ST of RA patients (307), and by ~55% of the $CCR4^{+}$ CD4 cells in the SF of JRA patients (305). Therefore, CCR5 might be relevant in the migration of $CCR4^{+}$ or $CXCR3^{+}$ Th1 cells to inflamed joints.

In addition, I found that the increased accumulation of Tc1 cells to inflamed paws in arthritic mice was CCR4-independent; the recruitment of $CCR4^{-/-}$ Tc1 cells to inflamed paws or lymphoid tissues was normal (Figure 25). It is possible that other CKRs, like CCR5 or CXCR6, contribute to the migration of CD8 T cells to inflamed synovium. This is suggested by the expression of CCR5 on most of the CD8 T cells in ST (307) or SF (135, 315, 316) of RA patients, and by the efficacy of CCR5 blockade in reducing the development of CIA in monkeys (319). CXCR6 is expressed on ~60-80% of the $CD69^{+}$ CD4 and CD8 cells (324, 377) and of cells in SF of RA patients (324). Its contribution is also suggested by its increased expression on CD8 cells of SF than in PB of RA patients and the inhibitory effect of CXCR6 ligand blockade on the development of CIA in mice (378). Still, CKRs required for Tc1 cells to migrate to arthritic joints are unknown.

Moreover, I have examined the effect of CCR4 deficiency on the development of collagen-induced arthritis, and found that $CCR4^{-/-}$ mice developed CIA at reduced incidence (Figure 26A) but normal severity, as assessed by the severity scores, changes in the body weight, weight of inflamed paws, and vascular permeability (Figure 26B-E).

This is the first study to examine the development of CIA in $CCR4^{-/-}$ mice (Figure 26). Although the underlying mechanism is unclear, I would speculate that an effect of CCR4 deficiency on the function of DCs in response to *M. tuberculosis* may lead to the observed reduction in incidence of CIA in $CCR4^{-/-}$ mice. This may influence the T cell and/or B cell responses during the induction of arthritis. DCs were shown to increase their activation markers (e.g. CD80, MHC II) in response to high but not low doses of *M. tuberculosis* (279). In fact, the increased expression of MHC II and CD80 was reported on $CD8\alpha^{+}CD11c^{+}$ DCs in the spleen of arthritic mice and the co-transfer of these DCs with CD4 T cells accelerated the onset of CIA (303). This might have been due to the proliferation and production of IL-17 and IFN-γ by CD4 T cells, which increased in co-cultures with $CD8\alpha^{+}CD11c^{+}$ DCs (303). Also, it may be related to an increase in B cell responses. In other words, the inability of IL-12 treatment to enhance the development of CIA in immunized C57Bl/6 mice (304) may have been linked to the lower levels of CII-specific IgG2a in immunized C57Bl/6 mice that do not develop arthritis (253). It is important to note that $CCR4^{-/-}$ DCs were shown to induce less IFN-γ from WT CD4 cells (171). Therefore, if $CCR4^{-/-}$ DC function is affected, the resultant T cell and/or B cell

responses may be impaired, leading to the delayed onset of CIA in $CCR4^{-/-}$ mice. It is possible that that $CCR4^{-/-}$ DCs do not upregulate of markers of activation (like CD80, MHC II) in response to *M. tuberculosis*. Alternatively, $CCR4^{-/-}$ mice may have a skewed population of DCs, with fewer or less responsive $CD8\alpha^{+}$ DCs, which would affect the development of CIA in $CCR4^{-/-}$ mice. Differences in the DC, T cell or B cell responses were not examined by my work, or by the other studies reporting reduced development of arthritis (310, 319, 378).

It was interesting to find fewer $CD25^{+}$ CD4 cells in the LNs draining site of immunization in collagen-immunized $CCR4^{-/-}$ mice (Figure 27). The reduced activation of CD4 cells, indicated by the reduced number of $CD25^{+}$ CD4 cells, may possibly reflect the effect of $CCR4^{-/-}$ DCs. It should be noted that $CCR4^{+}$ CD4 cells are found in T cell-DC clusters in the inflamed skin and LNs (234). Therefore, the formation of T cell-DC clusters may be impaired in $CCR4^{-/-}$ mice, resulting in fewer activated $CD25^{+}$ CD4 cells in the LNs (Figure 27). In addition to this, as discussed above, the resultant effect on T or B cell responses may be possibly linked to reduced incidence of CIA in $CCR4^{-/-}$ mice. However, since I have not fully identified the $CD25^{+}$ CD4 cells, there may have been less $CD25^{+}$ CD4 Treg cells in LNs of $CCR4^{-/-}$ mice. My work can not exclude this possibility.

In addition, I found that the inflamed paws of $CCR4^{-/-}$ mice recruit fewer Th1 cells than do WT mice, independent of whether transferred cells were from WT or $CCR4^{-/-}$ mice (Figure 28). This effect was not observed with WT Tc1 cells (Figure 29). I speculated an effect of CCR4 deficiency on DC function, T cell and/or B cell responses, which may have possibly influenced the activation of inflamed synovium. However, there are possible explanations; neither T nor B cell responses were affected in mice with conditional CXCR4 deficiency which had a reduced the incidence of CIA (325). The presence of CCR4 on other than activated T cells may contribute to the development of CIA and the recruitment of Th1 cells to joint inflammation. CCR4 may influence the development and/or the production of proinflammatory cytokines by macrophages, as suggested by studies of lethal peritonitis in $CCR4^{-/-}$ mice (173, 187-189). Therefore, it is possible that the CCR4 deficiency reduces the infiltration of monocytes/macrophages, since a third of those in the synovium are $CCR4^{+}$ (161), and their production of pro-inflammatory cytokines (187-189). This may lead to reduced recruitment of Th1 cells

that I observed (Figure 28). It should be noted that CCR4 is also present on 10-30% of the endothelial cells in inflamed synovial tissue (263, 313); the effect of CCR4 deficiency on their function during the development of arthritis is not identified. Therefore, I suggest that the effect of CCR4 on DCs, monocytes/macrophages and/or the endothelium may have influenced the induction of CIA, the number of $CD25^+$ CD4 cells in the draining LNs, and the infiltration of Th1 cells into inflamed paws.

In my opinion, several factors might affect the levels of CKs in the inflamed synovium, and these need to be considered in order to understand the complexity of the T cell migration to joint inflammation. The CK production by the activated synovial fibroblasts/synoviocytes (122, 323) and tissue-infiltrating monocytes or DCs (314) needs to be examined. In addition, the CK production by eosinophils (111), which can be recruited to the joints in the presence of type 2 T cells, as shown in CIA in mice (302) should be determined. Also, the formation of lymphoid aggregates in the inflamed synovium in chronic disease (353, 354) would affect the CKRs required for T cell recruitment and/or localization in inflamed joints.

In order to understand the requirement for CKRs in T cell recruitment to joint inflammation, there are several issues that should be studied in future studies. For instance, the role of CCR4 and CXCR3 in the recruitment of other T cell subsets can not be excluded by my work. It is possible that CXCR3 mediates the migration of memory T cells or activated CD8 T cells. Previous studies demonstrated the presence of $CXCR3^+$ T cells in inflamed joints in rats with AA, and the inhibitory effect of CXCR3 blockade on the recruitment of *in vivo* activated T cells (310). It is also worthwhile to examine the contribution of CCR4 in the recruitment of Treg cells to joint inflammation. The increased presence of CCR4 in ankles of AA rats after the appearance of symptoms (308) may be related to the recruitment of Treg cells in an attempt to control the local inflammation, as shown in CIA or AA models (30, 38), and may explain the presence of CCR4 on most of the Treg cells in SF of patients (151).

In addition, CXCR3 and CCR4 may contribute to other aspects of activated T cell accumulation that I have not examined. For instance, the CCR4- and CXCR3-independent recruitment of Th1 cells to joint inflammation can not be generalized to chronic phase of arthritis. A positive correlation between the number of $CCR4^+$ CD4 cells

and disease severity (312), or duration of arthritis in patients (311) may reflect the role of CCR4 on T cell infiltration during chronic inflammatory response. These CKRs may also contribute to recruitment of T cells during the initial phase of T cell recruitment or later when tertiary lymphoid tissue is present. The levels of CKs present in the joints are likely to vary during the different stages of disease, and may be influenced by the infiltration of innate immune cells, which was not examined.

In contrast to a role in T cell recruitment, an alternative role for CCR4 should be explored. This relates to the exit of T cells from the inflamed joints which may occur when the inflammation is resolving. Some studies reported low expression of CCR4 on T cells in ST or SF of RA patients (307, 315) or rodents with arthritis (263, 317), and an increased presence of $CCR4^{+}$ CD4 cells in the peripheral blood (PB) of RA patients (309, 312). This may implicate the contribution of CCR4 in the exit of T cells from the tissue to the blood. Interestingly, a higher proportion of CD4 cells in SF of JRA patients express CCR4 compared to PB, and these T cells were shown to produce IL-4 rather than IFN-γ (311). Thus, it is possible that, during the resolution of inflammatory response, synovial T cells express CCR4 and either leave to the blood or remain in tissue and produce IL-4.

Moreover, the role of CCR4 on innate immune cells and structural cells should be investigated. Expression of CCR4 on other cells is likely to account for the reduced induction of arthritis and the reduced recruitment of Th1 cells to inflamed paws of $CCR4^{-/-}$ mice. The relevance of CCR4 that is present on third of monocytes/macrophages (161), and on 10-30% of the endothelial cells in inflamed synovial tissue (263, 313) was not examined by my work. Also, I did not examine the effect of CCR4 deficiency on the function of DCs or macrophages, or their influence on the development of T cell and B cell responses during arthritis.

The CKRs that mediate the migration of Th1 and Tc1 cells in inflamed joints requires careful investigation. The involvement of other CKRs that are co-expressed with CCR4 and CXCR3 Th1 cells, like CCR5, should be studied as they may account for the CCR4- and CXCR3-independent migration of Th1 cells. Also, the knowledge base on the CKRs, like CCR5, CXCR3, CXCR6, that may be involved in the migration of Tc1 cells to joint inflammation is incomplete, and needs to be investigated. The presence of several CKRs on infiltrating T cells in the inflamed synovium and possible redundancies between

the different CKRs should be considered, as this may explain discrepancies reported with targeting individual CKRs. For instance, the reduced development of CIA in mice and monkeys after CCR5 blockade (317-319) contradicts the inability of CCR5 blockade to influence disease progression in patients with RA (379). Discrepancies were also reported on the development of CIA in $CCR5^{-/-}$ mice (320, 321), which may reflect the complexity in the functions played by the different CKRs.

Moreover, the broad expression of an individual CKR on different multiple cell types may explain its contribution to different functions. For example, CCR2 may contribute to the inflammatory response as shown by the efficacy of CCR2 blockade in reducing *in vitro* migration of Th1 cell clones to ST cell derived supernatants of patients with RA by 60-75% (296). However, the regulatory role of CCR2 was indicated by studies demonstrating the increased severity of CIA in $CCR2^{-/-}$ mice (321, 327). These differing functions may be apparent during different phases of arthritis, as illustrated by opposing effects of CCR2 blockade during initiation *vs*. during later phases of CIA (326).

Limited evidence is available for the requirement of different CKRs in the migration of activated T cells into the inflamed joints or out of the tissue into the circulation. From my studies and those of others (151, 296, 310, 317-319, 324, 326, 327, 378), one may postulate a model in which CKRs contribute to the recruitment of different T cell subsets, and would be affected by the composition of the inflamed tissue (Figure 40). The infiltration of Th1 cells into the inflamed joints does not require CXCR3 or CCR4, but may involve other CKRs, like CCR5, CXCR4 or CXCR6 (296, 306, 315-319, 324, 325). The migration of Tc1 and into inflamed paws is also CCR4-independent, but may utilize other CKRs that they express, like CXCR3, CCR5 or CXCR6 (135, 307, 315, 316, 324, 377, 378). It is possible that memory CD4 cell migration require CXCR3 and/or other CKRs co-expressed on $CXCR3^{+}$ cells (310).

Also, I may speculate that the effect of CCR4 on the function and/or infiltration of innate immune cells, such as monocytes/macrophages that express CCR4 (161, 187-189), or DCs which interact with $CCR4^{+}$ T cells (234). This would in turn influence in the induction of arthritis and the activation of the synovium. The presence of CK-producing innate immune cells may enhance the recruitment of T cells (e.g. Th1 cells) and affect the CKRs requirement for T cells. It is possible to envision that the presence of tertiary

lymphoid structures in chronic arthritis would warrant the involvement of other CKRs in the recruitment of T cells, like CCR7, CXCR3 or CXCR4.

In contrast, the high proportion of circulating $CCR4^{+}$ CD4 cells observed in patients with RA (309, 312), may suggest the contribution of CCR4 in the exit of T cells from tissue during later stages of the disease, when the local inflammatory response is subsiding. The recruitment of Treg cells into joint inflammation may involve CCR4 and/or CCR2 (151, 326, 327), and could contribute to the downregulation of the inflammatory process in arthritis.

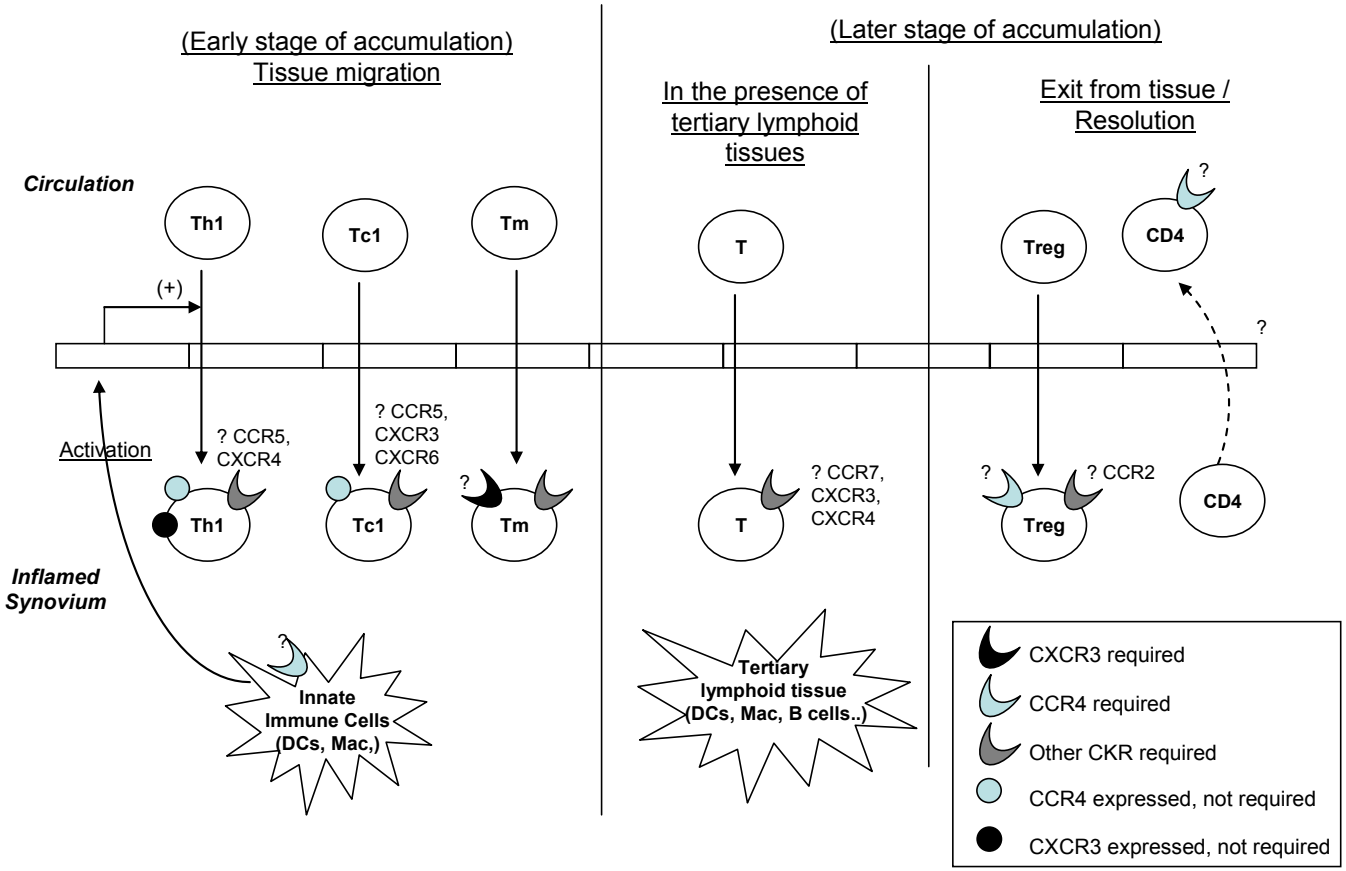

Figure 40. Model for the contribution of CCR4 and CXCR3 in the recruitment of T cells to joint inflammation. Different CKRs may be involved in the recruitment of T cells; Th1 cells express CCR4 and CXCR3 but are not required for this process. Th1 cells may use other CKRs, like CCR5, CXCR4 or CXCR6. Tc1 cell infiltration does not involve CCR4, but may use CXCR3, CCR5 or CXCR6. Memory CD4 cells (Tm) may use CXCR3 or other CKRs. The presence of CCR4 may influence the function and/or infiltration of innate immune cells, as macrophages or DCs, which would influence the activation of the synovium and the recruitment of Th1 cells. During later stage of accumulation, other factors may influence T cell recruitment. In the presence of tertiary lymphoid structures in chronic arthritis, T cell recruitment may involve CKRs, like CCR7, CXCR3 or CXCR4. During the resolution of inflammatory response, Treg cell recruitment may use CCR4 and/or CCR2, while other CD4 cells may possibly exit from the tissue and express CCR4.

8.3 Contribution of CCR4 and CXCR3 in the Homing of Th1 Cells to Lymph Nodes Draining Sites of Immunization

The CKRs required for T cell homing to reactive LNs is not understood. LNs draining sites of hapten-sensitization, antigen-immunization, or infection have increased expression of selectins (361), and ligands for CXCR3 and CCR4 (169, 196, 338, 339), but the contribution of CXCR3 and CCR4 in Th1 cell homing into draining LNs is not clear.

Most of the literature on T cell homing to lymphoid tissues suggests a requirement for L-selectin, CXCR4 and CCR7 (94, 328-330). The homing of T cells into the LNs was suggested to involve CCR7 and the egress of T cells may involve a downregulation of CCR7 with a gain in S1P responsiveness (380). Also, the migration of developing T cells through the thymus involves sequential changes in the expression of CXCR4, CCR9, CCR4, CCR7 and S1P receptor (334, 335). The role of CCR7 and CCR9 in the recruitment of cells to the thymus was highlighted by studies in $CCR9^{-/-}$ $CCR7^{-/-}$ mice, which have significantly fewer DN cells compared to DP cells (381).

However, this may not be the case for all CD4 cells, as shown by the homing of memory CD4 cells to draining LNs after L-selectin blockade (328). The expression of CLA and CCR7 by memory cells (136) may have contributed to this process, since the homing of antigen-specific CD4 T cells is reduced in E-selectin deficient mice (95). In addition, Th1 cell recruitment to draining LNs was found to only partially require CCR7 (344), so it is possible that other CKRs are involved in this process. CXCR3 ligands that are found on TNF stimulated endothelial venules may induce adhesion of monocytes (336), but its role for T cell migration is unclear. Also, the expression of CCR4 on thymocytes (138, 382) is thought to be related to the selection process (382), but its role in the recruitment of T cells in the lymphoid tissues is uncharacterized.

In order to examine the contribution of the CXCR3 and CCR4 in the migration of Th1 cells to reactive LNs, it was necessary for a homing assay to be developed. Increased numbers of ESL^{+}, $CXCR3^{+}$ and $CD25^{+}$ CD4 cells were observed in LNs draining the sites of OVA-CFA immunization compared to OVA-Alum immunization (Figure 30), and this increase was apparent after 3 days until 6 days after immunization (Figure 31). I

also found that labeled i.v. transferred Th1 cells could be recovered from the draining LNs in increasing numbers up to 72 h post-i.v. (Figure 32).

My studies show that CXCR3 deficiency reduced the accumulation of Th1 cells into draining LNs (Figure 33). CXCR3 ligands are produced by TNF-stimulated HEV (336) or by mature DCs (339), and they are increased in LNs draining sites of immunization or infection (169, 196, 338, 339). The initial accumulation of Th1 cells in draining LNs, during the first 24 h, was modest over control LNs and did not involve CXCR3 (Figure 33). Instead, the initial homing of Th1 cells into draining and control LNs may require CCR7, as the homing of $CCR7^{-/-}$ Th1 cells into LNs is reduced at 3 h after i.v. injection (344). In contrast, the increased accumulation in draining LNs at 72 h is mostly dependent on CXCR3 (Figure 33). This is accordance with studies demonstrating the efficacy of CXCR3 blockade in reducing the number of *in vivo* activated T cells in the LNs (339) and metastasis of $CXCR3^{+}$ tumor cell lines to the LNs in mice (338). It is interesting to note that CXCL12 increases the *in vitro* responsiveness of DCs to CXCR3 ligands (364, 383). If this applies to Th1 cells, then I would speculate that presence of other CKs in the LNs may increase the dependence on CXCR3 for Th1 cell homing and explain why it was observed only at 72 h post-i.v. injection.

In contrast to CXCR3 deficiency, I found that the CCR4 deficiency resulted in the increased accumulation of Th1 cells in the draining LNs at 72 h post-i.v. injection (Figure 34) and after they were introduced subcutaneously (Figure 37). Though most of the WT most of the Th1 cells recovered from the draining LNs at 72 h post-i.v. injection were $CCR4^{-}$ (Figure 36), the CCR4 deficiency increased the accumulation of several subpopulations of Th1 cells, namely ESL^{+}, PSL^{+} or $CXCR3^{+}$ Th1 cells (Figure 35). One of the possible explanations for the increased accumulation of $CCR4^{-/-}$ Th1 cells in the draining LNs might have been related to their resistance to apoptosis. However, apoptosis was equivalent for WT and $CCR4^{-/-}$ Th1 cells, as assessed after IL-2 withdrawal by trypan blue exclusion and the binding of Annexin V and 7AAD. (Figure 38). Hence, CCR4 deficient Th1 cells have increased the accumulation in the draining LNs, and this does not appear to be related to apoptosis as assessed *in vitro*. I did not examine other inducers of apoptosis, such as tumor necrosis factor related apoptosis inducing ligand

(TRAIL) that is expressed by DCs and neutrophils when stimulated with IFN-γ and/or mycobacterial components (384, 385).

I observed the increased accumulation of $CCR4^{-/-}$ Th1 cells in the draining LNs whether they were introduced via bloodstream or subcutaneous injection (Figures 34 and 37). The mechanism behind this is unclear, but I can postulate a few explanations. WT Th1 cells that are recovered from the LNs at 72 h have downregulated their surface expression of CCR4 and CXCR3 compared to the transferred cell population (Figure 36). The downregulation of CXCR3 on $CXCR3^{+}$ CD8 T cells activated *in vivo* was also observed in a model of GvHD (146), and the downregulation of CCR4 trancript levels was shown in MLNs draining peritonitis (188). However, more $CCR4^{-/-}$ Th1 cells express CXCR3 (~71%) than WT Th1 cells (~47%) at 72 h post-i.v., but the reason why $CCR4^{-/-}$ Th1 cells resist CXCR3 downregulation is unclear. Heterologous desensitization on T cells was reported for CCR5 and CXCR4; CCR5 ligands reduced the late stage chemotaxis of T cells to CXCR4 ligands (386). This may occur between CCR4 and CXCR3, such that CCR4 ligands desensitize and reduce the surface expression of CXCR3 on WT Th1 cells. This would not occur for $CCR4^{-/-}$ Th1 cells; resulting in the presence of CXCR3 on higher proportion of $CCR4^{-/-}$ Th1 cells than on WT cells.

Increased accumulation of $CCR4^{-/-}$ Th1 cells could be mediated by CXCR3, based on the reduced homing of $CXCR3^{-/-}$ Th1 cells (Figure 33), and on the ability of $CXCR3^{+}$ tumor cells to migrate via afferent lymphatics (338). A higher proportion of $CCR4^{-/-}$ Th1 cells express CXCR3, which may explain their increased accumulation compared to the WT Th1 cells. In addition to CXCR3, I cannot exclude the participation of other CKRs, like CCR5, in the accumulation of $CCR4^{-/-}$ Th1 cells. The increase in CD4 cells found in the LNs draining CHS in $CCR4^{-/-}$ mice coincided with the increased transcript levels of CCR5 (195). So, CCR5 may contribute to this process, as does CXCR3, since the expression of both CCR5 and CCR4 is induced in reactive LNs in mice (169).

Also, I postulate that the downregulation of CCR4 and/or CXCR3 on WT Th1 cells may be associated with increased responsiveness to egress signals. The downregulation of these CKRs is altered in $CCR4^{-/-}$ Th1 cells so that they may be less susceptible to egress signal and they remain in the LNs longer. One of the signals thought to be related to egress from LNs is Sphingosine-1-phosphate (S1P) (387). In fact, when

its receptor, S1P1, is downregulated by the FTY720 agonist, the accumulation of T cells in LNs was found to be increased (388, 389). Adoptively transferred $S1P1^{-/-}$ CD4 thymocytes were found to accumulate in LNs, but not recirculate through lymph or blood (390). Since the levels of S1P in the serum and lymph are known to be more significant than in the tissues (391), the cyclical modulation of S1P responsiveness by T cells is thought to be relevant in egress from LN.

Interestingly, the reciprocal relationship between responsiveness to S1P and CCR7 has been reported. T cells activated *in vivo* for 1 day downregulate the S1P1, and increase their responsiveness to CCR7 ligands. After 3 days of activation, they express less CCR7 and regain responsiveness to S1P (390). The downregulation of CCR7 by activated T cells and the responsiveness to S1P allows for egress from LNs (380). Also, S1P was shown to enhance or inhibit chemotaxis in a dose dependent manner; the exposure of CD4 T cells to S1P at low dose (0.1-1 μM) enhances the chemotaxis to CCL21, CXCL4 and CCL5, while at higher concentrations the S1P resulted in reduced chemotaxis to these CKs (392). Though FTY720 treatment, which reduces surface expression of S1P1, did not influence the number of transferred antigen-specific Th1 cells in the peripheral blood (393), it is important to consider the competing signals induced by CKs, which were not examined in this study (393).

It is possible that the downregulation of CXCR3 and/or CCR4 by Th1 cells in the LNs is associated with an increase in the expression of S1P1 and their egress from LNs. The evidence for this hypothesis is circumstantial; mainly based on reciprocal relationship between CCR7 and S1P (390), and the sequential expression of CCR4 and S1P on thymocytes (382, 387). It is interesting to note that developing thymocytes express CCR4, then later they lose CCR4 and become $S1P1^{+}$, and this appears to be negatively linked to CD69 expression; $CD4^{+}$ $CD8^{-}$ cells that are $CD69^{+}$ chemotax to CCL22, and do not express S1P1, while after developing into $CD69^{-}$ cells they no longer chemotax to CCL22 and gain responsiveness to S1P (382, 387). CD69 is shown to interact with S1P1, and facilitate its internalization and degradation, so the function of S1P and egress from LNs is inhibited in the presence of CD69 (394). In my studies, WT Th1 cells recovered from LNs have downregulated CXCR3 and CCR4 and are almost all $CD25^{-}$ compared to the injected cells, predicting that these cells may gain the ability to

upregulate S1P1. I have not examined whether the downregulation of these CKRs is linked to an upregulation of S1P1. If this holds true, CCR4$^{-/-}$ Th1 cells do not downregulate CKRs as readily, and possibly do not upregulate S1P1 or egress from the LN as quickly as do WT Th1 cells.

In addition, defects in other signals were shown to be related to an increased T cell accumulation in LNs. So, if CCR4 deficiency influences the function of these molecules, then an increased T cell accumulation may result. For instance, CTLA-4$^{-/-}$ Th1 cells were shown to have reduced *in vitro* migration to ligands of CXCR4 and CCR7, but normal migration to ligands of CXCR3 (395). The interaction of CD4 cells with mature DCs increases the expression of CTLA-4, which in turn enhances the recruitment of Th1 cells to LNs draining sites of OVA-CFA immunization (395). Other studies reported that the deficiency of Rac-specific guanine exchange factors (DOCK2 or Tiam) lead to an increased retention of T cells in LNs, as determined 24 h after entry was blocked using anti-L-selectin (396, 397). It is not clear if impaired function of these molecules increased the T cell accumulation or if it was caused by the reduced *in vitro* migration of these T cells to S1P and/or CCR7 ligands (396, 397). All in all, these studies show that the dysregulated signaling of other molecules (i.e. increased for CTLA-4 or impaired for DOCK2 and Tiam) may contribute to the increased accumulation of T cells in LNs. This phenotype is similar to what I observed with CCR4$^{-/-}$ Th1 cells; may be due to an effect for CCR4 deficiency on the functionality of these molecules, but my work did not examine this aspect.

In conclusion, the literature related to the migration of T cells to lymphoid tissue provides more questions than answers. I found that CXCR3 mediates most of the increased accumulation of Th1 cells to reactive LNs, while CCR4 appears to play an opposing role. Future studies should address several aspects to overcome the limitations of this study and to clarify the role of CKRs in this process. The CKRs required for T cell migration will likely vary depending on the T cell subset. I only examined Th1 cells, and it is possible that other cell types do not rely on CXCR3 to the same extent (195).

Moreover, I have only examined LNs draining a site of CFA-immunization, but the infiltration of T cells into reactive LNs is likely influenced by the type of stimuli. LNs draining chronic inflammatory sites, or those draining sites of type 2 related stimuli, such

as parasitic infections, are likely to have a different CK profile. The levels of CKs present in the LNs may also vary as the inflammatory response develops; tertiary lymphoid tissues may have different CKs according to their cellular composition. The relative contribution of other CKRs, e.g. CCR7, CXCR4, CCR5, should be examined since their ligands are known to be present in reactive LNs. It is also important to consider whether these CKRs contribute to homing or localization of T cells in the LNs.

In addition, studies that examine single time points cannot discern the entry and retention of cells into the LNs. The requirements for T cells to enter may differ in different phases, as shown by the CXCR3$^{-/-}$ Th1 cells, which accumulate normally at 24 h, but not at later time points (Figure 33). Likewise, it is unclear whether the cells that enter the LNs after 24 h require different CKRs than those retained in the LNs during that time. It would be of interest to compare T cells that are consequentively injected into animals with activated LNs to determine if they require different CKRs. This can be achieved by injecting differentially labeled T cells at several time points and then following their recruitment to reactive LNs, or by examining the antigen-specific CD4 T cells as they proliferate in the LNs over time. It is also important to consider that the kinetics of accumulation of T cells to LNs would differ according to the route of administration, as shown by my studies using i.v. and s.c. injections (Figures 32 and 37).

It is also of interest to examine the reason why CCR4$^{-/-}$ Th1 cells have increased accumulation in reactive LNs. As mentioned above, I have postulated that CCR4 signalling desensitizes CXCR3, and since CCR4$^{-/-}$ Th1 cells resist downregulation of CXCR3, I think that their increased expression of CXCR3 leads to their increased accumulation. In addition to examining this aspect, the involvement of other CKRs like CCR5 in increasing the recruitment of CCR4$^{-/-}$ Th1 cells should be determined. Also, it is of interest to examine the consequences of resisting CKR downregulation by CCR4$^{-/-}$ Th1 cells in terms of responsiveness to egress signals, like S1P. A reciprocal relationship, such as observed with S1P and CCR7, is not unlikely given their sequential expression on thymic CD4 cells. Studies should also examine the possible effect of CCR4 deficiency on other molecules, such as CTLA4 and Rac activators, which may have indirectly influenced the accumulation of CCR4$^{-/-}$ Th1 cells in the LNs.

The limited literature available on the contribution of CKRs in the recruitment of T cells to LNs, in addition to the postulated hypotheses and the results of my studies, suggest a model shown in Figure 41. CXCR3 mediates part of the accumulation of Th1 cells, but it does not contribute to the initial recruitment, which may be mediated by CCR7 (344). I cannot exclude the contribution of other CKRs whose ligands are present in reactive LNs like CCR5 (169, 195). CCR4 is not required for the Th1 cell accumulation in LNs, but I postulate that its downregulation may desensitize CXCR3. T cells that resist CXCR3 downregulation are retained and have increased accumulation in the LNs, as do $CCR4^{-/-}$ Th1 cells. I also postulate that downregulation of CCR4 and CXCR3 (and also CD25) by Th1 cells may allow for an increased responsiveness to S1P, as shown with CCR7 (380). In addition, it is possible that dysregulated function of other molecules, such as CTLA-4 or Rac activators (395-397), caused the increased accumulation of $CCR4^{-/-}$ Th1 cells.

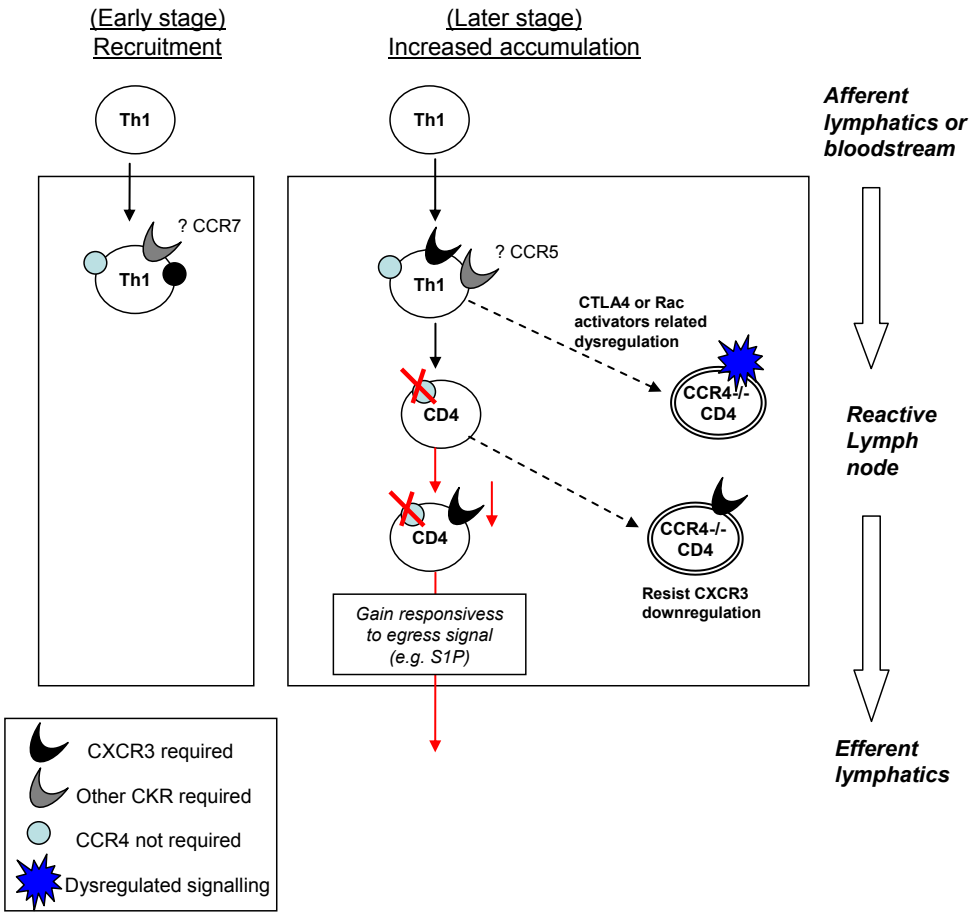

Figure 41. Model for the contribution of CCR4 and CXCR3 in the homing of T cells to draining LNs. The initial recruitment of Th1 cells does not depend on CXCR3 or CCR4, but may be mediated by other CKRs, like CCR7 (344). At later stage, the accumulation of Th1 cells is increased into the reactive LNs and this is mediated by CXCR3, not CCR4. I cannot exclude the contribution of other CKRs, like CCR5, if their ligands are present (169, 195). Th1 cells downregulate CCR4 completely and CXCR3 partially, which I postulate may allow for an increased responsiveness to egress signals, as shown with CCR7 and S1P (380). $CCR4^{-/-}$ Th1 cells resist CXCR3 downregulation, so they either have increased accumulation or are retained longer in the LNs. Also, if events that involve signaling of CTLA-4 or Rac activators are dysregulated, the homing of Th1 cells would be increased (395-397). I speculate that this may be related to the increased accumulation of $CCR4^{-/-}$ Th1 cells.

8.4 Implications for Targeting CCR4: Deficiency and Blockade Studies

The contribution of CCR4 to the recruitment of T cells into different tissues, to the function of innate immune cells and to the development of inflammation was researched in order to evaluate the efficacy of targeting CCR4 to control inflammatory responses. Even though some of these aspects have been examined in my studies and in the literature (summarized in Table 16), I think that the contribution of CCR4 to the functions of immune cells is not fully understood. In my opinion, further research is needed before CCR4 antagonism can be successfully applied to control specific inflammatory diseases.

My studies have examined the recruitment of $CCR4^{-/-}$ T cells to inflammatory sites in the skin, joints and LNs. The development of CIA in $CCR4^{-/-}$ mice and the ability of their inflamed paws to recruit T cells was also determined. In general, all of the memory CD4, Treg cells, type 1 T cells, and most of the type 2 T cells were recruited to dermal inflammation in a CCR4-independent manner. Similarly, the recruitment of type 1 T cells to inflamed paws and LNs draining immunization sites was not dependent on CCR4. In fact, my studies show that the deficiency of CCR4 leads to the increased accumulation of some of the T cells in inflammatory sites in the skin and LNs, namely for the memory CD4 cells and Treg cells in the skin and Th1 cells in LNs.

My studies and those of others suggest that the deficiency of CCR4 does not necessarily translate into a reduced inflammatory response. Though CCR4 ligands are increased in inflammatory conditions in the skin (110, 112, 196, 234), joints (311, 313), lungs (119, 174, 176, 178), and autoimmune diseases (179), the deficiency or blockade of CCR4 in mice does not change the severity of collagen-induced arthritis (Figure 26), or lung inflammation (172, 173). In fact, it lead to an increased infiltration of CD4 cells in the skin (195) or an increased resistance to lung inflammation (166, 167, 190), and lethal peritonitis (173, 187-189). The function of the CCR4 expressed on T cells, innate immune cells and structural cells is not fully understood. Since these cells modulate inflammatory process, it is difficult to envision successful application of a generalized blockade of CCR4 for precise control of the immune response.

Most of the studies that report an inhibitory effect for CCR4 deficiency on T cell accumulation are related to antigen-specific CD4 or Th2 cells in skin and lungs (170,

246, 247). It is possible that CCR4 does not mediate their migration to the inflamed tissues, instead CCR4 may be involved in their interaction with antigen and APCs and thus result in their accumulation / retention. Indeed, CCR4 expression is demonstrated in tissues with chronic inflammation (90, 229, 233, 308, 311), where T cell accumulation may occur over prolonged periods. Thus, CCR4 expression may relate to the events that occur after the T cell migration into the tissue.

I found that CCR4 expression is not required for activated T cells to migrate to joint and dermal inflammation, except for part of the type 2 T cell recruitment into eosinophil-recruiting lesions (Figures 20C and 21C). Increased accumulation of $CCR4^{-/-}$ T cells is a recurring theme; more $CCR4^{-/-}$ Th1 cells in LNs draining site of immunization (Figures 34 and 37), more $CCR4^{-/-}$ *in vivo* activated CD4 cells in LNs draining site of OXA sensitization (195), more $CCR4^{-/-}$ memory CD4 cells and Treg cells in ConA (Figures 15-17), and more $CCR4^{-/-}$ CD8 T cells in cardiac allografts (180). Still, the migration of Th1 cells was reduced in inflamed paws in $CCR4^{-/-}$ recipients (Figure 28), thus highlighting the contribution of CCR4 on non-T cells in modulating the inflammatory response in collagen-induced arthritis.

The function of CCR4 on innate immune cells should not be underestimated. CCR4 is present on CD4 cells within T cell-DC clusters (234), thus CCR4 may be involved in the interaction between CD4 and DCs. In addition, the development of inflammatory responses in peritoneum and lungs of $CCR4^{-/-}$ mice have suggested a role for CCR4 in the development and/or the function of innate immune cells, namely DCs and macrophages (171, 187, 189). $CCR4^{-/-}$ DCs were shown to induce less IFN-γ production by WT CD4 cells (171), while activated $CCR4^{-/-}$ macrophages were found to have an altered phenotype (167, 187, 189), produce less pro-inflammatory cytokines (189) and/or are retained in the peritoneum (173). The effect of CCR4 deficiency on innate immune cells was highlighted by the resistance of $CCR4^{-/-}$ mice to models of lethal peritonitis (173, 188, 189). It was also indicated by the inability of WT CD4 cells to reconstitute the impaired formation of type 1 granulomas in lungs or the production of IFN-γ, IL-5 and IL-13 in draining LNs of $CCR4^{-/-}$ mice (171). It is of interest to note the expression of CCR4 on the eosinophils (158), NK cells (163), dermal endothelial cells

(165) and keratinocytes (164), but the functionality of CCR4 expressed by these cells is unclear.

In viewing the literature related to CCR4, one can appreciate the attention gained by $CCR4^{+}$ Treg cells. Treg cells are present around tumors (183, 398) and DCs (399), which may produce CCR4 ligands (183, 185). $CCR4^{-/-}$ Treg cells have reduced accumulation in grafts (154) and in normal skin and lungs of mice (156). However CCR4 does not mediate their migration to inflamed skin; $CCR4^{-/-}$ Treg cells have increased accumulation in inflamed skin (Figure 17) (195). The increased accumulation of $CCR4^{-/-}$ Treg cells in draining LNs during OXA-CHS (195) and later phases of colitis (155) suggests that they may be still capable of downregulating the immune response, especially since they maintain their normal suppressive ability as observed *in vitro* (155, 156) and *in vivo* (154).

Studies have examined the efficacy of CCR4 antagonists, but have used general readouts to demonstrate proof-of-principle. These included the reduction in ear swelling (400), the increased levels of antigen-specific antibody IgG or the increased proliferation or IFN-γ production by splenocytes from immunized mice cells in response to antigens (401, 402). CCR4 antagonists were used to target CCR4 expressing tumors *in vitro* and *in vivo* (184, 186), however when this was followed for longer periods, the rate of mortality was only reduced (186), and CCR4-independent tumor regrowth were observed (184). Therefore, the successful application of CCR4 antagonism requires further knowledge with regards to the function of CCR4 on different T cells for their migration or rentention, as well as the their involvement in different phases of the inflammation. The possible role of CCR4 in the development and function of innate immune cells should also be considered.

Table 16. Summary of literature related to the contribution of CCR4 to immune cell functions.

Immune functions (reference)	**Is CCR4 required?**
Recruitment of T cells to tissues.	
Recruitment of Th1 or Tc1 cells to inflamed skin (TLR agonists, ConA, DTH) (Chapter 5)	NO
Recruitment of Th2 or Tc2 cells to inflamed skin (TLR agonists) (Chapter 5)	NO
Recruitment of Th2 or Tc2 cells to inflamed skin (ConA) (Chapter 5)	YES
Recruitment of antigen-specific Th2 cells to antigen-injected sites in lungs (170)	YES
Recruitment of Th1 or Tc1 cells to inflamed joints (CIA) (Chapter 6)	NO
Recruitment of Th1 cells to draining LNs (CFA immunization) (Chapter 7)	NO
Recruitment of memory CD4 cells to inflamed skin (TLR agonists, ConA, DTH) (Chapter 5)	NO
Recruitment of antigen-specific CD4 cells to antigen-injected sites in skin (6-10 day period) (246, 247).	YES
Recruitment of CD4 cells to CHS in skin (195)	
Recruitment of Treg cells to inflamed skin (TLR agonists, ConA, DTH) (Chapter 5)	NO
Recruitment of Treg cells to CHS in skin (195) or later phase of colitis (155)	NO
Recruitment of Treg cells to islet allografts (154)	YES
Recruitment of Treg cells to normal skin and lungs (156)	YES
Other functions of T cells.	
In vitro production of IFN-γ from Th1 cells (Chapter 5)	NO
In vitro production of IL-4 from Th2 cells (Chapter 5)	YES
Suppressive activity of Treg cells (154-156).	NO
Functions of innate immune cells.	
DC function (*in vitro* induction of IFN-γ from CD4 cells) (171)	YES
Macrophage development and function (production of proinflammatory cytokines) (187, 189)	YES
Development of inflammatory responses.	
Susceptibility to lethal peritonitis (167, 173, 187-189)	YES
Severity of arthritis (CIA) (Chapter 6)	NO
Incidence of arthritis (CIA), number of CD4 $CD25^+$ cells in draining LNs and number of Th1 cells in inflamed paws (CIA) (Chapter 6)	YES
Development of CHS in skin (195)	NO
Development of inflammatory response in lungs: NO (172, 173) or YES (167, 171)	NO/YES

8.5 Concluding Remarks

The expression of CXCR3 and CCR4 on different T cell subsets warrants the investigation for their contribution to the recruitment of T cells to inflammatory tissues. My studies have demonstrated that CXCR3 deficiency mediates part of the migration of unstimulated CD4 cells, $CCR4^+$ and $CCR4^-$ Th1 cells and Tc1 cells to inflamed skin (Figures 11-14). CXCR3 also mediates the accumulation of Th1 cells to LNs draining a site of CFA immunization, but does not mediate their infiltration into inflamed joints (Figures 23 and 33). This supports studies showing that CXCR3 blockade reduces the migration of T cells to dermal inflammation (93), but suggests that the effect of CXCR3 blockade on reducing the migration of *in vivo* activated T cells to inflamed joints (310) is not related to a direct effect on Th1 cells.

In contrast, CCR4 does not mediate the homing of memory CD4 or Treg cells to inflamed skin, but CCR4 deficiency instead resulted in increased accumulation especially into ConA sites (Figures 16 and 17). CCR4 mediated part of the migration of Th2 cells and Tc2 cells to ConA sites, but not other sites of dermal inflammation (Figures 20 and 21). CCR4 did not mediate the migration of Th1 or Tc1 cells to the inflamed skin or inflamed paws of arthritic mice (Figures 20, 21, 24 and 25). However, CCR4 deficiency resulted in the increased accumulation of Th1 cells, in particular those that are $CXCR3^+$, ESL^+, PSL^+, in LNs draining a site of CFA immunization whether they were administered i.v. or s.c. (Figures 34, 35 and 37).

My studies on the contribution of CCR4 in the migration of T cells to inflammatory sites in the skin, joints and draining LNs, demonstrated four themes in association with CCR4 deficiency:

1) No effect on the migration of Th1 and Tc1 cells to inflamed skin or inflamed paws (Figures 20, 21, 24 and 25). Other studies reported the infiltration of *in vivo* activated CD4 cells in CHS was unhindered by CCR4 deficiency (195, 237). It is likely that CXCR3 and other CKRs mediates the infiltration of Th1 and Tc1 cells, but the role of CCR4 on type 1 T cells remains uncharacterized.

2) An increased accumulation of CD4 cells in inflammatory sites in the skin and draining LNs, as observed for memory CD4 cells and Treg cells at 20 h in the skin, and for activated Th1 cells at 72 h in the draining LNs (Figures 16, 17, 34 and 37).

Interestingly, CCR4 deficiency has been associated with an exacerbated accumulation of CD4 cells and Treg cells in CHS skin reactions and the associated draining LNs (195). This contradicts the reduced accumulation of $CCR4^{-/-}$ memory CD4 cells to antigen-injected sites over 6 days (246, 247). Thus, it is possible that CCR4 may does not mediate the recruitment of memory CD4 cells to the inflamed skin site, and instead $CCR4^{-/-}$ CD4 cells are not as efficient in leaving the inflamed skin site. However, when antigen specific $CCR4^{+}$ CD4 cells interacts with APCs in the local tissue, CCR4 is involved in the retention of these cells, so that $CCR4^{-/-}$ CD4 cells are not retained as well in antigen injected sites.

3) A selective reduction in the infiltration of Th2 and Tc2 cells to ConA sites in the skin (Figures 20 and 21). It is possible that this is related to the eosinophil-recruiting ability of ConA (203). Th2 cells can migrate to CCR4 ligands in the skin (145, 245), and $CCR4^{-/-}$ Th2 cells had reduced migration to allergic lung inflammation (170). It is interesting note that $CCR4^{+}$ CD4 cells are correlated with the severity of eosinophil recruiting diseases, such as eosinophilc pneumonia (174), and atopic dermatitis (110 , 229, 232).

4) There is a role for CCR4 on non T cells, such as innate immune cells or stromal cells, as highlighted by the studies of CIA. $CCR4^{-/-}$ mice had reduced incidence of CIA, reduced number of $CD25^{+}$ CD4 cells in the draining LNs, and reduced recruitment of Th1 cells in the inflamed paws (Figures 26-28).

The contribution of CKRs, like CCR4 and CXCR3, should be further examined. Investigations into the role of CCR4 and CXCR3 on different T cell subsets, innate immune cells and structural cells should be considered. Also, the effect of CCR4 on T cells migration *versus* retention or exit from the tissue should be examined. It is also worthwhile to assess the contribution of these CKRs in the accumulation of T cells during different stages of the inflammation in different tissues.

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