Targeting inflammation
with
autoantigen-specific T cells

Autoantigeen-specifieke T cellen in de aanval tegen ontsteking
(met een samenvatting in het Nederlands)

Proefschrift

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CONTENTS

ABBREVIATIONS

-/-	homozygous knockout
Ag	antigen
AIO	assistent in opleiding
-APC	allophycocyanin-labeled
APC	antigen presenting cell
BCR	B cell receptor
B_{eff}	effector B cell
BM-DC	bone marrow derived dendritic cell
B_{reg}	regulatory B cell
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary deoxy ribonucleic acid
CDR	complementary-determining region
CFSE	5-(and-6)-carboxyfluorescein diacetate succinimidyl ester
CIA	type II collagen-induced arthritis
ConA	concavalin A
CPM	counts per minute
DC	dendritic cell
DDA	dimethyl-dioctadecyl-ammoniumbromide
EAE	experimental autoimmune encephalomyelitis (murine multiple sclerosis model)
ELISA	enzyme linked immuno sorbent assay
FACS	fluorescence activated cell sorter
F_c	constant and crystallizable fragment of antibody
F_cR	F_c receptor
FCS	fetal calf serum
-FITC	fluoresceine isothyocyanate labeled
Foxp3	forkhead box protein P3
FSC	forward scatter
GFP	green fluorescent protein
HLA	human leukocyte antigen
hPG	human cartilage proteoglycan
-HRP	horse radish peroxidise labeled
HSP	heat shock protein
ICOS	inducible costimulator
IFN-γ	interferon-γ
Ig	immunoglobulin (antibody)
IL-	interleukin-
IL-1RA	IL-1β receptor antagonist
i.p.	intraperitoneal
IRES	interribosomal entry site
i.v.	intravenous
LN	lymph node
LPS	lipopolysacharide
LTR	long terminal repeat
mAb	monoclonal antibody
MACS	magnetic adsorbent cell sorter
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mPG	mouse cartilage proteoglycan
mRNA	messenger ribonucleic acid
MSCV	mouse stem cell virus
OVA	ovalbumin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
-PE	phycoerythrin labeled
-PerCP	peridinin-chlorophyll-protein labeled
PG	proteoglycan
PGIA	proteoglycan-induced arthritis
PRR	pattern recognition receptor
RA	rheumatoid arthritis
rGM-CSF	recombinant granulocyte-macrophage colony-stimulating factor
SCID	severe combined immunodeficiency
SEM	standard error of the mean
SI	stimulation index
SSC	side scatter
T_c	cytotoxic T cell ($CD8^+$)
TCR	T cell receptor
T_{eff}	effector T cell
Tg	transgenic
T_{GFP}	GFP-transduced ($CD4^+$) T cell
TGF-β	transforming growth factor-β
T_h	T helper cell ($CD4^+$)
T_{IL-10}	IL-10/GFP-transduced ($CD4^+$) T cell
T_{IL-1RA}	IL-1RA/GFP-transduced ($CD4^+$) T cell
T_{IL-4}	IL-4/GFP-transduced ($CD4^+$) T cell
TLR	Toll like receptor
TNF-αR-Ig	TNF-α receptor-Ig
TNF-α	tumor necrosis factor-α
T_{R1}	regulatory T cell type 1
T_{reg}	regulatory T cell
T_{resp}	responder/responding T cell
$T_{TNF-R-Ig}$	TNF-R-Ig/GFP-transduced ($CD4^+$) T cell
WT	wild type

CHAPTER 1

General Introduction

THE IMMUNE SYSTEM

A sophisticated army defending the body from unwanted intruders

In everyday life the body has to deal with many different pathogenic micro-organisms, toxins or possible growth of malignant tumor cells. An army of cells fighting these harmful and redundant materials to defend the body comprises one of the most complex organs: the immune system. This defense system contains a broad repertoire of different white blood cells (also called leukocytes), each with its own specialized function but acting in concert with other cells for efficient eradication and neutralization of harmful micro-organisms and other unwanted materials. White blood cells use the blood stream as a highway to travel throughout the body, and they leave the blood flow to enter tissues where their action as safety guard is (or is expected to be) needed. Moreover, immune cells unite in lymph nodes. These structures are organized to sample body fluids from the different tissues in search of harmful materials. They function as meeting points for different immune cells and harmful substances.

Like a sophisticated army, immune cells have to meet some requirements in order to properly fight unwanted intruders. They must recognize the “enemy” and must therefore be able to discriminate between its “own soldiers” and the “other forces”. At the same time, they must distinguish between “dangerous enemies” and “innocent or collaborating crews”, and discern in what shape these appear. This must all be done in order to decide when to act and in what manner to respond in order to efficiently eliminate the enemy and not destroy the own harmless forces, *i.e.* there is no use for sending out submarines to fight a battle in the desert and placing hidden mines often does not eliminate only the enemy forces. Another indispensable skill for successful function is good co-operation between the different troops, which requires sufficient communication. Like armies have developed many different devices and strategies for defense over the past thousands of years, the immune system has evolved into a diverse organ during the struggle with evolution of the high variety of micro-organisms over the past millions of years.

Leukocytes carry receptors that function as detectors to specifically sense common patterns in either pathogenic or harmless materials. These receptors are therefore called pattern-recognition receptors (PRRs). Interaction between these PRRs and their ligands (the structures that are specifically detected by receptors) will provide signals to leukocytes to instruct them what action to undertake in response to detected materials. Such responses can range from “neglect or a friendly conversation” to an “aggressive and deadly gun fire”. Two types of leukocytes carry receptors that allow them to recognize structures in more detail than PRRs do. The cells that express such receptors are the B cells and the T cells (lymphocytes). The restricted structures (epitopes) of substances that are detected with extreme precision by these receptors are called antigens, because they *gen*erate action against (*anti*) such structures by the antigen-specific B- or T cells. In terms of warfare; PRRs sense general features that allow cells to discriminate enemies from innocent bystanders and activate a first line of defense. Additionally, B- and T cells recognize names and other characteristics of the individual soldiers with their antigen-specific receptors and create a sophisticated form of precision warfare.

Upon activation through recognition of their antigens, lymphocytes sustain ongoing immune responses and will form immunological memory that allows for quicker responses during a next encounter with that same pathogen or otherwise antigenic material. In general,

T cells recognize their specific antigens with their T cell receptor only when presented via the major histocompatibilty complex (MHC in animals, or human leukocyte antigen (HLA) complex in humans) by other cells. B cells can recognize antigens with their B cell receptor without processing and presentation via MHC by other cells. However, B cells themselves can process and efficiently present antigens to T cells via their MHC. Moreover, B cells can target pathogens over a huge distance by the action of secreted antigen-specific antibodies that specifically recognize particular pathogens.

For effective co-operation cells communicate with each other by interaction of ligand-receptor pairs on cell surfaces during physical cell-cell contact and with cytokines. Cytokines are messenger molecules that act over a rather short distance between cells and instruct cells that carry receptors for these cytokines to either stimulate or inhibit particular responses of cells. Individual immune signals can in its simplest form be divided in either immune activators or immune suppressors, but the combination of all individual signals will determine the net result of immune stimulation. Thus, the complex mixture of the types of cytokine signals that are sent out by a cell, together with signaling by cell-cell contact and antigenic- and/or PRR-mediated signals, provide the information that sets the "tone" of action by responding cells.

When the action gets out of hand

Different leukocytes and the molecules they secrete must act in concert in a balanced manner to set the correct tone of an immune response to fight unwanted materials, while avoiding damage to the host (1, 2). Sometimes this delicate balance is lost and, as a result, the immune system will react aberrantly to normally innocuous materials. This is the case in allergies, where the immune system overreacts to environmental antigens. On the other hand, when the immune system overreacts to body's own tissues, autoimmune diseases like diabetes, multiple sclerosis or rheumatoid arthritis can develop. The exact cause of such misbalanced immune responses that lead to pathology has not been clarified, but many human and animal studies have revealed that auto-immune pathologies are mediated by B and helper T (T_h) cells with a pro-inflammatory appearance (phenotype) that specifically recognize tissue antigens (3).

IMMUNITY IN RHEUMATOID ARTHRITIS

Rheumatoid arthritis

Inflammation that is mainly targeted to a particular (set of) tissue(s) of the body characterizes autoimmune disorders. Although different target organs are affected in different autoimmune disorders respectively, mechanistically these disorders share fundamental features in terms of the (auto)immune responses that participate in inflammation. Examples of autoimmune disorders that are seen frequently in Western-societies are insulin-dependent diabetes mellitus (affecting the pancreatic islets), multiple sclerosis (inflicting damage to the central nervous system) and rheumatoid arthritis. Rheumatoid arthritis (RA) is a chronic inflammatory disorder that primarily affects the diarthrodial joints and occurs in 1% of the adult human population in the Western world. The polyarticular inflammation features joint swelling, pain and stiffness, can be accompanied by fever and leads to irreversible deformity and ankylosis (4, 5).

Morphologically, RA expresses abundant hyperplasia of the intimal lining of the synovium which results from a massive increase in fibroblast-like synoviocytes. The function of these fibroblast-like cells will be explained later on. Accumulation, or so called pannus formation, of invaded cells in the synovial membrane that extends to cartilage and bone is characteristic of RA. In addition, during RA the otherwise relatively a-cellular synovium is abundantly invaded by macrophages, B cells and $CD4^+$ T cells that sometimes organize into lymphoid aggregates with germinal centers (6). Also mast cells, abundant numbers of neutrophils and many other cell types have been found in the inflamed tissue and are considered to have destructive effects in RA (reviewed in (7, 8)). The arthritic synovium contains excessive amounts of pro-inflammatory cytokines, among which TNF-α and IL-1β (4, 5, 9). The etiology of common autoimmune disorders like RA is unknown, but genetic factors (10-12) as well as environmental factors like infection or smoking (13-15) may contribute to pathogenesis.

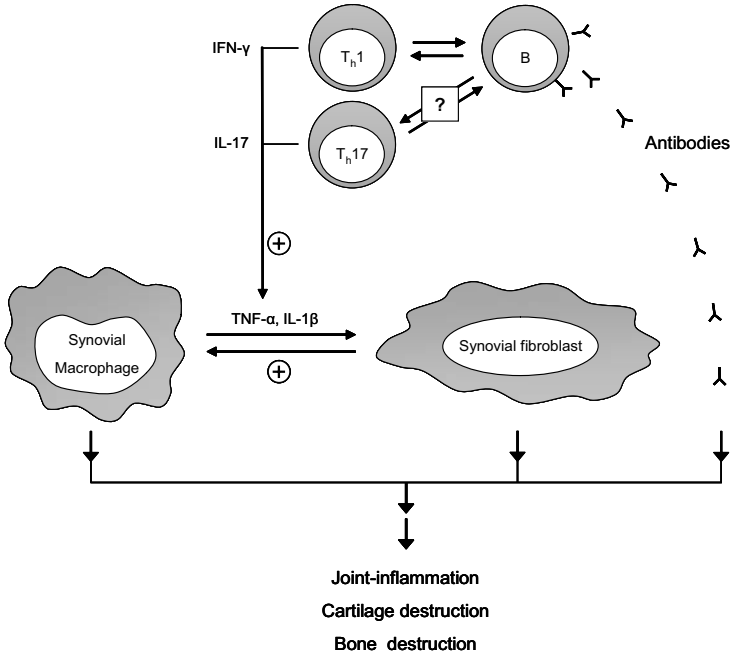

FIGURE 1. Simplified schematic representation of ubiquitous pro-inflammatory cells and their respective mediators in arthritis. Macrophages and fibroblast-like cells belong to the most prominent cells present in the synovium of the inflamed joint and secrete pro-inflammatory cytokines like TNF-α and IL-1β. These cytokines can activate macrophages and fibroblast-like cells to express more pro-inflammatory cytokines and cartilage- and bone destructive proteolytic enzymes. $T_h1$or T_h17 cells contribute to inflammation by activating innate cells in the joint through their respective hallmark cytokines IFN-γ or IL-17. Moreover, pro-inflammatory cytokines have been reported to induce mechanisms that attract leukocytes into the inflamed joint and to activate tissue-destructive immune responses in other cells that localize in the inflamed joint. T_h1 cells also sustain B cells to produce pathogenic antibodies that can activate pro-inflammatory complement pathways and can form immune complexes with cartilage antigens. These complexes activate pro-inflammatory leukocyte responses through F_c receptors expressed by leukocytes in the joint. In addition, cartilage-specific B cells are efficient antigen presenting cells for cartilage-specific T cells. The role of B-T_h17 interaction is unclear. The depicted interactions can participate in many inflammatory reactions and are not necessarily unique for rheumatoid arthritis. Adapted from Firestein *et al.* (4) and McInnes and Schett (9).

The involvement of immune cells and many of their pro-inflammatory and anti-inflammatory mediators in rheumatoid arthritis has been described extensively (4, 5, 9), but the complex network of interactions between these cells and their respective cytokines still remains far from understood. Although it seems an impossible challenge to find coherence in all ever growing knowledge on cellular interactions, we can use simplified models of these processes to fit data in order to understand them. Some of the major cell types involved in RA and their interactions are schematically represented in figure 1 and briefly introduced in the next section.

Synovial fibroblasts

Synovial fibroblasts are thought to have a prominent role in the pro-inflammatory processes that lead to joint-tissue damage as they release pro-inflammatory effector molecules that act on a variety of cells (reviewed in (9, 16)). Synovial fibroblasts can be activated by cytokines like TNF-α and IL-1β and through binding of IgG to $F_c\gamma RI$ (type I receptor for IgG antibodies) on their membrane (17).

Activated synovial fibroblasts show an enormous capacity to proliferate, to produce matrix degrading molecules like collagenase and gelatinase, and they have been reported to produce the pro-inflammatory cytokines TNF-α, IL-1-β and IL-6 that induce the production of catabolic enzymes such as MMPs (which cleave cartilage collagen fibres) and aggrecanases (18, 19). Furthermore, synovial fibroblasts from inflamed joints of RA patients promote migration and expansion of B cells (20) and T cells (21) by producing chemokines and cytokines, and they produce abundant quantities of macrophage inhibitory factor (MIF). This is a pro-inflammatory cytokine that can induce T cell activation and promote phagocytotic activity and production of TNF-α by macrophages (22).

Apart from producing pro-inflammatory cytokines in arthritis, synovial fibroblasts can also produce mediators that inhibit matrix degradation and stimulate bone formation under conditions of the normal steady state of cartilage and bone turn-over. In addition, cultured fibroblasts have been shown to constitutively produce the anti-inflammatory cytokine IL-10, which could be enhanced by TNF-α and IL-1β. IL-10 is also present at high levels in inflamed joints of RA patients, and addition of exogenous IL-10 to rheumatoid synovial membrane suppressed IL-1β and TNF-α production (23). This suggests that IL-10 is produced in response to inflammatory conditions to control inflammation. However, the IL-10 in the inflamed joints is not sufficient to completely suppress inflammation, indicating a relative deficiency of expression of anti-catabolic or anti-inflammatory cytokines in the inflamed joint during RA.

Macrophages

Macrophages that are present in the inflamed synovial membrane and at the cartilage-pannus junction have a highly activated appearance. At the site of tissue destruction these innate cells abundantly express IL-1β, TNF-α, GM-CSF and proteases like matrix metalloproteinases (MMP) (24-27). In addition, macrophages show over-expression of MHC class II molecules, which can be up-regulated by IFN-γ (28), indicating positive feedback interactions of macrophages with T_h1 cells. Interaction of macrophages with activated T cells induces production of metalloproteinases, TNF-α and IL-1β, but also IL-10, which is known for its many immunosuppressive actions (29). Another indication for T cell-macrophage interaction comes from data showing pro-inflammatory effects of IL-17 on cytokine

expression of human macrophages (30). IL-17, which is predominantly produced by T cells, is often found in synovial cells, present in T cell rich areas of RA synovial samples and strongly stimulates macrophages to produce IL-1β and TNF-α (30).

In addition, interaction of macrophages with fibroblasts has been demonstrated to elicit production of pro-inflammatory molecules like IL-6 (31). Moreover, production of TNF-α and IL-1β by macrophages suggests that these cells can induce activation of fibroblasts to produce pro-inflammatory and joint-destructive mediators since these cytokines have been described to activate synovial fibroblasts as described in the previous section.

B lymphocytes in rheumatoid arthritis

The concept of autoantigen-specific immunity in disease arose from the discovery of autoantibodies in diseases like RA in the early 20th century (32). A well known autoantibody that is often -though not uniquely- found in sera of RA patients is rheumatoid factor. This antibody is specific for F_c of self IgG. It was only in the 1970's when pathogenic potential of rheumatoid factor in RA as an initiator of immune complex-mediated disease was hypothesized (33). This hypothesis stated that immune complexes formed by rheumatoid factors fix complement and release chemotactic factors that attract inflammatory cells to the rheumatic joint. These cells, such as neutrophils, accumulate in the synovial fluid where they engulf immune complexes and get activated to release proteolytic enzymes. Later on, antibodies against autoantigens such as collagen type II, cartilage aggrecan and citrullinated peptides in synovial tissue were found in RA patients (34-37), and their presence correlated with disease (34, 35, 37).

Arthritic potential of B cells and autoantibodies has now been demonstrated by animal studies. For example, transfer of arthritis with sera or antibodies of arthritic donor animals or RA patients and depletion of antibody secretion have confirmed the arthritogenic potential of cartilage antigen-specific antibodies (38-43). Complementary to the role of the B cell as producer of pro-inflammatory antibodies is its potential as antigen-presenting cell to activate T cells, which is required to induce autoimmune disease like arthritis (3, 38, 44-49).

$CD4^+$ T lymphocytes in rheumatoid arthritis

A strong indication for involvement of $CD4^+$ T cells in RA came from studies showing association of HLA-DR genes, which determine what epitopes are presented to $CD4^+$ T cells, and risk for development of RA (50-52). In addition, the presence in the inflamed joint of $CD4^+$ T cells showing clonal restriction of TCR variable (V)-gene usage (53, 54) and T cell proliferation in response to cartilage antigens collagen type-II (55, 56), human cartilage glycoprotein 39 (57) and proteoglycan (58-61) found in human studies have also pointed to an active role of $CD4^+$ T cells in RA. Studies on expression profiles of T cell-associated cytokines in joint-tissue and $CD4^+$ T cells in synovial tissue and peripheral blood of RA patients indicated dominance of IFN-γ expression, suggesting a dominant role for the T_h1 cell phenotype in RA (62-66).

T cell transfer studies in animal models have mechanistically shown that $CD4^+$ T cells are critical for induction and exacerbation of arthritis and especially T_h1-like responses have been found to be arthritogenic (67-71). Although arthritis has long been considered a T_h1-mediated disorder, recent studies implicate IL-17 produced by T_h17 cells as crucial T cell-mediated effector cytokine in cartilage breakdown, induction of pro-inflammatory cytokines like IL-6 in macrophages and fibroblasts and induction of RA (30, 72-76). In addition to cell-

contact-mediated activation of macrophages and fibroblasts producing pro-inflammatory cytokines in the joints, pathogenic $CD4^+$ T cells help to induce arthritogenic B cell responses (Fig. 1 and reviewed in (9)).

Because $CD4^+$ T cells have a central role in pathogenesis of autoimmune disease as they have the possibility to antigen-specifically interact with many cells involved in RA, they can be employed as a useful tool to study autoantigen-specific immune responses in RA.

LYMPHOCYTE'S INTERPLAY

The studies presented in this thesis focuses mainly on autoimmunity that causes inflammation. Particularly T and B cell biology will therefore be addressed in more detail. Antigen-specific B and T cells and cytokines they produce have been demonstrated to contribute to pathogenesis of arthritis and other autoimmune disorders. During ontogeny most highly self-reactive lymphocytes are excluded from the final lymphocyte repertoire by clonal deletion. Still, many antigen-specific lymphocytes escape this process of elimination and enter the periphery to participate in immune responses (77-79). Therefore, tolerating mechanisms must exist that prevent harmful responses to autoantigens (or harmless environmental antigens) by these lymphocytes. T cells derive stimulatory signals from other cells through presentation of antigens via MHC together with co-stimulatory signals and cytokines. A well exemplified and important subset of such antigen presenting cells (APCs) that interact with T cells are dendritic cells (DCs), which are considered professional APCs that scavenge the body for antigens to be presented to T cells. In turn, T cells and their cytokines provide signals to APCs during APC-T cell interaction. Other cells that interact with T cells as APC in RA are macrophages and B cells. B-T cell interaction has been shown to be required for effective activation to generate effector phenotypes of B cells and T cells (3, 38, 46, 47, 80-84). On the other hand, some phenotypes of T and B cells (Table I, Fig. 2) will prevent rather than induce inflammatory effector responses (85, 86), indicating that not the mere activation but rather differentiation of cells and balance between effector and regulatory phenotypes of lymphocytes are detrimental to induction and regulation of inflammation (2, 65). This implicates that therapeutic interference in immune responses should not aim only at suppressing pro-inflammatory responses, but also on boosting the natural regulatory functions that down-modulate these inflammatory responses.

Table I. Some characteristics of defined $CD4^+$ T and B cell populations

	Origin	Phenotype	Mode of action
Effector cells			
T_h1	Periphery	IFN-γ, IL-2	IFN-γ
B_e1	Periphery (?)	IFN-γ	IFN-γ (?)
T_h2	Periphery	IL-4, IL-13, IL-5	IL-4, IL-13, IL-5
B_e2	Periphery (?)	IL-4	IL-4 (?)
T_h17	Periphery	IL-17	IL-17
Regulatory cells			
T_h3	Periphery	TGF-β and/or IL-10	TGF-β and/or IL-10
$CD4^+CD25^+$ T_{reg}	Thymus, periphery	Foxp3, constant $CD25^+$	IL-10, TGF-β, cell-cell contact
T_r1	Periphery	IL-10, IFN-γ	IL-10 and TGF-β
B_{reg}	Periphery (?)	IL-10	IL-10

T_h1/T_h2-mediated lymphocyte biology

Effector phenotypes of $CD4^+$ cells have long been subdivided into the two distinct lineages of T_h1 cells and T_h2 cells as first described in 1986 by Tim Mosmann (87). T_h1 cells are characterized by production of IFN-γ and IL-2, but no IL-4. T_h1 cells generally have been described to contribute to cellular responses to intracellular pathogens and autoimmunity. T_h2 cells are characterized by production of IL-4, IL-13 and IL-5, but little production of IFN-γ. In general, T_h2 cells have been described to contribute to responses against extracellular pathogens and to allergic reactions and were initially described as main effectors in B cell immunoglobulin synthesis (1, 88, 89). However, both T_h1 and T_h2 cells can stimulate B cells to enhance switching of different antibody classes (90). T_h1- and T_h2 cell-derived cytokines were found to be mutually exclusive as IFN-γ promoted development of T_h1 cells but prevented development of T_h2 cells, whereas IL-4 promoted development of T_h2 cells and prevented development of T_h1 cells (91-93) (Fig. 2). Regarding these opposing T_h cell phenotypes, a breakdown of the T_h1/T_h2 balance had become an explanation for the pathological outcome of T cell mediated disorders like allergies or autoimmune disease (89).

The interplay of T_h1 and T_h2 cytokines and T cell differentiation can be extended to B cell biology (94). T_h1 cells polarize differentiation of B cells into B_e1 cells, characterized by high IFN-γ production and T_h2 cells provide a context that induces B_e2 cells, which are characterized by low IFN-γ but high expression of IL-4. In turn, B_e1 and B_e2 cells presenting cognate antigen to $CD4^+$ T cells induce T_h1 cells and T_h2 cells respectively (Fig. 2).

Although classification of T cell phenotypes and functions into either T_h1 or T_h2 subsets has provided a valuable model to explain and investigate many immune reactions, this highly restricted and rigid classification may give a flawed and incomplete view on complexity of T cell responses (95). The identification of T cells with other phenotypes has now extended our view on T cell biology and helped to better understand the processes that are mediated by T cells as we will discuss later on.

Regulatory T cells

The paradigm of dichotomous T cell polarization was challenged by the discovery of $CD4^+$ T cells that could suppress differentiation of effector functions of both T_h1 and T_h2 cells (Fig. 2). Interest in this T cell mediated regulation that does not restrict to the T_h1/T_h2 classification has exploded by the discovery of a subset of $CD25^+$ (IL-2 receptor α-chain) $CD4^+$ T cells that maintain immune tolerance (96). T cells that are specialized to regulate T cell responses which may otherwise cause inflammation are designated regulatory T (T_{reg}) cells. $CD25^+CD4^+$ T_{reg} cells, which are induced and characterized by expression of the transcription factor Foxp3 (97-99), are generated in the thymus as a functionally distinct population (100) and are therefore often called natural T_{reg} cells. Besides only suppressing T cell responses, $CD4^+CD25^+$ T_{reg} cells inhibit autoantibody responses (101). $CD25^+CD4^+$ T_{reg} cells are anergic *in vitro* unless an excess of the growth factor IL-2 is provided. *In vivo*, however, these T_{reg} cells can replicate rather efficiently (102-104). For active regulation these T_{reg} cells require expression of IL-10, although this is not necessarily to be produced by the $CD25^+CD4^+$ T_{reg} cells themselves, as IL-10 producing neighboring cells can mediate the regulatory actions (105-107). Another feature of natural $CD4^+CD25^+$ T_{reg} cells is the requirement for expression of TGF-β to suppress immune responses (108), although TGF-β may not necessarily be produced by the natural $CD4^+CD25^+$ T_{reg} cells themselves. In addition to the regulatory action of immunosuppressive cytokines, T_{reg} cells may require cell-cell contact with T cells that are

to be suppressed by interaction of molecules on cell membranes like CTLA-4 and B7-family members that provide negative co-stimulation upon interaction (109).

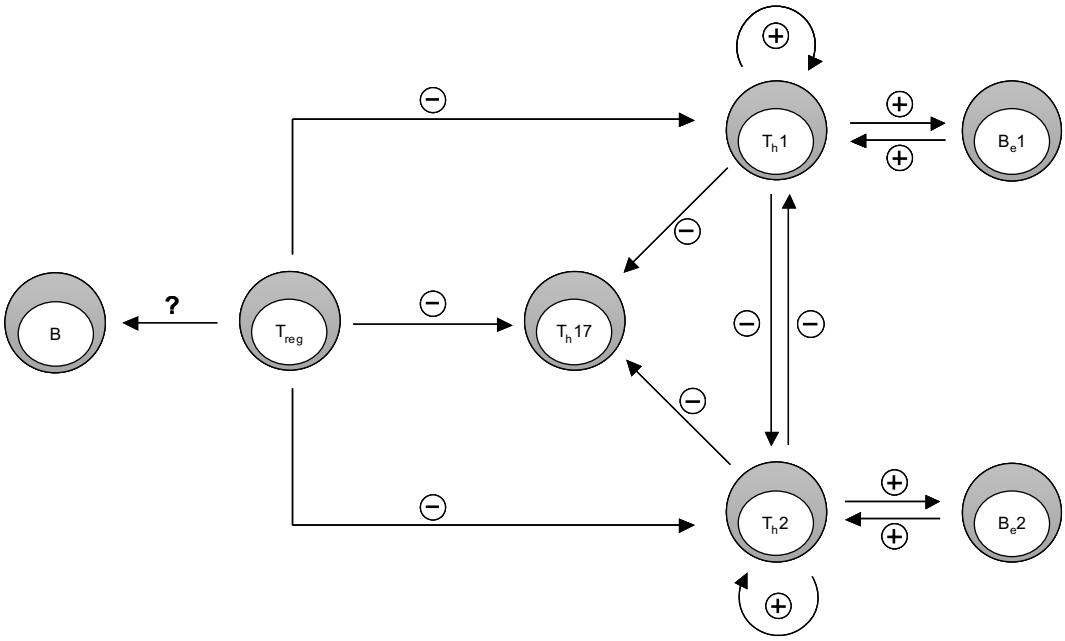

FIGURE 2. Simplified representation of interplay between B cells and helper T cells in lymphocyte differentiation. T_h1 and T_h2 cells and their characteristic cytokines, IFN-γ and IL-4 respectively, counteract on each other's differentiation while favoring differentiation of their own phenotype. T_h1 cells that interact with B cells in the presence of antigenic stimulation induce IFN-γ production in these activated effector B cells (B_e1 cells) and *vice versa*. T_h2 cells that interact with B cells in the presence of antigenic stimulation induce IL-4 production in these activated effector B cells (B_e2 cells) and *vice versa*. T_h1 and T_h2 derived cytokines have been reported to inhibit differentiation of the T_h17 cell lineage. Regulatory T (T_{reg}) cells have been described to suppress effector responses and differentiation of T_h1, T_h2 and T_h17 cells. T_{reg} mediated suppression is in many cases mediated by expression of IL-10. However, the effect of IL-10 produced by antigen-specific T_{reg} cells on the B cell response is poorly understood. In addition, the effect of IL-10 produced by antigen-specific T_{reg} cells in arthritis has not been well described.

Besides natural $CD25^+CD4^+$ T_{reg} cells, different subsets of T_{reg} cells exist that can be induced *in vitro* and *in vivo* under particular conditions of antigen and cytokine exposure (2, 86). Among those T_{reg} cells, T_{reg} type 1 (T_r1) and helper T type 3 (T_h3) cells are well studied. T_r1 cells can be generated by antigenic stimulation in the presence of IL-10 *in vitro* and suppress antigen-induced proliferation naive $CD4^+$ T cells and T cell-mediated colitis in a murine model for inflammatory bowel disease (110, 111). T_r1 cells characteristically produce high levels of IL-10 and low levels of IL-2. Such IL-10 T_{reg} cells can also be induced by co-stimulation via CD46 (112) or immunosuppressive drugs (113). This induction could be enhanced by neutralization of typical T_h1- and T_h2-inducing cytokines, such as IFN-γ and IL-4 respectively (113). Another subset of T_{reg} cells that can be distinguished is the T_h3 cell population. Cytokine expression in T_h3 cells has characteristically shifted to a high level of regulatory TGF-β and sometimes IL-10 and the mucosal immune system is an important environment for induction of this subset of T_{reg} cells (114-116). *In vitro* studies have shown

that differentiation of T_h3 cells is enhanced by culture in the presence of exogenous IL-10 and TGF-β (116).

TGF-β and IL-10 have been shown not only to enhance differentiation of T_r1 cells or T_h3 cells, but TGF-β and IL-10 have also been shown to enhance numbers of $Foxp3^+$ and $CD25^+CD4^+$ T cells with regulatory capacities (117-121). However, this can only occur as long as abundant levels of the pro-inflammatory cytokines are absent (122), as will be explained later on in more detail. The capacity of IL-10 and TGF-β to induce T_{reg} cell phenotypes adds to their well known immunosuppressive functions such as down regulation of antigen presentation, T cell proliferation and release of pro-inflammatory cytokines (29, 123) and underscore the complexity of immune regulation by T_{reg} cells and their cytokines. Although extensive efforts have been made to delineate functions of the cytokines IL-10 and TGF-β and the transcription factor $Foxp3^+$ in T_{reg} cells (124), data on the induction of these molecules in T cells by T_{reg} cells that produce IL-10 are scarce.

Regulatory B cells

B cells are typically known for their ability to produce antibodies and their antigen-presenting functions that activate $CD4^+$ T cells. As with T cells, the B cell compartment of the immune system contains distinct subsets of effector cells (94). Recent studies add to different effector functions of B cells as they describe critical regulatory capacities of B cells (85). Regulatory B (B_{reg}) cells have been described to suppress chronic inflammation and T_h1-type responses in models for a wide range of chronic inflammatory disorders, among which experimental encephalomyelitis (125), colitis (126) and arthritis (127-129). In many studies, B_{reg} cells have been described to produce large amounts of IL-10, but also TGF-β^+ B_{reg} cells have been identified (85). Although the origin of such B_{reg} cells may not be fully understood, the expression of IL-10 by B cells seems crucial for regulation of inflammation (125, 128).

Induction of a regulatory IL-10^+ phenotype in B cells could be actively induced by stimulation via the B cell receptor in the presence of an agonistic anti-CD40 monoclonal antibody (128), whereas TGF-β^+ B_{reg} cells could be induced with LPS (130, 131). B_{reg} cells were also induced by apoptotic cells in an IL-10 dependent manner, and these B cells were able to affect the cytokine profile of antigen-specific $CD4^+$ T cells (129). Also naive B cells, which previously had been considered ineffective antigen-presenting cells for $CD4^+$ T cells, have been demonstrated to induce T_{reg} cells upon close B-T cell interaction (132). These data indicate close collaboration of antigen-presenting B cells and responding T cells that is needed to induce IL-10 production and concomitant regulatory activity in $CD4^+$ T lymphocytes preventing inflammation. However, the role of T_{reg} cells that produce IL-10 in differentiation of cytokine responses of B cells, especially the induction of IL-10, in a pro-inflammatory setting remains to be elucidated.

T_h17 cells

Another identified phenotype that has expanded the repertoire of T_h cells, is the IL-17 producing T_h17 cell (133, 134). This T cell subset has been discovered only quite recently, but emerging data suggest a highly inflammatory and prominent role for these cells in the induction of arthritis (73) and other inflammatory autoimmune diseases (135). Although T_h17 and T_h1 cells were initially thought to derive from a common pre-T_h1 precursor, today T_h17 cells are considered to be a separate lineage of helper T cells that develop from naïve $CD4^+$ T cells (133, 136, 137). Differentiation of T_h17 cells is induced by TGF-β in the presence of

pro-inflammatory cytokines like IL-6 or IL-1β and growth of the T_h17 cell population is further promoted by IL-23 (138-142). Moreover, the lymphocyte growth factor IL-2 ultimately leads to expansion of the T_h17 cell population, though this cytokine inhibits the early differentiation of T_h17 cells (141-143). Differentiation of T_h17 cells can be inhibited by T_h1- and T_h2 cell-derived cytokines (136, 137, 143) and it has also been postulated that $Foxp3^+$ T_{reg} cells may block differentiation of T_h17 cells (144, 145). The role of T_h17 cells in B cell differentiation and autoantibody production, however, has remained unclear.

IMMUNOTHERAPY FOR AUTOIMMUNE DISEASE

Many forms of pre-clinical and clinical immune therapies for autoimmune diseases aim to specifically antagonize or eliminate the inflammatory cells or cytokines by use of biological immune mediators. Together with yielding curative effects, such interventions provide insight in ongoing inflammatory processes.

Targeting of pro-inflammatory cytokines has been established as a feasible and widespread method for treating chronic inflammatory disease. Blockade of a broad range of different pro-inflammatory cytokines with biological agents specific for these cytokines has been studied in animal models and are now being tested in clinical trials and used for clinical treatment of RA. The most successful agents used are agents like TNF-specific antibodies or TNF-receptor F_c fusion protein and interleukin-1 receptor antagonist (IL-1RA) that target TNF-α and IL-1 respectively (9, 146). However, because repeated systemic application of such agents results in systemic inactivation of effector immune responses, such therapy increases the risk of serious infection (147).

As explained in the previous paragraphs, B cells provide signals to T cells through antigen presentation and cytokines and T cells provide help to B cells through delivery of cytokines and cell-surface-ligands. These interactions are central in pathogenesis of autoimmune disease. Several therapies therefore aim at depleting pathogenic B cells by use of anti-CD20 monoclonal antibodies (32) or T cells by use of anti-CD3 monoclonal antibodies (148). Despite successful application of such agents, unwanted side effects may occur because therapy is not restricted to the immune responses involved in the autoimmune-mediated inflammation.

Antigen-specific intervention

To overcome unwanted non-specific side effects, current research aims at more antigen-specific intervention. An extensively studied approach is the induction of antigen-specific regulatory T cells by application of defined autoantigens or allergens via the mucosal route. Such studies have indicated that mucosal lymph nodes are a microenvironment that supports the induction of antigen-specific T_{reg} cells (149, 150). This approach requires definition of the autoantigens involved in the inflammatory immune responses. Although this method has been proven successful in animal models for autoimmunity, translation to the clinic is difficult.

An alternative but promising approach to correct immune balance by restoring the regulatory T cell compartment is treatment with heat shock proteins (HSPs) (151). HSPs are highly conserved between animals and bacteria and application of HSP has been demonstrated to induce regulatory T cells in several immune-mediated models. Expression of HSP increases at sites of inflammation and may lead to enhanced presentation of epitopes that are recognized locally by HSP-specific T_{reg} cells and therefore mediate immune regulation at these sites of inflammation (152, 153).

Adoptive cellular gene therapy

Since the introduction of recombinant DNA technology the use of gene therapy in prevention and treatment of many diseases has evolved rapidly. Cloning of genes that code for anti-inflammatory molecules has created the possibility to introduce long time expression and provision of theoretically any recombinant biological agent expressed by genes introduced in tissues of patients as has now been successfully shown in many inflammatory models, among which arthritis (154). Transfer of active genes to eukaryotic cells to establish stable expression of such genes in these cells can be done by infection of cells with (retro)viruses that contain the transgene (Fig. 3). Such viral systems have been developed to eliminate pathogenic characteristics of viruses while the capability to efficiently transfer selected genes into the genome of the host cell has been left intact.

Because $CD4^+$ T cells are antigen-specific, play a central role in autoimmune disease and accumulate in inflammatory lesions, *ex vivo* modification of autoantigen specific T cells by gene transfer in order to express immunosuppressive biologicals may be a refined method for treatment of autoimmune disease. This so called adoptive cellular gene therapy to interfere specifically in antigen-specific pathological immune responses has been demonstrated successfully first in experimental encephalomyelitis, a murine model to study multiple sclerosis (155). Thereafter, many studies have demonstrated the efficacy of cellular gene therapy with $CD4^+$ T cells expressing different biologicals in restoring immune balance in different models of disease like RA (156), MS, diabetes and allergy (157).

Although success of cellular gene therapy with T cells was claimed in most of these studies, many of these studies were done with T cell hybridoma's instead of primary T cells, with T cells with undefined specificity or with antigens that are not naturally expressed in inflamed tissues. In the studies presented in this thesis we aimed to explore *ex vivo* manipulated primary $CD4^+$ T cells with different phenotypes but defined specificity for an antigen that is naturally expressed in the inflamed joint tissue in a chronic model of RA; proteoglycan-induced arthritis. This may not only indicate what approach may be useful for treatment of disease, but may also provide insight in the function of certain T cell phenotypes mechanistically.

PROTEOGLYCAN-INDUCED ARTHRITIS

Proteoglycan-induced arthritis (PGIA) is a chronic relapsing-remitting arthritis that is induced in BALB/c mice by immunization with human cartilage proteoglycan. An extensive overview of this model can be found in Glant *et al.* (158). In brief, PGIA is a chronic model that represents many features of RA (159). The arthritis is dominated by T_h1 responses and can be prevented by T_h2-like mediators (69, 160-162), but activation of both B and $CD4^+$ T cells is required for full blown disease. The arthritogenic antigen-specific immune response is based on cross-reactivity to both immunizing (human) and self (mouse) proteoglycan (38, 67, 68, 163, 164). B cell epitopes of proteoglycan have been defined in the regions that are hidden by keratan sulphate chains, whereas arthritogenic $CD4^+$ T cell epitopes have been identified within regions that are hidden by keratan sulphate side chains (165). Moreover, immunodominant arthritogenic $CD4^+$ T cell epitopes have been identified especially within the G1 N-terminal domain of proteoglycan (48, 165). B cells serve a dual purpose because they are required as antigen-presenting cells to stimulate T cells and as producers of arthritogenic antibodies that provoke inflammatory effects through activation of F_c antibody receptors (38, 44, 166).

Altogether, this model provides a system to study chronic B and T cell mediated inflammation that is directed to a well defined tissue (self) antigen. In addition, an arthritogenic proteoglycan specific T cell hybridoma (68) is a premise to initiate studies using cartilage-antigen specific $CD4^+$ T cell mediated intervention in this model.

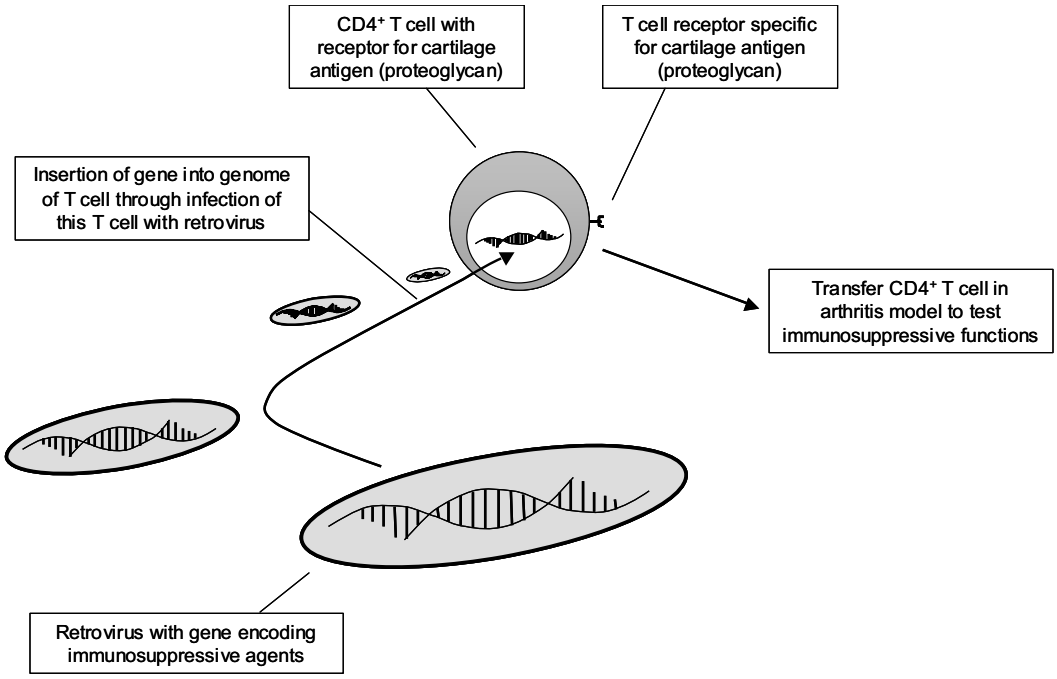

FIGURE 3. Schematic representation of the use of retrovirally transduced T cells expressing immunosuppressive agents as vectors to target autoimmunity in arthritis. Active genes coding for known immunosuppressive agents were inserted into the genome of $CD4^+$ T cells expressing a T cell receptor that specifically recognizes the cartilage antigen proteoglycan. This gene transduction was done through infection with retroviral particles that have encapsulated the respective genes. Upon infection the gene is released from the viral particles, stably integrated into the T cell genome and thus expressed by this T cell. Due to the specificity of such T cells for the arthritogenic cartilage antigen T cells have the ability to interfere with the arthritogenic response and are therefore supposed to specifically target the pathogenic pro-inflammatory immune response that leads to arthritis. As such, proteoglycan-specific $CD4^+$ T cells were tested in a B and $CD4^+$ T cell mediated arthritis model that is induced by generation of an immune response against cartilage derived antigen proteoglycan in mice.

OUTLINE AND AIM OF THIS THESIS

The aim of this thesis was to explore cartilage-antigen specific $CD4^+$ T cells specifically targeting inflammation in rheumatoid arthritis with immunomodulatory agents to ameliorate inflammation in arthritis. For this purpose cartilage-specific $CD4^+$ T cells were provided with active genes encoding immunomodulatory agents through retroviral transduction and were then transferred in proteoglycan-induced arthritis (Fig. 3); a chronic B and T cell-mediated model for rheumatoid arthritis in mice.

Chapter 2 describes the generation of a T cell receptor transgenic mouse expressing a T cell receptor that is specific for the immunodominant epitope of cartilage proteoglycan on its T cells. These mice served as donor for proteoglycan specific $CD4^+$ T cells to be used in antigen specific interventions in PGIA.

Chapter 3 describes how genes encoding different agents that previously have been shown to regulate arthritis were expressed by proteoglycan-specific $CD4^+$ T cells through retroviral transduction and how these T cells were tested in PGIA.

Chapter 4 shows how cartilage antigen-specific $CD4^+$ T cells expressing a transduced IL-10 gene regulate the cytokine and antibody response in the chronic phase of arthritis, and how these T cells require recognition of their cognate antigen to ameliorate arthritis. Moreover, the requirement for IL-10 expression by the treated host to regulate arthritis was determined as we hypothesized that induction of endogenous IL-10 expression is part of the regulatory mechanism that is induced by $IL\text{-}10^+$ T cells.

Chapter 5 concerns the effect of cartilage antigen-specific T cells expressing a transduced IL-10 gene on early differentiation of (both pro-inflammatory and immunosuppressive) cytokine expression of B cells and $CD4^+$ T cells and $CD4^+$ T cell proliferation during the early arthritogenic immune response.

Finally, in *chapter 6*, the data presented in this thesis are discussed in the context of current knowledge on T cell mediated regulation of immune differentiation.

REFERENCES

1. Mills, K. H. 2004. Regulatory T cells: friend or foe in immunity to infection? *Nat Rev Immunol* 4:841-855.
2. von Herrath, M. G., and L. C. Harrison. 2003. Antigen-induced regulatory T cells in autoimmunity. *Nat Rev Immunol* 3:223-232.
3. Shlomchik, M. J., J. E. Craft, and M. J. Mamula. 2001. From T to B and back again: positive feedback in systemic autoimmune disease. *Nat Rev Immunol* 1:147-153.
4. Firestein, G. S. 2003. Evolving concepts of rheumatoid arthritis. *Nature* 423:356-361.
5. Lee, D. M., and M. E. Weinblatt. 2001. Rheumatoid arthritis. *Lancet* 358:903-911.
6. Weyand, C. M., J. J. Goronzy, S. Takemura, and P. J. Kurtin. 2000. Cell-cell interactions in synovitis. Interactions between T cells and B cells in rheumatoid arthritis. *Arthritis Res* 2:457-463.
7. Edwards, S. W., and M. B. Hallett. 1997. Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. *Immunol Today* 18:320-324.
8. Woolley, D. E. 2003. The mast cell in inflammatory arthritis. *N Engl J Med* 348:1709-1711.
9. McInnes, I. B., and G. Schett. 2007. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* 7:429-442.
10. MacGregor, A. J., H. Snieder, A. S. Rigby, M. Koskenvuo, J. Kaprio, K. Aho, and A. J. Silman. 2000. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis Rheum* 43:30-37.
11. Crawley, E., S. Kon, and P. Woo. 2001. Hereditary predisposition to low interleukin-10 production in children with extended oligoarticular juvenile idiopathic arthritis. *Rheumatology (Oxford)* 40:574-578.
12. van der Helm-van Mil, A. H., J. Z. Wesoly, and T. W. Huizinga. 2005. Understanding the genetic contribution to rheumatoid arthritis. *Curr Opin Rheumatol* 17:299-304.
13. Klareskog, L., L. Padyukov, and L. Alfredsson. 2007. Smoking as a trigger for inflammatory rheumatic diseases. *Curr Opin Rheumatol* 19:49-54.
14. Benoist, C., and D. Mathis. 2001. Autoimmunity provoked by infection: how good is the case for T cell epitope mimicry? *Nat Immunol* 2:797-801.
15. Ermann, J., and C. G. Fathman. 2001. Autoimmune diseases: genes, bugs and failed regulation. *Nat Immunol* 2:759-761.
16. Ritchlin, C. 2000. Fibroblast biology. Effector signals released by the synovial fibroblast in arthritis. *Arthritis Res* 2:356-360.
17. Pap, T. 2005. Direct interaction of immunoglobulins with synovial fibroblasts: a missing link in the pathogenesis of rheumatoid arthritis? *Arthritis Res Ther* 7:44-46.
18. Ritchlin, C., E. Dwyer, R. Bucala, and R. Winchester. 1994. Sustained and distinctive patterns of gene activation in synovial fibroblasts and whole synovial tissue obtained from inflammatory synovitis. *Scand J Immunol* 40:292-298.
19. Bucala, R., C. Ritchlin, R. Winchester, and A. Cerami. 1991. Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. *J Exp Med* 173:569-574.
20. Burger, J. A., N. J. Zvaifler, N. Tsukada, G. S. Firestein, and T. J. Kipps. 2001. Fibroblast-like synoviocytes support B-cell pseudoemperipolesis via a stromal cell-derived factor-1- and CD106 (VCAM-1)-dependent mechanism. *J Clin Invest* 107:305-315.
21. Harada, S., M. Yamamura, H. Okamoto, Y. Morita, M. Kawashima, T. Aita, and H. Makino. 1999. Production of interleukin-7 and interleukin-15 by fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Arthritis Rheum* 42:1508-1516.
22. Leech, M., C. Metz, P. Hall, P. Hutchinson, K. Gianis, M. Smith, H. Weedon, S. R. Holdsworth, R. Bucala, and E. F. Morand. 1999. Macrophage migration inhibitory factor in rheumatoid arthritis: evidence of proinflammatory function and regulation by glucocorticoids. *Arthritis Rheum* 42:1601-1608.
23. Katsikis, P. D., C. Q. Chu, F. M. Brennan, R. N. Maini, and M. Feldmann. 1994. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. *J Exp Med* 179:1517-1527.
24. Tetlow, L. C., M. Lees, Y. Ogata, H. Nagase, and D. E. Woolley. 1993. Differential expression of gelatinase B (MMP-9) and stromelysin-1 (MMP-3) by rheumatoid synovial cells in vitro and in vivo. *Rheumatol Int* 13:53-59.
25. Burmester, G. R., B. Stuhlmuller, G. Keyszer, and R. W. Kinne. 1997. Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? *Arthritis Rheum* 40:5-18.
26. Stuhlmuller, B., U. Ungethum, S. Scholze, L. Martinez, M. Backhaus, H. G. Kraetsch, R. W. Kinne, and G. R. Burmester. 2000. Identification of known and novel genes in activated monocytes from patients with rheumatoid arthritis. *Arthritis Rheum* 43:775-790.
27. Firestein, G. S., J. M. Alvaro-Gracia, and R. Maki. 1990. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J Immunol* 144:3347-3353.
28. Hessian, P. A., J. Highton, and D. G. Palmer. 1989. Quantification of macrophage cell surface molecules in rheumatoid arthritis. *Clin Exp Immunol* 77:47-51.
29. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.
30. Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, F. C. Jolicoeur, Y. He, M. Zhang, F. Mineau, and J. P. Pelletier. 1998. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* 160:3513-3521.
31. Chomarat, P., M. C. Rissoan, J. J. Pin, J. Banchereau, and P. Miossec. 1995. Contribution of IL-1, CD14, and CD13 in the increased IL-6 production induced by in vitro monocyte-synoviocyte interactions. *J Immunol* 155:3645-3652.
32. Edwards, J. C., and G. Cambridge. 2006. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. *Nat Rev Immunol* 6:394-403.
33. Zvaifler, N. J. 1973. The immunopathology of joint inflammation in rheumatoid arthritis. *Adv Immunol* 16:265-336.
34. Cook, A. D., M. J. Rowley, I. R. Mackay, A. Gough, and P. Emery. 1996. Antibodies to type II collagen in early rheumatoid arthritis. Correlation with disease progression. *Arthritis Rheum* 39:1720-1727.
35. van Boekel, M. A., E. R. Vossenaar, F. H. van den Hoogen, and W. J. van Venrooij. 2002. Autoantibody systems in rheumatoid arthritis: specificity, sensitivity and diagnostic value. *Arthritis Res* 4:87-93.
36. Karopoulos, C., M. J. Rowley, M. Z. Ilic, and C. J. Handley. 1996. Presence of antibodies to native G1 domain of aggrecan core protein in synovial fluids from patients with various joint diseases. *Arthritis Rheum* 39:1990-1997.

37. Schellekens, G. A., H. Visser, B. A. de Jong, F. H. van den Hoogen, J. M. Hazes, F. C. Breedveld, and W. J. van Venrooij. 2000. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum* 43:155-163.
38. O'Neill, S. K., M. J. Shlomchik, T. T. Glant, Y. Cao, P. D. Doodes, and A. Finnegan. 2005. Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis. *J Immunol* 174:3781-3788.
39. Stuart, J. M., M. A. Cremer, A. S. Townes, and A. H. Kang. 1982. Type II collagen-induced arthritis in rats. Passive transfer with serum and evidence that IgG anticollagen antibodies can cause arthritis. *J Exp Med* 155:1-16.
40. Stuart, J. M., and F. J. Dixon. 1983. Serum transfer of collagen-induced arthritis in mice. *J Exp Med* 158:378-392.
41. Petkova, S. B., K. N. Konstantinov, T. J. Sproule, B. L. Lyons, M. A. Awwami, and D. C. Roopenian. 2006. Human antibodies induce arthritis in mice deficient in the low-affinity inhibitory IgG receptor Fc gamma RIIB. *J Exp Med* 203:275-280.
42. Wooley, P. H., H. S. Luthra, S. K. Singh, A. R. Huse, J. M. Stuart, and C. S. David. 1984. Passive transfer of arthritis to mice by injection of human anti-type II collagen antibody. *Mayo Clin Proc* 59:737-743.
43. Rademacher, T. W., P. Williams, and R. A. Dwek. 1994. Agalactosyl glycoforms of IgG autoantibodies are pathogenic. *Proc Natl Acad Sci U S A* 91:6123-6127.
44. Brennan, F. R., K. Mikecz, E. I. Buzas, D. Ragasa, G. Cs-Szabo, G. Negroiu, and T. T. Glant. 1995. Antigen-specific B cells present cartilage proteoglycan (aggrecan) to an autoreactive T cell hybridoma derived from a mouse with proteoglycan-induced arthritis. *Clin Exp Immunol* 101:414-421.
45. Brennan, F. R., G. Negroiu, E. I. Buzas, C. Fulop, K. Hollo, K. Mikecz, and T. T. Glant. 1995. Presentation of cartilage proteoglycan to a T cell hybridoma derived from a mouse with proteoglycan-induced arthritis. *Clin Exp Immunol* 100:104-110.
46. Yan, J., B. P. Harvey, R. J. Gee, M. J. Shlomchik, and M. J. Mamula. 2006. B cells drive early T cell autoimmunity in vivo prior to dendritic cell-mediated autoantigen presentation. *J Immunol* 177:4481-4487.
47. Takemura, S., P. A. Klimiuk, A. Braun, J. J. Goronzy, and C. M. Weyand. 2001. T cell activation in rheumatoid synovium is B cell dependent. *J Immunol* 167:4710-4718.
48. Glant, T. T., E. I. Buzas, A. Finnegan, G. Negroiu, G. Cs-Szabo, and K. Mikecz. 1998. Critical roles of glycosaminoglycan side chains of cartilage proteoglycan (aggrecan) in antigen recognition and presentation. *J Immunol* 160:3812-3819.
49. Serreze, D. V., and P. A. Silveira. 2003. The role of B lymphocytes as key antigen-presenting cells in the development of T cell-mediated autoimmune type 1 diabetes. *Curr Dir Autoimmun* 6:212-227.
50. Nepom, G. T. 1998. Major histocompatibility complex-directed susceptibility to rheumatoid arthritis. *Adv Immunol* 68:315-332.
51. Nepom, G. T., P. Byers, C. Seyfried, L. A. Healey, K. R. Wilske, D. Stage, and B. S. Nepom. 1989. HLA genes associated with rheumatoid arthritis. Identification of susceptibility alleles using specific oligonucleotide probes. *Arthritis Rheum* 32:15-21.
52. Stastny, P. 1976. Mixed lymphocyte cultures in rheumatoid arthritis. *J Clin Invest* 57:1148-1157.
53. Kobari, Y., Y. Misaki, K. Setoguchi, W. Zhao, Y. Komagata, K. Kawahata, Y. Iwakura, and K. Yamamoto. 2004. T cells accumulating in the inflamed joints of a spontaneous murine model of rheumatoid arthritis become restricted to common clonotypes during disease progression. *Int Immunol* 16:131-138.
54. Stamenkovic, I., M. Stegagno, K. A. Wright, S. M. Krane, E. P. Amento, R. B. Colvin, R. J. Duquesnoy, and J. T. Kurnick. 1988. Clonal dominance among T-lymphocyte infiltrates in arthritis. *Proc Natl Acad Sci U S A* 85:1179-1183.
55. Kim, H. Y., W. U. Kim, M. L. Cho, S. K. Lee, J. Youn, S. I. Kim, W. H. Yoo, J. H. Park, J. K. Min, S. H. Lee, S. H. Park, and C. S. Cho. 1999. Enhanced T cell proliferative response to type II collagen and synthetic peptide CII (255-274) in patients with rheumatoid arthritis. *Arthritis Rheum* 42:2085-2093.
56. He, X., A. H. Kang, and J. M. Stuart. 2000. Accumulation of T cells reactive to type II collagen in synovial fluid of patients with rheumatoid arthritis. *J Rheumatol* 27:589-593.
57. Cope, A. P., S. D. Patel, F. Hall, M. Congia, H. A. Hubers, G. F. Verheijden, A. M. Boots, R. Menon, M. Trucco, A. W. Rijnders, and G. Sonderstrup. 1999. T cell responses to a human cartilage autoantigen in the context of rheumatoid arthritis-associated and nonassociated HLA-DR4 alleles. *Arthritis Rheum* 42:1497-1507.
58. Goodstone, N. J., M. C. Doran, R. N. Hobbs, R. C. Butler, J. J. Dixey, and B. A. Ashton. 1996. Cellular immunity to cartilage aggrecan core protein in patients with rheumatoid arthritis and non-arthritic controls. *Ann Rheum Dis* 55:40-46.
59. Guerassimov, A., Y. Zhang, S. Banerjee, A. Cartman, J. Y. Leroux, L. C. Rosenberg, J. Esdaile, M. A. Fitzcharles, and A. R. Poole. 1998. Cellular immunity to the G1 domain of cartilage proteoglycan aggrecan is enhanced in patients with rheumatoid arthritis but only after removal of keratan sulfate. *Arthritis Rheum* 41:1019-1025.
60. Li, N. L., D. Q. Zhang, K. Y. Zhou, A. Cartman, J. Y. Leroux, A. R. Poole, and Y. P. Zhang. 2000. Isolation and characteristics of autoreactive T cells specific to aggrecan G1 domain from rheumatoid arthritis patients. *Cell Res* 10:39-49.
61. Guerassimov, A., Y. Zhang, A. Cartman, L. C. Rosenberg, J. Esdaile, M. A. Fitzcharles, and A. R. Poole. 1999. Immune responses to cartilage link protein and the G1 domain of proteoglycan aggrecan in patients with osteoarthritis. *Arthritis Rheum* 42:527-533.
62. Bucht, A., P. Larsson, L. Weisbrot, C. Thorne, P. Pisa, G. Smedegard, E. C. Keystone, and A. Gronberg. 1996. Expression of interferon-gamma (IFN-gamma), IL-10, IL-12 and transforming growth factor-beta (TGF-beta) mRNA in synovial fluid cells from patients in the early and late phases of rheumatoid arthritis (RA). *Clin Exp Immunol* 103:357-367.
63. Dolhain, R. J., A. N. van der Heiden, N. T. ter Haar, F. C. Breedveld, and A. M. Miltenburg. 1996. Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joints of patients with rheumatoid arthritis. *Arthritis Rheum* 39:1961-1969.
64. Morita, Y., M. Yamamura, M. Kawashima, S. Harada, K. Tsuji, K. Shibuya, K. Maruyama, and H. Makino. 1998. Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. *Arthritis Rheum* 41:1669-1676.
65. Yudoh, K., H. Matsuno, F. Nakazawa, T. Yonezawa, and T. Kimura. 2000. Reduced expression of the regulatory CD4+ T cell subset is related to Th1/Th2 balance and disease severity in rheumatoid arthritis. *Arthritis Rheum* 43:617-627.

66. Skapenko, A., J. Leipe, P. E. Lipsky, and H. Schulze-Koops. 2005. The role of the T cell in autoimmune inflammation. *Arthritis Res Ther* 7 Suppl 2:S4-14.
67. Bardos, T., K. Mikecz, A. Finnegan, J. Zhang, and T. T. Glant. 2002. T and B cell recovery in arthritis adoptively transferred to SCID mice: antigen-specific activation is required for restoration of autopathogenic CD4+ Th1 cells in a syngeneic system. *J Immunol* 168:6013-6021.
68. Buzas, E. I., F. R. Brennan, K. Mikecz, M. Garzo, G. Negroiu, K. Hollo, G. Cs-Szabo, E. Pintye, and T. T. Glant. 1995. A proteoglycan (aggrecan)-specific T cell hybridoma induces arthritis in BALB/c mice. *J Immunol* 155:2679-2687.
69. Finnegan, A., K. Mikecz, P. Tao, and T. T. Glant. 1999. Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. *J Immunol* 163:5383-5390.
70. Maffia, P., J. M. Brewer, J. A. Gracie, A. Ianaro, B. P. Leung, P. J. Mitchell, K. M. Smith, I. B. McInnes, and P. Garside. 2004. Inducing experimental arthritis and breaking self-tolerance to joint-specific antigens with trackable, ovalbumin-specific T cells. *J Immunol* 173:151-156.
71. Wang, D., J. A. Hill, A. M. Jevnikar, E. Cairns, and D. A. Bell. 2002. Induction of transient arthritis by the adoptive transfer of a collagen II specific Th1 clone to HLA-DR4 (B1*0401) transgenic mice. *J Autoimmun* 19:37-43.
72. Furuzawa-Carballeda, J., M. I. Vargas-Rojas, and A. R. Cabral. 2007. Autoimmune inflammation from the Th17 perspective. *Autoimmun Rev* 6:169-175.
73. Hirota, K., M. Hashimoto, H. Yoshitomi, S. Tanaka, T. Nomura, T. Yamaguchi, Y. Iwakura, N. Sakaguchi, and S. Sakaguchi. 2007. T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17+ Th cells that cause autoimmune arthritis. *J Exp Med* 204:41-47.
74. Lubberts, E., M. I. Koenders, and W. B. van den Berg. 2005. The role of T-cell interleukin-17 in conducting destructive arthritis: lessons from animal models. *Arthritis Res Ther* 7:29-37.
75. Sato, K., A. Suematsu, K. Okamoto, A. Yamaguchi, Y. Morishita, Y. Kadono, S. Tanaka, T. Kodama, S. Akira, Y. Iwakura, D. J. Cua, and H. Takayanagi. 2006. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* 203:2673-2682.
76. Kotake, S., N. Udagawa, N. Takahashi, K. Matsuzaki, K. Itoh, S. Ishiyama, S. Saito, K. Inoue, N. Kamatani, M. T. Gillespie, T. J. Martin, and T. Suda. 1999. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest* 103:1345-1352.
77. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49:273-280.
78. Ota, K., M. Matsui, E. L. Milford, G. A. Mackin, H. L. Weiner, and D. A. Hafler. 1990. T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* 346:183-187.
79. Chen, C., Z. Nagy, M. Z. Radic, R. R. Hardy, D. Huszar, S. A. Camper, and M. Weigert. 1995. The site and stage of anti-DNA B-cell deletion. *Nature* 373:252-255.
80. Crawford, A., M. Macleod, T. Schumacher, L. Corlett, and D. Gray. 2006. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. *J Immunol* 176:3498-3506.
81. Toellner, K. M., S. A. Luther, D. M. Sze, R. K. Choy, D. R. Taylor, I. C. MacLennan, and H. Acha-Orbea. 1998. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. *J Exp Med* 187:1193-1204.
82. Fulcher, D. A., A. B. Lyons, S. L. Korn, M. C. Cook, C. Koleda, C. Parish, B. Fazekas de St Groth, and A. Basten. 1996. The fate of self-reactive B cells depends primarily on the degree of antigen receptor engagement and availability of T cell help. *J Exp Med* 183:2313-2328.
83. Guay, H. M., J. Larkin, 3rd, C. C. Picca, L. Panarey, and A. J. Caton. 2007. Spontaneous autoreactive memory B cell formation driven by a high frequency of autoreactive CD4+ T cells. *J Immunol* 178:4793-4802.
84. Stockinger, B., T. Zal, A. Zal, and D. Gray. 1996. B cells solicit their own help from T cells. *J Exp Med* 183:891-899.
85. Mizoguchi, A., and A. K. Bhan. 2006. A case for regulatory B cells. *J Immunol* 176:705-710.
86. Shevach, E. M. 2006. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* 25:195-201.
87. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:2348-2357.
88. O'Shea, J. J., A. Ma, and P. Lipsky. 2002. Cytokines and autoimmunity. *Nat Rev Immunol* 2:37-45.
89. Liew, F. Y. 2002. T(H)1 and T(H)2 cells: a historical perspective. *Nat Rev Immunol* 2:55-60.
90. Stevens, T. L., A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann, and E. S. Vitetta. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 334:255-258.
91. Fernandez-Botran, R., V. M. Sanders, T. R. Mosmann, and E. S. Vitetta. 1988. Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. *J Exp Med* 168:543-558.
92. Openshaw, P., E. E. Murphy, N. A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J Exp Med* 182:1357-1367.
93. Gajewski, T. F., and F. W. Fitch. 1988. Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J Immunol* 140:4245-4252.
94. Harris, D. P., L. Haynes, P. C. Sayles, D. K. Duso, S. M. Eaton, N. M. Lepak, L. L. Johnson, S. L. Swain, and F. E. Lund. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol* 1:475-482.
95. Allen, J. E., and R. M. Maizels. 1997. Th1-Th2: reliable paradigm or dangerous dogma? *Immunol Today* 18:387-392.
96. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-1164.
97. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.
98. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.
99. Martin, B., A. Banz, B. Bienvenu, C. Cordier, N. Dautigny, C. Becourt, and B. Lucas. 2004. Suppression of CD4+ T lymphocyte effector functions by CD4+CD25+ cells in vivo. *J Immunol* 172:3391-3398.
100. Sakaguchi, S. 2003. The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery. *J Clin Invest* 112:1310-1312.
101. Fields, M. L., B. D. Hondowicz, M. H. Metzgar, S. A. Nish, G. N. Wharton, C. C. Picca, A. J. Caton, and J. Erikson. 2005. CD4+ CD25+ regulatory T cells inhibit the maturation but not the initiation of an autoantibody response. *J Immunol* 175:4255-4264.

102. Fisson, S., G. Darrasse-Jeze, E. Litvinova, F. Septier, D. Klatzmann, R. Liblau, and B. L. Salomon. 2003. Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. *J Exp Med* 198:737-746.
103. Walker, L. S., A. Chodos, M. Eggena, H. Dooms, and A. K. Abbas. 2003. Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J Exp Med* 198:249-258.
104. Tang, Q., K. J. Henriksen, M. Bi, E. B. Finger, G. Szot, J. Ye, E. L. Masteller, H. McDevitt, M. Bonyhadi, and J. A. Bluestone. 2004. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 199:1455-1465.
105. Mekala, D. J., R. S. Alli, and T. L. Geiger. 2005. IL-10-dependent infectious tolerance after the treatment of experimental allergic encephalomyelitis with redirected CD4+CD25+ T lymphocytes. *Proc Natl Acad Sci U S A* 102:11817-11822.
106. Kearley, J., J. E. Barker, D. S. Robinson, and C. M. Lloyd. 2005. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* 202:1539-1547.
107. Dieckmann, D., C. H. Bruett, H. Ploettner, M. B. Lutz, and G. Schuler. 2002. Human CD4(+)CD25(+) regulatory, contact-dependent T cells induce interleukin 10-producing, contact-independent type 1-like regulatory T cells [corrected]. *J Exp Med* 196:247-253.
108. Jonuleit, H., E. Schmitt, H. Kakirman, M. Stassen, J. Knop, and A. H. Enk. 2002. Infectious tolerance: human CD25(+) regulatory T cells convey suppressor activity to conventional CD4(+) T helper cells. *J Exp Med* 196:255-260.
109. Sansom, D. M., and L. S. Walker. 2006. The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. *Immunol Rev* 212:131-148.
110. Roncarolo, M. G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M. K. Levings. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* 212:28-50.
111. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737-742.
112. Kemper, C., A. C. Chan, J. M. Green, K. A. Brett, K. M. Murphy, and J. P. Atkinson. 2003. Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* 421:388-392.
113. Barrat, F. J., D. J. Cua, A. Boonstra, D. F. Richards, C. Crain, H. F. Savelkoul, R. de Waal-Malefyt, R. L. Coffman, C. M. Hawrylowicz, and A. O'Garra. 2002. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 195:603-616.
114. Levings, M. K., R. Sangregorio, C. Sartirana, A. L. Moschin, M. Battaglia, P. C. Orban, and M. G. Roncarolo. 2002. Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J Exp Med* 196:1335-1346.
115. Chen, Y., V. K. Kuchroo, J. Inobe, D. A. Hafler, and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265:1237-1240.
116. Weiner, H. L. 2001. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* 182:207-214.
117. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198:1875-1886.
118. Goudy, K. S., B. R. Burkhardt, C. Wasserfall, S. Song, M. L. Campbell-Thompson, T. Brusko, M. A. Powers, M. J. Clare-Salzler, E. S. Sobel, T. M. Ellis, T. R. Flotte, and M. A. Atkinson. 2003. Systemic overexpression of IL-10 induces CD4+CD25+ cell populations in vivo and ameliorates type 1 diabetes in nonobese diabetic mice in a dose-dependent fashion. *J Immunol* 171:2270-2278.
119. Rao, P. E., A. L. Petrone, and P. D. Ponath. 2005. Differentiation and expansion of T cells with regulatory function from human peripheral lymphocytes by stimulation in the presence of TGF-{beta}. *J Immunol* 174:1446-1455.
120. Zheng, S. G., J. H. Wang, J. D. Gray, H. Soucier, and D. A. Horwitz. 2004. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol* 172:5213-5221.
121. Carrier, Y., J. Yuan, V. K. Kuchroo, and H. L. Weiner. 2007. Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. *J Immunol* 178:179-185.
122. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 299:1033-1036.
123. Taylor, A., J. Verhagen, K. Blaser, M. Akdis, and C. A. Akdis. 2006. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. *Immunology* 117:433-442.
124. Wan, Y. Y., and R. A. Flavell. 2006. The roles for cytokines in the generation and maintenance of regulatory T cells. *Immunol Rev* 212:114-130.
125. Fillatreau, S., C. H. Sweenie, M. J. McGeachy, D. Gray, and S. M. Anderton. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 3:944-950.
126. Mizoguchi, A., E. Mizoguchi, H. Takedatsu, R. S. Blumberg, and A. K. Bhan. 2002. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* 16:219-230.
127. Evans, J. G., K. A. Chavez-Rueda, A. Eddaoudi, A. Meyer-Bahlburg, D. J. Rawlings, M. R. Ehrenstein, and C. Mauri. 2007. Novel suppressive function of transitional 2 B cells in experimental arthritis. *J Immunol* 178:7868-7878.
128. Mauri, C., D. Gray, N. Mushtaq, and M. Londei. 2003. Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* 197:489-501.
129. Gray, M., K. Miles, D. Salter, D. Gray, and J. Savill. 2007. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci U S A* 104:14080-14085.
130. Parekh, V. V., D. V. Prasad, P. P. Banerjee, B. N. Joshi, A. Kumar, and G. C. Mishra. 2003. B cells activated by lipopolysaccharide, but not by anti-Ig and anti-CD40 antibody, induce anergy in CD8+ T cells: role of TGF-beta 1. *J Immunol* 170:5897-5911.
131. Tian, J., D. Zekzer, L. Hanssen, Y. Lu, A. Olcott, and D. L. Kaufman. 2001. Lipopolysaccharide-activated B cells down-regulate Th1 immunity and prevent autoimmune diabetes in nonobese diabetic mice. *J Immunol* 167:1081-1089.
132. Reichardt, P., B. Dornbach, S. Rong, S. Beissert, F. Gueler, K. Loser, and M. Gunzer. 2007. Naive B cells generate regulatory T cells in the presence of a mature immunologic synapse. *Blood* 110:1519-1529.

133. Wynn, T. A. 2005. T(H)-17: a giant step from T(H)1 and T(H)2. *Nat Immunol* 6:1069-1070.
134. Stockinger, B., and M. Veldhoen. 2007. Differentiation and function of Th17 T cells. *Curr Opin Immunol* 19:281-286.
135. Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8:345-350.
136. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6:1123-1132.
137. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.
138. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179-189.
139. Wilson, N. J., K. Boniface, J. R. Chan, B. S. McKenzie, W. M. Blumenschein, J. D. Mattson, B. Basham, K. Smith, T. Chen, F. Morel, J. C. Lecron, R. A. Kastelein, D. J. Cua, T. K. McClanahan, E. P. Bowman, and R. de Waal Malefyt. 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 8:950-957.
140. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235-238.
141. Acosta-Rodriguez, E. V., G. Napolitani, A. Lanzavecchia, and F. Sallusto. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8:942-949.
142. Laurence, A., C. M. Tato, T. S. Davidson, Y. Kanno, Z. Chen, Z. Yao, R. B. Blank, F. Meylan, R. Siegel, L. Hennighausen, E. M. Shevach, and J. O'Shea J. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26:371-381.
143. Amadi-Obi, A., C. R. Yu, X. Liu, R. M. Mahdi, G. L. Clarke, R. B. Nussenblatt, I. Gery, Y. S. Lee, and C. E. Egwuagu. 2007. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. *Nat Med* 13:711-718.
144. Sakaguchi, S., and F. Powrie. 2007. Emerging challenges in regulatory T cell function and biology. *Science* 317:627-629.
145. Lohr, J., B. Knoechel, J. J. Wang, A. V. Villarino, and A. K. Abbas. 2006. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *J Exp Med* 203:2785-2791.
146. Prakken, B. J., S. Albani, and W. van Eden. 2007. Translating immunological tolerance into therapy. *Eur J Immunol* 37:2360-2363.
147. Weisman, M. H. 2002. What are the risks of biologic therapy in rheumatoid arthritis? An update on safety. *J Rheumatol Suppl* 65:33-38.
148. Chatenoud, L., and J. A. Bluestone. 2007. CD3-specific antibodies: a portal to the treatment of autoimmunity. *Nat Rev Immunol* 7:622-632.
149. Hauet-Broere, F., W. W. Unger, J. Garssen, M. A. Hoijer, G. Kraal, and J. N. Samsom. 2003. Functional CD25- and CD25+ mucosal regulatory T cells are induced in gut-draining lymphoid tissue within 48 h after oral antigen application. *Eur J Immunol* 33:2801-2810.
150. Faria, A. M., and H. L. Weiner. 2005. Oral tolerance. *Immunol Rev* 206:232-259.
151. van Eden, W., R. van der Zee, and B. Prakken. 2005. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol* 5:318-330.
152. Wieten, L., F. Broere, R. van der Zee, E. K. Koerkamp, J. Wagenaar, and W. van Eden. 2007. Cell stress induced HSP are targets of regulatory T cells: a role for HSP inducing compounds as anti-inflammatory immuno-modulators? *FEBS Lett* 581:3716-3722.
153. Hauet-Broere, F., L. Wieten, T. Guichelaar, S. Berlo, R. van der Zee, and W. Van Eden. 2006. Heat shock proteins induce T cell regulation of chronic inflammation. *Ann Rheum Dis* 65 Suppl 3:iii65-68.
154. van de Loo, F. A., R. L. Smeets, and W. B. van den Berg. 2004. Gene therapy in animal models of rheumatoid arthritis: are we ready for the patients? *Arthritis Res Ther* 6:183-196.
155. Kramer, R., Y. Zhang, J. Gehrmann, R. Gold, H. Thoenen, and H. Wekerle. 1995. Gene transfer through the blood-nerve barrier: NGF-engineered neuritogenic T lymphocytes attenuate experimental autoimmune neuritis. *Nat Med* 1:1162-1166.
156. Nakajima, A. 2006. Application of cellular gene therapy for rheumatoid arthritis. *Mod Rheumatol* 16:269-275.
157. Tarner, I. H., A. J. Slavin, J. McBride, A. Levicnik, R. Smith, G. P. Nolan, C. H. Contag, and C. G. Fathman. 2003. Treatment of autoimmune disease by adoptive cellular gene therapy. *Ann N Y Acad Sci* 998:512-519.
158. Glant, T. T., A. Finnegan, and K. Mikecz. 2003. Proteoglycan-induced arthritis: immune regulation, cellular mechanisms, and genetics. *Crit Rev Immunol* 23:199-250.
159. Glant, T. T., K. Mikecz, A. Arzoumanian, and A. R. Poole. 1987. Proteoglycan-induced arthritis in BALB/c mice. Clinical features and histopathology. *Arthritis Rheum* 30:201-212.
160. Cao, Y., F. Brombacher, M. Tunyogi-Csapo, T. T. Glant, and A. Finnegan. 2007. Interleukin-4 regulates proteoglycan-induced arthritis by specifically suppressing the innate immune response. *Arthritis Rheum* 56:861-870.
161. Hollo, K., T. T. Glant, M. Garzo, A. Finnegan, K. Mikecz, and E. Buzas. 2000. Complex pattern of Th1 and Th2 activation with a preferential increase of autoreactive Th1 cells in BALB/c mice with proteoglycan (aggrecan)-induced arthritis. *Clin Exp Immunol* 120:167-173.
162. Kaplan, C., J. C. Valdez, R. Chandrasekaran, H. Eibel, K. Mikecz, T. T. Glant, and A. Finnegan. 2002. Th1 and Th2 cytokines regulate proteoglycan-specific autoantibody isotypes and arthritis. *Arthritis Res* 4:54-58.
163. Glant, T. T., G. Cs-Szabo, H. Nagase, J. J. Jacobs, and K. Mikecz. 1998. Progressive polyarthritis induced in BALB/c mice by aggrecan from normal and osteoarthritic human cartilage. *Arthritis Rheum* 41:1007-1018.
164. Mikecz, K., T. T. Glant, and A. R. Poole. 1987. Immunity to cartilage proteoglycans in BALB/c mice with progressive polyarthritis and ankylosing spondylitis induced by injection of human cartilage proteoglycan. *Arthritis Rheum* 30:306-318.
165. Leroux, J. Y., A. Guerassimov, A. Cartman, N. Delaunay, C. Webber, L. C. Rosenberg, S. Banerjee, and A. R. Poole. 1996. Immunity to the G1 globular domain of the cartilage proteoglycan aggrecan can induce inflammatory erosive polyarthritis and spondylitis in BALB/c mice but immunity to G1 is inhibited by covalently bound keratan sulfate in vitro and in vivo. *J Clin Invest* 97:621-632.

166. Kaplan, C. D., Y. Cao, J. S. Verbeek, M. Tunyogi-Csapo, and A. Finnegan. 2005. Development of proteoglycan-induced arthritis is critically dependent on Fcgamma receptor type III expression. *Arthritis Rheum* 52:1612-1619.

CHAPTER 2

Increased Arthritis Susceptibility in Cartilage Proteoglycan-Specific T Cell Receptor-Transgenic Mice

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ABSTRACT

To better understand the role of antigen (arthritogenic epitope)-specific T cells in the development of autoimmune arthritis, a transgenic (Tg) mouse expressing the T cell receptor (TCR) Vα1.1 and Vβ4 chains specific for a dominant arthritogenic epitope (designated 5/4E8) of human cartilage proteoglycan (hPG) aggrecan was generated. This TCR-Tg mouse strain was backcrossed into the PG-induced arthritis (PGIA)-susceptible BALB/c strain and tested for arthritis incidence and severity. $CD4^+$ TCR-Tg T cells carried functionally active TCR specific for a dominant arthritogenic epitope of hPG (5/4E8). T cells of naive TCR-Tg mice were in an activated stage, since the *in vitro* response to hPG or to peptide stimulation induced interferon-γ and interleukin-4 production. TCR-Tg mice uniformly, without exception, developed severe and progressive polyarthritis, even without adjuvant. Inflamed joints showed extensive cartilage degradation and bone erosions, similar to that seen in the arthritic joints of wild type BALB/c mice with PGIA. Spleen cells from both naive and hPG-immunized arthritic TCR-Tg mice could adoptively transfer arthritis when injected into syngeneic BALB/c.SCID recipient mice. Thus, TCR-Tg BALB/c mice display increased arthritis susceptibility and develop aggravated disease upon *in vivo* antigen stimulation. This model using TCR-Tg mice is a novel and valuable research tool for studying mechanisms of antigen (arthritogenic epitope)-driven regulation of arthritis and understanding how T cells recognize autoantigen in the joints. This type of mouse could also be used to develop new immunomodulatory strategies in T cell-mediated autoimmune diseases.

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common human autoimmune diseases, characterized by chronic inflammation of the synovium of diarthrodial joints. Although the etiology of RA is unknown, accumulating evidence indicates that it is a T cell-mediated and autoantibody-dependent disease in which both genetic and environmental factors play crucial roles (1-3). The RA synovium is infiltrated with $CD4^+$ T cells of the T_h1 phenotype (4, 5), and antibodies are also involved in the pathologic mechanisms of joint inflammation and progression of the disease (6, 7). The demonstrated therapeutic efficacy of agents that block T cell activation (8) or deplete B cells (6) in RA patients has confirmed the critical role of the adaptive immune system. Among the candidate autoantigens, the cartilage proteoglycan (PG) aggrecan is one of the target autoantigens in RA joints (9-14). PG is a complex macromolecule consisting of a large core protein (>2,200 amino acids) to which more than 100 glycosaminoglycan and oligosaccharide side chains are covalently attached (15, 16). The core protein of aggrecan is heavily degraded by proteases released during either degenerative or inflammatory processes, resulting in the loss of normal function of articular cartilage (17).

Immunization of BALB/c mice with human cartilage PG (hPG) induces chronic progressive polyarthritis (18). This PG-induced arthritis (PGIA) has many similarities to human RA, as indicated by clinical assessments, radiographic analyses, scintigraphic bone scans, laboratory tests, and histopathologic examination of the peripheral joints (18-21). The development of the disease is based on the development of T and B cell responses that are cross-reactive between the immunizing hPG and self (mouse) cartilage PG (mPG). This cross-reactivity, most likely achieved through epitope spreading, could explain why these T cells home to mouse joints to initiate arthritis (22, 23).

Several lines of evidence indicate T cell involvement in the pathogenesis of PGIA. For example, $CD4^+$ T cells selectively proliferate in response to hPG (24, 25), and arthritis can be prevented when $CD4^+$ T cells are depleted either *in vivo* (26) or *in vitro* prior to adoptive transfer to naive mice (27, 28). A PG-specific T cell hybridoma clone, 5/4E8, can induce arthritis in BALB/c mice (29). Furthermore, $CD4^+$ T cells from arthritic animals are resistant to activation-induced cell death (30). Finally, susceptibility to PGIA is associated with the class II major histocompatibility complex (MHC) ($H\text{-}2^d$ haplotype in BALB/c) (20). In addition, immunization of BALB/c mice with PG induces a dominant T_h1 cell response, and treatment of arthritic mice with interleukin-4 (IL-4) can prevent disease development by inducing a switch from the originally T_h1-polarized response to a T_h2-polarized response (20, 31). The importance of $CD4^+$ T_h1 cells was further supported by the observation that IL-4-deficient BALB/c mice develop a significantly more severe form of the disease than that seen in wild type BALB/c mice (32).

The arthritogenic 5/4E8 T cell hybridoma has a $CD4^+$ T_h1 phenotype and expresses the T cell receptor (TCR) α and β chains (29). These hybridoma cells secrete IL-2 and interferon-γ (IFN-γ), but not IL-4, upon stimulation with hPG, and the antigen-specific response is class II MHC ($I\text{-}A^d$) restricted (29). The epitope recognized by 5/4E8 cells is located in the G1 domain of hPG and has been identified in previous mapping studies as an immunodominant, and possibly the most arthritogenic, T cell epitope of hPG (15, 29). The 5/4E8 hybridoma shows cross-reactivity with a homologous epitope of mPG (15).

In this study, to gain more insight into the role of antigen-specific T cells in the development of autoimmune arthritis, we generated transgenic (Tg) mice expressing the

TCR of the 5/4E8 hybridoma. We found that a single PG injection provoked a severe form of PGIA in TCR-5/4E8-Tg (hereafter referred to as TCR-Tg) BALB/c mice. Our experiments demonstrated that splenocytes from both naive TCR-Tg mice, after *in vivo* activation, and hPG-immunized arthritic TCR-Tg mice could adoptively transfer arthritis into BALB/c.SCID recipient mice.

MATERIALS AND METHODS

Isolation, amplification, and cloning of the α- and β-chains of TCR-5/4E8

T cell hybridoma 5/4E8 (29), a $CD4^+$ T_h1 cell line, recognizes the most dominant arthritogenic peptide sequence, ^{70}ATEGRVRVNSAYQDK^{84} (referred to as peptide P70; underline indicates the core sequence), in the G1 domain of hPG, and cross-reacts with the mouse homolog sequence, ATEGQVRVNS*/*YQDK (mPG P70; italics indicate the substitutions). T cell hybridoma 5/4E8 carries Vα1.1 chain and Vβ4 chain of the TCR (GeneBank accession no. AY823583 and U19234, respectively).

Genomic DNA was isolated from 5/4E8 hybridoma cells to obtain full-length, rearranged TCRα and TCRβ DNA, including leader and intron sequences, and the corresponding regions were amplified by polymerase chain reaction (PCR). The 2 PCR fragments were cloned into the pGEM-T Easy Vector (Promega, Madison, WI), introduced in *Escherichia coli* DH5α cells (Invitrogen, Breda, The Netherlands), and sequenced. The *Xma* I- and *Not* I-released DNA fragment containing the TCR-5/4E8 α-chain was recloned into the pTα cassette, and the *Xho* I- and *Sac* II-digested and purified TCR-5/4E8 β-chain fragment was inserted into the pTβ cassette (33). Both of the *in vivo* expression plasmid constructs (pTα and pTβ) were generous gifts from C. Benoist and D. Mathis (Brigham and Women's Hospital, Boston, MA). Subsequently, XL10-Gold cells (Stratagene, La Jolla, CA) were transfected with the constructs, and the correct sequences were confirmed.

Generation of TCRα/β-Tg mice

Linearized pTα TCR-5/4E8 and pTβ TCR-5/4E8 DNA fragments were purified by electroelution, phenol extraction, and ethanol precipitation. Both TCR fragments were coinjected in equal amounts into the pronuclei of fertilized eggs of (CBAxC57BL/6)F_1 mice (Charles River Laboratories, Sulzfeld, Germany). TCR-Tg founders were identified by PCR analysis of tail genomic DNA. The TCR α1 chain was genotyped by PCR using forward primer 5'-TGC TCC AGG CTA ATG GTA CA-3' and reverse primer 5'-CGC TCT CCT GAC TAG GGA TG-3'; the Vβ4 chain was detected using forward primer 5'-CTC GAG CAC TGC TAT GGG CTC CAT-3' and reverse primer 5'-CCC AAT CCC GCG GAG AAC-3'. The expression of TCR-Vβ4 was confirmed by flow cytometric analysis on blood lymphocytes. Unfortunately, flow cytometric analysis of the Vα1.1 chain was impossible due to a lack of Vα1-specific antibody.

Since PGIA was restricted to the susceptible BALB/c strain and the $CD4^+$ hybridoma 5/4E8 was of H-2^d class II MHC (BALB/c origin), the TCR-Tg founders were backcrossed onto BALB/c (H-2^d). A marker-assisted genome screening process was used (20) until the pure BALB/c genomic background was achieved (backcross 8). This was confirmed by using 244 simple sequence-length polymorphic markers as described previously (20). Throughout the backcross processes with the TCR-Tg mice, the coexpression of Vα1.1 and Vβ4 chains was always detected in one Tg mouse line by genotyping, and all heterozygous TCR-Tg mice were tested for PGIA susceptibility at each backcross level (see below). Finally, male and female heterozygous TCR-Tg BALB/c mice were intercrossed to select homozygous offspring.

Antigens, animals, immunization, and experimental groups

The use of human cartilage, obtained from joint replacement surgeries, for isolation of PG was approved by the Institutional Review Board of Rush University Medical Center (Chicago, IL). hPG aggrecan was extracted with 4M guanidinium chloride as described in detail previously (21, 34). For immunoassays (T cell proliferation or antibody measurements), hPG and mPG were further purified on cesium chloride gradient centrifugation prior to treatment with chondrotinase ABC and endo-β-galactosidase (18, 21, 35).

All animal experiments were approved by the Institutional Animal Care and Use Committee of Rush University Medical Center and by the Animal Experiment Committee of Utrecht University (Utrecht, The Netherlands). hPG was used for immunization of 16-26-week-old TCR-Tg mice or their wild type littermates and age-matched female BALB/c mice (National Cancer Institute (NCI), Kingston Colony, NY), using a standard immunization protocol as described earlier (19, 21). Briefly, the antigen injection (100 µg hPG protein in 100 µl PBS, pH 7.2) was administered intraperitoneally, with or without 2 mg of the synthetic adjuvant dimethyl-dioctadecyl-ammonium bromide (DDA), on days 0 and 21. hPG-immunized TCR-Tg and wild type littermate mice were killed within 3-9 days after the onset of primary PGIA. Spleen cells were isolated for transfer experiments and *in vitro* tests, and sera were collected for antibody and cytokine measurements. Female SCID mice of the BALB/c background (NCI/NCrC.B-17-scid/scid; herein designated BALB/c.SCID) were purchased from the NCI and maintained under germ-free conditions.

Transfer of arthritis using spleen cells from naive and hPG-immunized wild type or TCR-Tg BALB/c mice with arthritis

Approximately 60 BALB/c.SCID mice were used for adoptive transfer experiments (summarized in Table I) to optimize the cell number, time interval between injections (if repeated), and dose of hPG peptide P70 or hPG. For the adoptive transfer experiments, BALB/c.SCID mice received unseparated spleen cells (30 or $15x10^6$ intraperitoneally) from either naive or hPG-immunized arthritic mice. Spleen cells were injected first with 100 µg peptide P70 or 100 µg hPG, or were injected without antigen (see Table I), as described previously (27).

Assessment of arthritis

The paws of mice were examined daily to record abnormalities due to arthritic changes of the joints. The onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of the paws (19, 21). The degree of joint swelling for each paw (scored from 0 to 4) was assessed and expressed as the cumulative arthritis score of 4 paws, with a possible maximum severity index of 16 per animal. The first day of the clinical appearance of paw swelling was recorded as the onset of arthritis. At the end of the experiments, the limbs of arthritic and nonarthritic mice were dissected, fixed, decalcified, and sectioned. The tissue sections were then stained with hematoxylin and eosin for histopathologic examination.

Flow cytometric analysis

Single-cell suspensions of the thymus and spleens of naive or hPG-immunized arthritic wild type and TCR-Tg mice were separated and washed with PBS containing 0.5% bovine serum albumin and 0.01% sodium azide (FACS buffer). Cells were stained with phycoerythrin (PE)-

conjugated anti-Vβ4, anti-CD8, or anti-CD25, fluorescein isothiocyanate (FITC)-conjugated anti-CD19, anti-CD44, or anti-Vβ4, allophycocyanin-conjugated anti-CD4 or peridinin chlorophyll protein-conjugated anti-CD3 monoclonal antibodies (mAb), or identically labeled relevant IgG isotypes as controls (BD Biosciences PharMingen, San Diego, CA) for 30 minutes on ice. After incubation, cells were washed twice with FACS buffer and analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences Immunocytometry, San Jose, CA).

Measurement of antigen-specific T cell responses

Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (Bodinco, Alkmaar, the Netherlands), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and $5x10^{-5}$ M β-mercaptoethanol was used as culture medium. Single-cell suspensions of spleens were cultured in triplicate in 96-well flat-bottom plates (Corning Life Sciences, Schiphol-Rijk, the Netherlands) at $2x10^5$ cells per well, in the presence or absence of peptide P70 (0.1 μg/ml), hPG (10 μg protein/ml), or control mouse peptide P70 (50 μg/ml). Proliferation was determined by overnight incorporation of ^{3}H-thymidine (0.4 μCi per well; Amersham Biosciences Europe, Roosendaal, the Netherlands) and measured using a liquid scintillation counter (Microbeta; Perkin-Elmer, Boston, MA). The magnitude of the proliferative response was expressed as the change in counts per minute, calculated by subtracting the cpm in nonstimulated cultures from the cpm in stimulated cultures.

Cytokine analysis

Supernatants were collected for cytokine assays after 72 hours of culture and analyzed for IL-4 and IFN-γ simultaneously using the Luminex 100 system (Becton Dickinson, Mountain View, CA) according to the method described by Carson and Vignali (36). Briefly, fluoresceinated microbeads coated with enzyme-linked immunosorbent assay (ELISA) capture antibodies (BD Biosciences PharMingen) were added to 50 μl of culture supernatant. Beads were centrifuged after a 45-minute incubation period at room temperature and then washed twice. Biotinylated detection antibodies (BD Biosciences PharMingen) were added, incubated for 15 minutes, and washed, and PE-labeled streptavidin was subsequently added. Cytokines were measured using a Luminex model 100 (Luminex, Austin, TX), and results were analyzed using LMAT software (Becton Dickinson).

Measurement of antigen (PG)-specific antibodies

PG-specific antibodies were measured by ELISA as described previously (19, 27). The 96-well ELISA plates (Corning) were coated overnight with hPG (0.1 μg protein/well) or mPG (0.15 μg protein/well), and the free binding sites were blocked with 1% fat-free milk in PBS. Sera were applied at increasing dilutions, and both total anti-PG antibodies and isotypes of PG-specific antibodies were determined using peroxidase-conjugated goat anti-mouse IgG (Accurate Chemical & Scientific, Westbury, NY) or rat mAb to mouse IgG_1 or IgG_{2a} (BD Biosciences PharMingen) as secondary antibodies (27). Serum antibody levels were calculated relative to the corresponding mouse IgG isotype (IgG_1 or IgG_{2a}) standards (all from BD Biosciences PharMingen) or mouse serum Ig fractions (Sigma-Aldrich, St. Louis, MO) (19, 27).

Statistical analysis

Data are expressed as the mean ± SEM or mean ± SD, as indicated. Statistical analyses were carried out with the nonparametric Mann-Whitney U test, using Prism software (version 3.0; Graphpad Software, San Diego, CA). The significance level was set at a P value less than 0.05.

RESULTS

In vivo expression of the transgenic TCR

The $CD4^+$ cell ratio was higher in the thymus of TCR-Tg mice than in their wild type littermates, and almost all thymocytes (mean ± SEM 91.8 ± 4.0%) from TCR-Tg mice displayed the transgenic TCR-Vβ4 chain (Fig. 1A). In addition, spleen cells from naive TCR-Tg and wild type (littermate) mice were analyzed for surface marker expression. The CD4:CD8 ratio in the spleen cells of TCR-Tg mice was significantly higher (9:1) than that in wild type animals (2:1) (Fig. 1B).

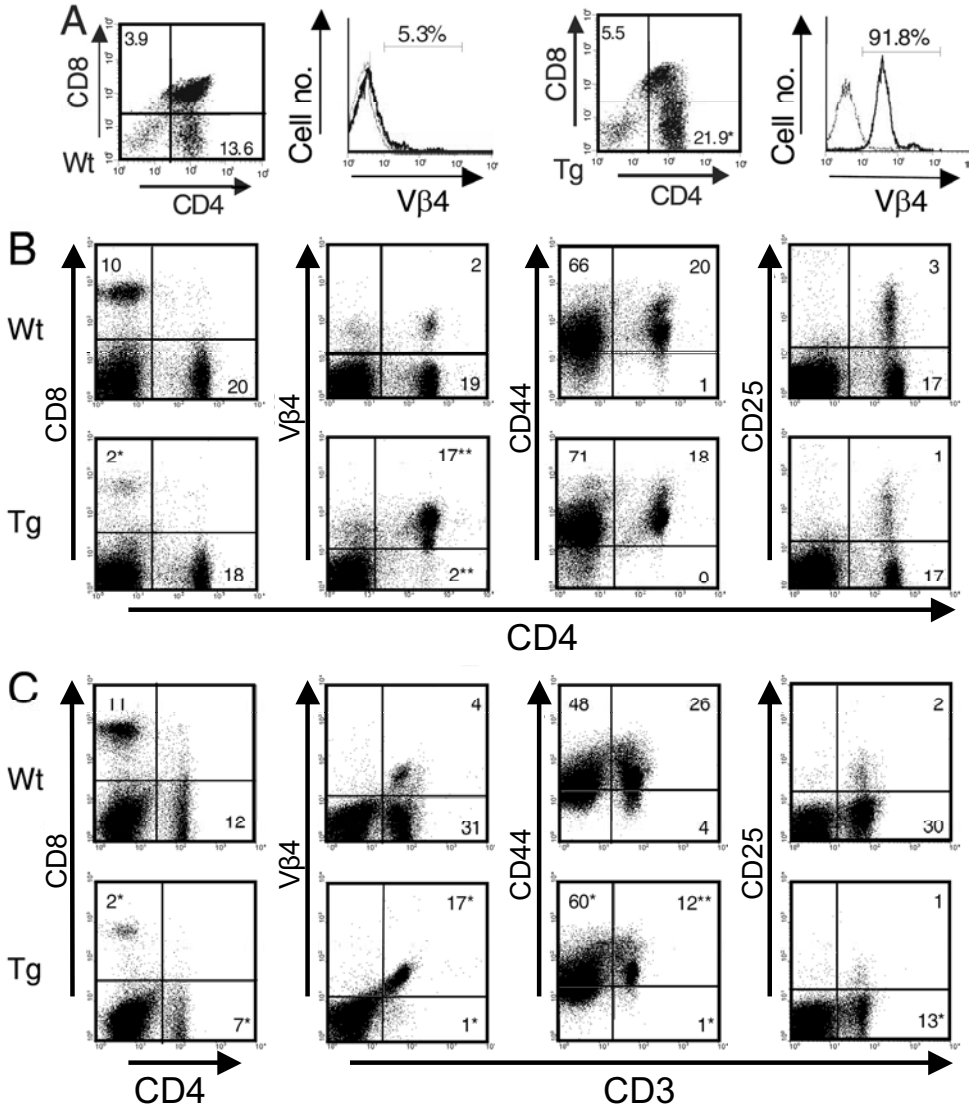

FIGURE 1. Flow cytometric analysis of the expression of cell surface molecules in T cell receptor-transgenic (TCR-Tg) mice. A-C, Cells were isolated from the thymus (A) and spleen (B) of naive (nonimmunized) mice or the spleen (C) of human cartilage proteoglycan-immunized arthritic TCR-Tg mice and their wild type (wt) littermates. The percentages of single- or double-positive cells (the mean values of at least 5 independent assays in A and B, and summary of 8 flow cytometry results in C) are indicated in the quadrants of the scatter plots or in the histograms. * = $P < 0.05$; ** = $P < 0.01$ versus wt mice, by nonparametric Mann-Whitney U test.

Nearly all $CD4^+$ T cells in the TCR-Tg mice expressed the Vβ4 chain, whereas the $CD4^+$:$V\beta4^+$ cell ratio was low (mean ± SEM 2.15 ± 0.93%) in the wild type littermates (Fig. 1B). The T cell:B cell ratio, measured as $CD4^+$:$CD19^+$ expression, was comparable in TCR-Tg and wild type mice (data not shown). Moreover, the expression levels of CD25 and CD44 were comparable in both the spleen cells (Fig. 1B) and popliteal lymph node cells (data not shown) of TCR-Tg and wild type mice, and the Vβ4 chain was present in the majority of TCR-Tg $CD3^+$ T cells (Fig. 1C).

Arthritis in TCR-Tg mice

Since TCRα/β-5/4E8 was already present in heterozygous TCR-Tg mice, and because these $CD4^+$ T cells were hPG reactive (without priming) and also cross-reacted with mPG, we were interested in determining whether the peripheral T cells in Tg mice were spontaneously activated by endogenous mPG. Spontaneous activation of the self-reactive T cells might cause autoimmune arthritis in TCR-Tg mice. Indeed, during the backcrossing process to BALB/c ($H\text{-}2^d$), some TCR-Tg mice spontaneously developed arthritis (<15%). However, the majority of nonimmunized TCR-Tg mice, even on a BALB/c background, remained symptom-free.

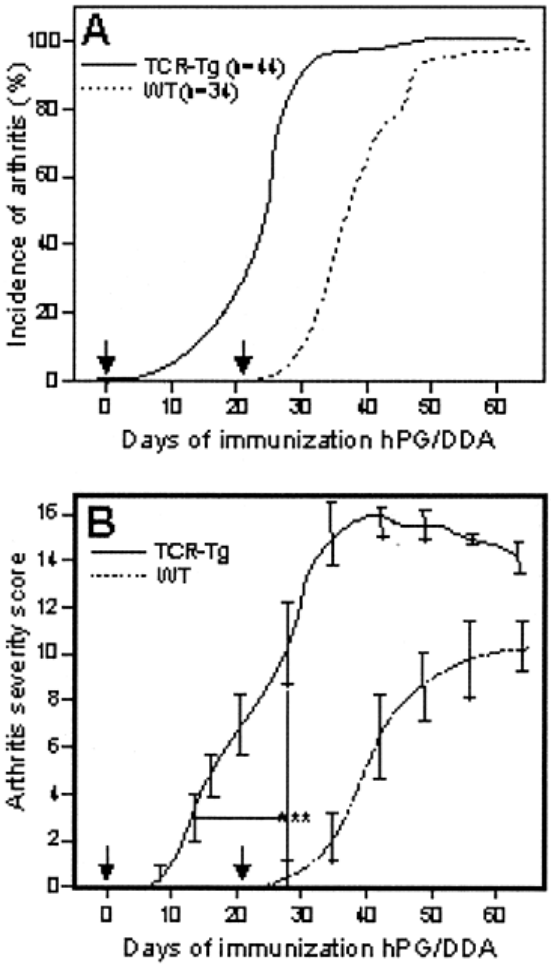

FIGURE 2. Incidence and severity of arthritis in T cell receptor-transgenic (TCR-Tg) and wild type (wt) littermate BALB/c mice. Heterozygous TCR-Tg and wt mice (20-26 weeks old) were immunized with human cartilage proteoglycan in dimethyl-dioctadecyl-ammonium bromide (hPG/DDA) on days 0 and 21 (arrows). A, Incidence of arthritis is expressed as the percentage of arthritic animals relative to the total number of TCR-Tg or wt mice. B, Disease severity is expressed as the mean ± SEM cumulative arthritis score in 2 independent experiments in arthritic animals only, using heterozygous TCR-Tg mice and their littermates after 8 backcrosses to BALB/c (representing the same groups as shown in A). The arthritis score was determined 3-4 times a week by 2 independent investigators, but only the weekly (cumulative) results are shown. *** = $P < 0.001$ between groups from day 27 until the end of the experiment (day 67).

We injected TCR-Tg and wild type BALB/c mice with hPG in DDA adjuvant intraperitoneally on day 0 and, if necessary, on day 21, using a standard immunization protocol (19, 21). Approximately 25-30% of the TCR-Tg mice developed arthritis after the first hPG-DDA injection, whereas in the wild type littermates, onset of arthritis occurred 8-10 days after the second hPG-DDA injection (Fig. 2A). When unaffected TCR-Tg mice and their wild type littermates were boosted on day 21, the incidence of arthritis increased quickly and, in the TCR-Tg mice, reached 100% within a few days, with development of more severe disease than that in their wild type littermates (Fig. 2A and B). Nevertheless, 95-96% of age-matched wild type littermates also developed arthritis, but with later onset (after the second hPG-DDA injection) and milder clinical symptoms than in the TCR-Tg mice (Fig. 2A and B).

We also tested whether hPG alone (without adjuvant) could induce arthritis in TCR-Tg mice, especially in aging animals (older than 20-26 weeks). These experiments indicated that hPG without adjuvant could induce arthritis in TCR-Tg mice, but not in wild type animals (Fig. 3). Although disease severity was lower in the TCR-Tg mice injected with hPG alone as compared with the TCR-Tg mice that received hPG in DDA adjuvant, our results suggest that constitutive (over)expression of TCRα/β-5/4E8 partially bypassed the requirement for adjuvant in the induction of arthritis.

The clinical appearance and histopathologic features of arthritis in the TCR-Tg mice, as indicated by comparable arthritis scores and patterns of onset (Fig. 4B), were very similar to those seen in the primary form of PGIA (18, 20) in wild type BALB/c mice. Joint inflammation resulted in pannus formation and extensive cartilage and bone erosions, leading to massive ankylosis and deformities of the peripheral joints in TCR-Tg mice (compare Fig. 4A with Fig. 4B), as has been reported for PGIA in wild type BALB/c mice (18-20).

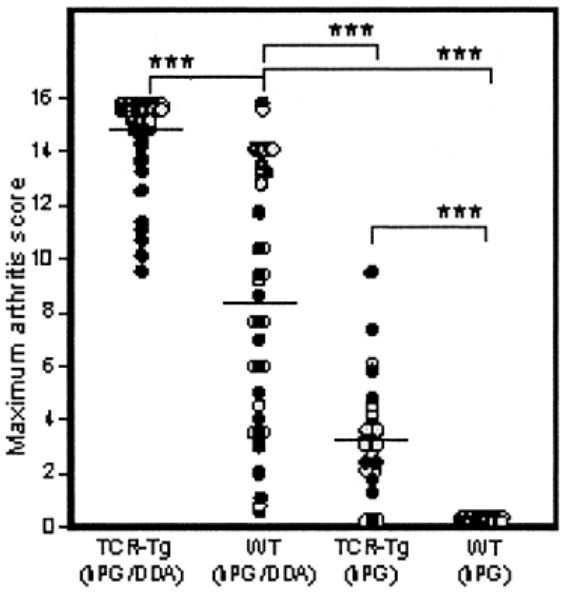

FIGURE 3. Immunization of T cell receptor-transgenic (TCR-Tg) mice with human cartilage proteoglycan (hPG) with or without adjuvant dimethyl-dioctadecyl-ammonium bromide (DDA) (at backcross level 8) in relation to severity of arthritis, as compared with wild type (wt) littermates. Each circle (either solid or open) represents 1 animal. The first 2 groups represent the same animals as in the longitudinal study shown in Figure 2. Mean values are indicated with horizontal lines. The mean ± SEM maximum arthritis scores were 14.6 ± 1.9 (n = 44), 8.6 ± 4.9 (n = 34), and 3.2 ± 2.1 (n = 24) in the the TCR-Tg and wt groups receiving hPG/DDA and the TCR-Tg group receiving hPG alone, respectively; this was negligible in the wt group receiving hPG alone (n = 24). *** = P < 0.001 between groups, by nonparametric Mann-Whitney U test.

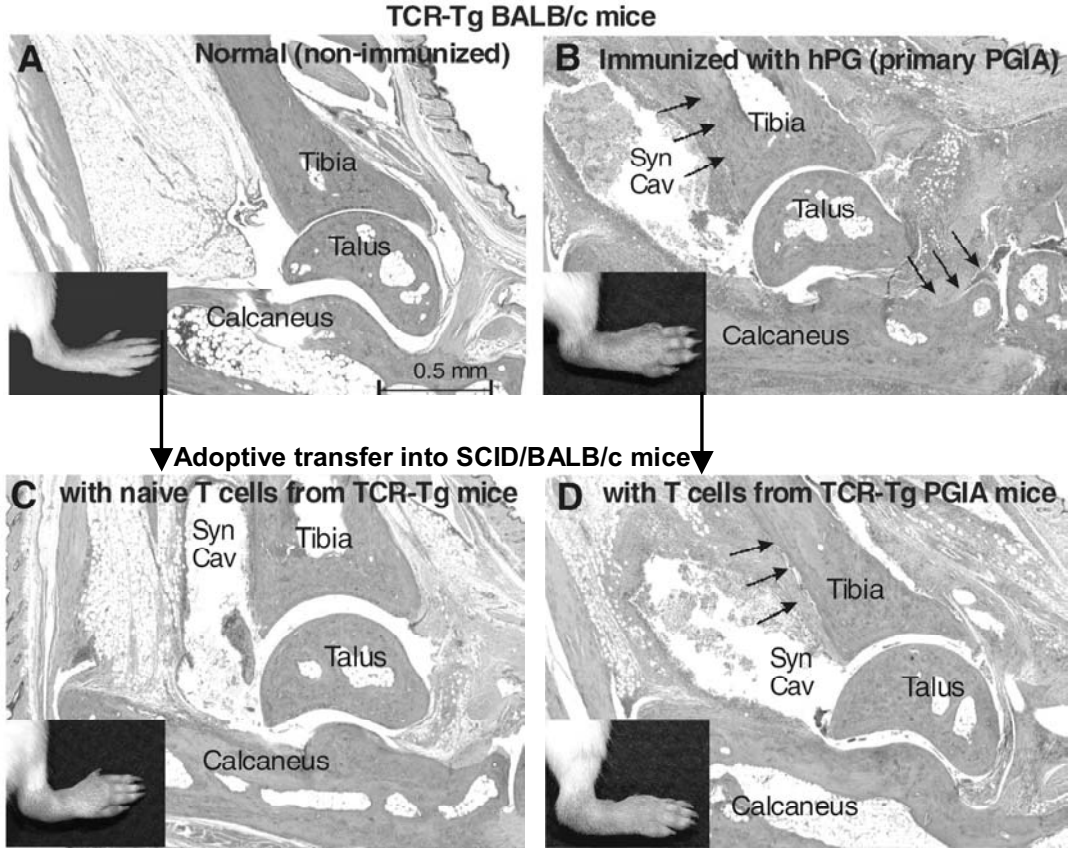

FIGURE 4. Histological analysis of inflammation in ankle joints. A-D, Histologic analysis of the ankle joints (insets) of naive (nonimmunized) mice (A) and human cartilage proteoglycan (hPG)-immunized T cell receptor-transgenic (TCR-Tg) BALB/c mice (B), and after adoptive transfer into BALB/c.SCID mice (C and D). These BALB/c.SCID mice received spleen cells intraperitoneally ($30x10^6$ and $15x10^6$ cells, 1 week apart) from naive TCR-Tg mice (C) or from hPG-immunized arthritic TCR-Tg mice (D). The first cell injection was administered along with 100 µg of hPG, and spleen cells were harvested from acutely arthritic (having arthritis for <1 week) or naive TCR-Tg mice. Arthritic mice were killed 6-7 days after the onset of arthritis, when the joints showed massive cartilage and bone erosions (arrows). SynCav indicates the expanded synovial cavity filled with a large volume of synovial fluid and inflammatory cells. Synovial hyperplasia accompanied by infiltrating cells and pannus formation is evident in all arthritic joints (B-D). The overall histopathologic features of joint inflammation and tissue destruction in a BALB/c.SCID mouse receiving spleen cells from naive TCR-Tg mice (C) are similar, but the joint damage is less extensive, than in another BALB/c.SCID mouse that received spleen cells from arthritic TCR-Tg mice (D). Sections are stained with hematoxylin and eosin. PGIA = proteoglycan-induced arthritis.

Recognition of peptide P70 and hPG by peripheral T cells of TCR-Tg mice

Peripheral T cells of Tg mice were examined for *in vitro* responses to peptide P70 and to hPG. Spleen cells from naive TCR-Tg mice proliferated vigorously when stimulated with peptide P70 or hPG (Fig. 5A), but did not respond to the control peptide (data not shown). A hPG-specific response was not detected in spleen cell cultures from naive wild type littermates (Fig. 5A). Although T cell responses to hPG or peptide P70 were higher in naive TCR-Tg mice than in hPG-immunized arthritic TCR-Tg or wild type mice, T cell proliferation in the presence of self (mouse) peptide P70 (5/4E8 epitope homolog) was detectable only in cell cultures of arthritic mice (Fig. 5A).

IL-4 and IFN-γ production was detected in naive TCR-Tg mice after *in vitro* stimulation with peptide P70, which then dramatically shifted toward the T_h1 bias (IL-4 < IFN-

γ) at the time of onset of arthritis (Fig. 5B). In contrast, the production of IFN-γ after *in vitro* stimulation was much higher in hPG-DDA-immunized arthritic TCR-Tg mice. These data indicate that T cells from naive Tg mice were not tolerized; instead, they appeared to be differentiated *in vivo* because they responded vigorously to peptide P70 by proliferation, as well as by production of IFN-γ and IL-4.

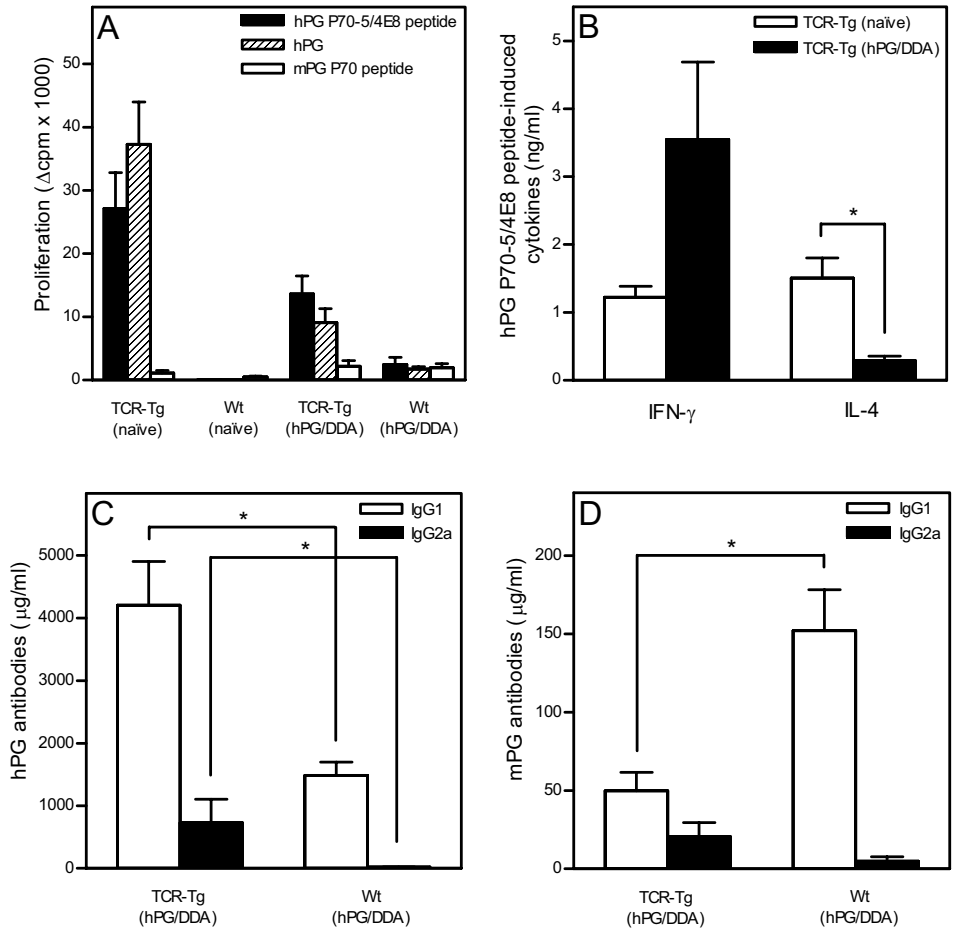

FIGURE 5. Analysis of antigen-specific T and B cell responses in naive and arthritic T cell receptor-transgenic (TCR-Tg) mice. The human cartilage proteoglycan-dimethyl-dioctadecyl-ammonium bromide (hPG/DDA)-immunized arthritic TCR-Tg and wild type (wt) mice were killed 9 days after arthritis onset (at ~31 days). Naive TCR-Tg and wt mice were used as controls. Bars show the mean and SEM of 4-10 mice per group. A, Proliferation assays involving spleen cells (2×10^5/well) cultured in the presence of peptide P70 (0.1 μg/ml), hPG (10 μg/ml), or murine PG (mPG) P70 peptide (50 μg/ml). B, Cytokine production in supernatants of peptide P70-stimulated spleen cell cultures of naive and hPG-immunized (arthritic) TCR-Tg mice. Cells were harvested after 72 hours and assayed by Luminex analysis for interferon-γ (IFN-γ) and interleukin-4 (IL-4). C and D, Production of serum PG-specific antibodies to immunizing hPG (C) and mPG (D) in the sera of arthritic TCR-Tg and wt mice. * = $P < 0.01$.

PG-specific antibodies could not be detected in nonimmunized mice but were present at high levels in hPG-immunized wild type and TCR-Tg mice. Most of the antibodies were specific for the immunizing hPG (Fig. 5C), although there was a clear antibody response against self PG (mPG) (Fig. 5D). The amounts of hPG-specific IgG_1 and IgG_{2a} antibodies

were significantly higher in TCR-Tg mice than in wild type mice ($P < 0.01$). While antibodies of the IgG_1 isotype were predominantly against either hPG (Fig. 5C) or mPG (Fig. 5D) in both TCR-Tg and wild type BALB/c mice, the IgG_{2a}:IgG_1 ratios of antibodies to either hPG or mPG were ~10 times higher in Tg mice than in wild type control mice.

Adoptive transfer to BALB/c.SCID mice

To test whether spleen cells of TCR-Tg mice could transfer arthritis adoptively, we injected unseparated spleen cells from naive and arthritic TCR-Tg mice and from arthritic wild type (BALB/c) mice into syngeneic BALB/c.SCID recipient mice. The results of these transfer experiments are summarized in Table I. When SCID mice were injected with naive TCR-Tg cells alone (without activation), no arthritis was adopted. However, when these naive TCR-Tg cells were injected together with either peptide P70 or hPG, some of the recipient animals developed arthritis (Table I). The onset of arthritis was relatively late (~27 days after transfer), with a maximum incidence of 57% (Table I). In contrast, spleen cells from arthritic TCR-Tg mice could induce arthritis upon a single transfer, which was even faster and more severe when cells from arthritic mice were coinjected with either peptide P70 or hPG. Remarkably, in this case, only 1 cell transfer was sufficient to induce arthritis in the BALB/c.SCID recipient mice (Table I), which cannot be easily achieved using spleen cells from arthritic wild type BALB/c mice (27).

The clinical appearance of the disease and the histopathologic characteristics of the acutely inflamed joints of TCR-Tg and wild type mice with adoptively transferred arthritis were similar (Fig. 4C and D) and indistinguishable from those described in primary PGIA (18, 20). Synovial hyperplasia accompanied by infiltrating cells and pannus formation were evident in all arthritic joints. The overall histopathologic features of joint inflammation and tissue destruction in the BALB/c.SCID mice that received spleen cells from naive TCR-Tg mice were somewhat less extensive than in the BALB/c.SCID mouse that received spleen cells from arthritic TCR-Tg mice (Fig. 4C and D).

Table I. Summary of adoptive transfer experiments using spleen cells from wild type and TCR-5/4E8-Tg mice into syngeneic BALB/c.SCID mice

Source of donor cells	Number of cells ($x10^6$) per transfer[a]	Challenging antigen (peptide or hPG)[a]	No. of arthritic animals/ total no. of animals	Incidence (%)	Arthritis onset (days)		Maximum arthritis severity score[b] (mean ± SD)
					Earliest	Mean (± SD)	
Naive TCR-Tg Mice	up to 4x30	None	0/8	0	NA	NA	NA
	30 + 15	P70-5/4E8	2/10	20	24	28.0 ± 5.7	3.25 ± 1.77
	30 + 15	hPG	8/14	57	22	26.3 ± 4.0	5.43 ± 2.99
Arthritic TCR-Tg mice (PGIA)	30 + 15	None	7/10	70	14	18.4 ± 4.6	4.14 ± 4.68
	30	P70-5/4E8	8/9	89	16	18.6 ± 2.8	9.75 ± 4.98
	30	hPG	13/15	87	6	9.8 ± 2.9	8.85 ± 2.94
Arthritic wild type (BALB/c) mice (PGIA)	30 + 15	None	1/15	6.7	42	42	3
	30 + 15	P70-5/4E8	1/9	11	36	36	4
	30 + 15	hPG	15/17	88	7	12.1 ± 3.3	9.53 ± 4.63

[a]Spleen cells ($30x10^6$) were injected intraperitoneally alone or with 100 µg human cartilage proteoglycan (hPG) P70-5/4E8 peptide (peptide P70; ^{70}ATEGRVRVNSAYQDK) or 100 µg hPG alone. A second spleen cell transfer ($15x10^6$ cells), if indicated, was administered 1 week later without peptide or hPG The first group received 4 times $30x10^6$ spleen cells from naive (nonimmunized) T cell receptor-transgenic (TCR-Tg) mice. NA = not applicable. [b]Animals were scored daily for arthritis symptoms (maximum score of 16 per animal), and all were killed on days 49-52 after the first transfer. Adoptive transfer experiments were performed after the backcrossing process into the BALB/c background was completed.

DISCUSSION

PGIA is a T cell-dependent and antibody (B cell)-mediated autoimmune model of RA (20). We herein describe a novel and exaggerated model of PGIA, wherein TCR-5/4E8-Tg mouse T cells, mostly $CD4^+$, respond only to a single arthritogenic epitope of hPG. These TCR-Tg mice represent a unique source of naive antigen (arthritogenic epitope)-specific T cells that are capable of inducing progressive chronic arthritis. Histologic analysis of the inflamed joints showed extensive cartilage and bone erosions similar to those seen in arthritic joints of wild type BALB/c mice, and these features were reminiscent of the histopathologic appearance of RA-affected joints. Arthritis onset, however, was much faster and the disease was more severe in the TCR-Tg mice compared with their wild type littermates.

$CD4^+$ T cells of the TCR-Tg mice carrying the Vα1.1 and Vβ4 chains of the TCR with class II MHC-restricted specificity for the consensus sequence of ^{73}GRVRVNSAY of hPG (15) were positively selected in the thymus and exported to the periphery, where they constituted the vast majority of T cells (Fig. 1). The dominant arthritogenic peptide P70 (the consensus sequence flanked with 3 amino acids at both sides) induced T cell proliferation (Fig. 5A), indicating that a functional TCR was indeed generated in the TCR-Tg mice. In contrast to the classic (original) form of PGIA in wild type BALB/c mice, which required multiple immunizations with hPG in adjuvant (18, 20, 21), a single dose of hPG, even in the absence of adjuvant, produced disease in TCR-Tg mice, whereas injection of the peptide P70 or adjuvant DDA alone did not induce arthritis. Altogether, this new model of PGIA is much more efficient, in the sense that it is epitope restricted. Moreover, since more than 90% of $CD4^+$ T cells carry peptide P70-specific TCRs (Fig. 1A), this transgenic model offers an excellent opportunity to test T cell activation events via a single epitope-specific TCR.

Flow cytometric analysis of cells from TCR-Tg mice showed a marked reduction in the $CD8^+$ thymocyte population (Fig. 1A). This reduction, however, was expected on the basis that the 5/4E8 epitope was class II MHC-restricted. Shrinkage of the $CD8^+$ T cell pool has also been observed in other class II MHC-restricted TCR-Tg mice expressing TCR specific for self antigens, such as type II collagen (37-39) or myelin basic protein (40). In addition to the reduced number of $CD8^+$ cells and the expression of the TCR-Vβ4 chain on almost all $CD4^+$ T cells in TCR-Tg mice, the expression of all other surface markers tested on naive T cells was comparable between TCR-Tg and wild type mice. *In vitro* studies (Fig. 5), however, showed extensive proliferation of TCR-Tg T cells in response to either peptide P70 or hPG, without clear evidence of activation-induced cell death (AICD).

Both IL-4 and IFN-γ cytokine-producing cells were present, in either naive or hPG-immunized Tg mice prior to the onset of inflammation (data not shown). However, the IL-4:IFN-γ ratio shifted significantly toward a T_h1 dominance at the time of onset of arthritis, illustrating a dynamic polarization during *in vivo* T cell activation. The presence of antibodies to both hPG and mPG in sera of arthritic TCR-Tg mice demonstrated that although $CD4^+$ Tg T cells were capable of providing sufficient help *in vivo* for PG-specific B cells (41), and vice versa, B cells probably can transfer signals for T cell activation. Autoantibodies, such as those produced against mPG, could play a role in initiating inflammation in the joints by binding to the cartilage surface (42), and by inducing chemokine and complement-dependent leukocyte recruitment (43).

A remarkable observation from this study was that we could transfer the disease into syngeneic BALB/c.SCID recipients using splenocytes from naive TCR-Tg mice, upon activation of these cells not only with hPG, but also with peptide P70. Moreover, spleen cells

of arthritic TCR-Tg donor mice were able to induce arthritis in recipient BALB/c.SCID mice without exogenous hPG or specific peptide, which was not possible using splenocytes from arthritic wild type donor BALB/c mice (20, 27, 28). In addition, adoptive transfer of PGIA was achieved by the injection of cells from arthritic TCR-Tg mice (Table I). It is conceivable that T cells from arthritic TCR-Tg mice migrate to the joints upon adoptive transfer and become reactivated by mPG in the mouse joint, where self peptides are released during the normal turnover of the cartilage matrix. Production of mPG peptides might be increased when PG degradation occurs in the inflamed joint, thus amplifying the inflammatory response.

T and B cell responses against cartilage matrix components are known to occur in RA, at least in a subset of patients, although the pathogenic role of these autoimmune responses is controversial (1, 44, 45). Among these matrix components, hPG could be a candidate autoantigen in humans. Relevant to this, we previously demonstrated that several predicted epitopes within the core protein of hPG were recognized by T cells in the context of human class II MHC in HLA-DR4- and HLA-DQ8-Tg mice (34). Collectively, the results of the present and earlier studies suggest that hPG, especially the 5/4E8 epitope, not only plays a critical role in PGIA, but more importantly, may become a target of disease-associated T cell responses in patients with RA (12-14).

There are a number of fundamental questions regarding autoimmunity, including the processes seen in RA, which cannot be answered with the use of currently available technology. By using these TCR-Tg mice as a unique source of antigen (epitope)-specific arthritogenic $CD4^+$ T cells, many of these questions, at least in part, can be answered. One of our immediate goals using these TCR-Tg mice is to understand the mechanisms of AICD, which, if defective, may lead to uncontrolled proliferation of antigen-specific peripheral T cells, creating an autoimmune homeostasis. Several lines of evidence indicate that a defect in the apoptotic process is a key component of autoimmunity (46, 47). However, the mechanisms of T cell apoptosis in the breakdown of peripheral T cell tolerance in RA are poorly understood. Each lymphocyte produces only one type of TCR from billions of possibilities (48), and therefore the activation mechanisms of a relatively low number of self-reactive T cells cannot be studied. Consequently, AICD is studied *in vitro* by an indirect method using immobilized anti-CD3 and soluble anti-CD28 antibodies, which activate all T cells in a nonspecific manner. Although the activation of all T cells by anti-CD3/CD28 antibodies is an artificial approach, this has been the only, and widely used, method to monitor intracellular events of activated T cells.

We have detected AICD in our PGIA model (30), which is characteristic of T cells in RA patients (49), but the mechanisms could be studied only through this nonspecific and general T cell activation, i.e., via all TCRs of all T lymphocytes. The high expression of antigen (peptide)-specific TCR of the $CD4^+$ T cells of these TCR-Tg mice allows us to directly study AICD via an arthritogenic epitope-specific mechanism. Recently, we generated HLA-$DR4^+$/$DQ8^+$ (and human $CD4^+$), humanized BALB/c.SCID Tg knockout mice, which do not have their own MHC ($H\text{-}2^d$). These humanized immunodeficient mice are being intercrossed with TCR-Tg animals to create a highly specific autoimmune environment, in which additional compounds (antibodies, B cells of different specificity, peptide P70 with altered sequence) can be used in a highly controlled environment (50).

In summary, based on the clinical and histopathologic features and the autoimmune aspects shared between PGIA and RA (20), we believe that these TCR-Tg mice are valuable tools for further analysis of the mechanisms associated with the initiation and pathogenesis

of autoimmune arthritis, and more specifically, for the analysis of the role of antigen-specific T cells in disease development. Our TCR-Tg model also supports the hypothesis that antigen-specific T cells play a critical role in the initiation of arthritis, since disease could be induced in the absence of adjuvant. Given the paucity of relevant animal models for RA, this novel TCR-Tg mouse model is a valuable addition to the arthritis research armamentarium. Furthermore, these TCR-Tg mice may be useful for developing new immunomodulating agents, which might provide further insights into the immunopathogenic mechanisms of RA.

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REFERENCES

1. Goronzy, J. J., and C. M. Weyand. 2005. Rheumatoid arthritis. *Immunol Rev* 204:55-73.
2. Klareskog, L., and H. McDevitt. 1999. Rheumatoid arthritis and its animal models: the role of TNF-alpha and the possible absence of specific immune reactions. *Curr Opin Immunol* 11:657-662.
3. Weyand, C. M., J. J. Goronzy, S. Takemura, and P. J. Kurtin. 2000. Cell-cell interactions in synovitis. Interactions between T cells and B cells in rheumatoid arthritis. *Arthritis Res* 2:457-463.
4. Dolhain, R. J., A. N. van der Heiden, N. T. ter Haar, F. C. Breedveld, and A. M. Miltenburg. 1996. Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joints of patients with rheumatoid arthritis. *Arthritis Rheum* 39:1961-1969.
5. van der Graaff, W. L., A. P. Prins, B. A. Dijkmans, and R. A. van Lier. 1998. Prognostic value of Th1/Th2 ratio in rheumatoid arthritis. *Lancet* 351:1931.
6. Edwards, J. C., L. Szczepanski, J. Szechinski, A. Filipowicz-Sosnowska, P. Emery, D. R. Close, R. M. Stevens, and T. Shaw. 2004. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med* 350:2572-2581.
7. van Boekel, M. A., E. R. Vossenaar, F. H. van den Hoogen, and W. J. van Venrooij. 2002. Autoantibody systems in rheumatoid arthritis: specificity, sensitivity and diagnostic value. *Arthritis Res* 4:87-93.
8. Dumont, F. J. 2004. Technology evaluation: abatacept, Bristol-Myers Squibb. *Curr Opin Mol Ther* 6:318-330.
9. Glant, T., J. Csongor, and T. Szucs. 1980. Immunopathologic role of proteoglycan antigens in rheumatoid joint disease. *Scand J Immunol* 11:247-252.
10. Golds, E. E., I. B. Stephen, J. M. Esdaile, H. Strawczynski, and A. R. Poole. 1983. Lymphocyte transformation to connective tissue antigens in adult and juvenile rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, systemic lupus erythematosus, and a nonarthritic control population. *Cell Immunol* 82:196-209.
11. Goodstone, N. J., M. C. Doran, R. N. Hobbs, R. C. Butler, J. J. Dixey, and B. A. Ashton. 1996. Cellular immunity to cartilage aggrecan core protein in patients with rheumatoid arthritis and non-arthritic controls. *Ann Rheum Dis* 55:40-46.
12. Guerassimov, A., Y. Zhang, S. Banerjee, A. Cartman, J. Y. Leroux, L. C. Rosenberg, J. Esdaile, M. A. Fitzcharles, and A. R. Poole. 1998. Cellular immunity to the G1 domain of cartilage proteoglycan aggrecan is enhanced in patients with rheumatoid arthritis but only after removal of keratan sulfate. *Arthritis Rheum* 41:1019-1025.
13. Li, N. L., D. Q. Zhang, K. Y. Zhou, A. Cartman, J. Y. Leroux, A. R. Poole, and Y. P. Zhang. 2000. Isolation and characteristics of autoreactive T cells specific to aggrecan G1 domain from rheumatoid arthritis patients. *Cell Res* 10:39-49.
14. Zou, J., Y. Zhang, A. Thiel, M. Rudwaleit, S. L. Shi, A. Radbruch, R. Poole, J. Braun, and J. Sieper. 2003. Predominant cellular immune response to the cartilage autoantigenic G1 aggrecan in ankylosing spondylitis and rheumatoid arthritis. *Rheumatology (Oxford)* 42:846-855.
15. Glant, T. T., E. I. Buzas, A. Finnegan, G. Negroiu, G. Cs-Szabo, and K. Mikecz. 1998. Critical roles of glycosaminoglycan side chains of cartilage proteoglycan (aggrecan) in antigen recognition and presentation. *J Immunol* 160:3812-3819.
16. Rosenberg, L. C., and J. A. Buckwalter. 1986. Cartilage proteoglycans. *In: Kuettner KE , Schleyerbach R , Hascall VC , editors. Articular cartilage biochemistry. New York: Raven Press*:39-57.
17. Poole, A. R., M. Ionescu, A. Swan, and P. A. Dieppe. 1994. Changes in cartilage metabolism in arthritis are reflected by altered serum and synovial fluid levels of the cartilage proteoglycan aggrecan. Implications for pathogenesis. *J Clin Invest* 94:25-33.
18. Glant, T. T., K. Mikecz, A. Arzoumanian, and A. R. Poole. 1987. Proteoglycan-induced arthritis in BALB/c mice. Clinical features and histopathology. *Arthritis Rheum* 30:201-212.
19. Hanyecz, A., S. E. Berlo, S. Szanto, C. P. Broeren, K. Mikecz, and T. T. Glant. 2004. Achievement of a synergistic adjuvant effect on arthritis induction by activation of innate immunity and forcing the immune response toward the Th1 phenotype. *Arthritis Rheum* 50:1665-1676.
20. Glant, T. T., A. Finnegan, and K. Mikecz. 2003. Proteoglycan-induced arthritis: immune regulation, cellular mechanisms, and genetics. *Crit Rev Immunol* 23:199-250.
21. Glant, T. T., and K. Mikecz. 2004. Proteoglycan aggrecan-induced arthritis: a murine autoimmune model of rheumatoid arthritis. *Methods Mol Med* 102:313-338.
22. Mikecz, K., and T. T. Glant. 1994. Migration and homing of lymphocytes to lymphoid and synovial tissues in proteoglycan-induced murine arthritis. *Arthritis Rheum* 37:1395-1403.
23. Gal, I., E. Bajnok, S. Szanto, B. Sarraj, T. T. Glant, and K. Mikecz. 2005. Visualization and in situ analysis of leukocyte trafficking into the ankle joint in a systemic murine model of rheumatoid arthritis. *Arthritis Rheum* 52:3269-3278.
24. Buzas, E. I., K. Mikecz, F. R. Brennan, and T. T. Glant. 1994. Mediators and autopathogenic effector cells in proteoglycan-induced arthritic and clinically asymptomatic BALB/c mice. *Cell Immunol* 158:292-304.
25. Zhang, Y., A. Guerassimov, J. Y. Leroux, A. Cartman, C. Webber, R. Lalic, E. de Miguel, L. C. Rosenberg, and A. R. Poole. 1998. Arthritis induced by proteoglycan aggrecan G1 domain in BALB/c mice. Evidence for t cell involvement and the immunosuppressive influence of keratan sulfate on recognition of t and b cell epitopes. *J Clin Invest* 101:1678-1686.
26. Banerjee, S., C. Webber, and A. R. Poole. 1992. The induction of arthritis in mice by the cartilage proteoglycan aggrecan: roles of CD4+ and CD8+ T cells. *Cell Immunol* 144:347-357.
27. Bardos, T., K. Mikecz, A. Finnegan, J. Zhang, and T. T. Glant. 2002. T and B cell recovery in arthritis adoptively transferred to SCID mice: antigen-specific activation is required for restoration of autopathogenic CD4+ Th1 cells in a syngeneic system. *J Immunol* 168:6013-6021.
28. Mikecz, K., T. T. Glant, E. Buzas, and A. R. Poole. 1990. Proteoglycan-induced polyarthritis and spondylitis adoptively transferred to naive (nonimmunized) BALB/c mice. *Arthritis Rheum* 33:866-876.
29. Buzas, E. I., F. R. Brennan, K. Mikecz, M. Garzo, G. Negroiu, K. Hollo, G. Cs-Szabo, E. Pintye, and T. T. Glant. 1995. A proteoglycan (aggrecan)-specific T cell hybridoma induces arthritis in BALB/c mice. *J Immunol* 155:2679-2687.
30. Zhang, J., T. Bardos, K. Mikecz, A. Finnegan, and T. T. Glant. 2001. Impaired Fas signaling pathway is involved in defective T cell apoptosis in autoimmune murine arthritis. *J Immunol* 166:4981-4986.
31. Finnegan, A., K. Mikecz, P. Tao, and T. T. Glant. 1999. Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. *J Immunol* 163:5383-5390.

32. Finnegan, A., M. J. Grusby, C. D. Kaplan, S. K. O'Neill, H. Eibel, T. Koreny, M. Czipri, K. Mikecz, and J. Zhang. 2002. IL-4 and IL-12 regulate proteoglycan-induced arthritis through Stat-dependent mechanisms. *J Immunol* 169:3345-3352.
33. Kouskoff, V., K. Signorelli, C. Benoist, and D. Mathis. 1995. Cassette vectors directing expression of T cell receptor genes in transgenic mice. *J Immunol Methods* 180:273-280.
34. Szanto, S., T. Bardos, Z. Szabo, C. S. David, E. I. Buzas, K. Mikecz, and T. T. Glant. 2004. Induction of arthritis in HLA-DR4-humanized and HLA-DQ8-humanized mice by human cartilage proteoglycan aggrecan but only in the presence of an appropriate (non-MHC) genetic background. *Arthritis Rheum* 50:1984-1995.
35. Zhang, J., K. Mikecz, A. Finnegan, and T. T. Glant. 2000. Spontaneous thymocyte apoptosis is regulated by a mitochondrion-mediated signaling pathway. *J Immunol* 165:2970-2974.
36. Carson, R. T., and D. A. Vignali. 1999. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. *J Immunol Methods* 227:41-52.
37. Brand, D. D., L. K. Myers, K. B. Whittington, K. A. Latham, J. M. Stuart, A. H. Kang, and E. F. Rosloniec. 2002. Detection of early changes in autoimmune T cell phenotype and function following intravenous administration of type II collagen in a TCR-transgenic model. *J Immunol* 168:490-498.
38. Mori, L., H. Loetscher, K. Kakimoto, H. Bluethmann, and M. Steinmetz. 1992. Expression of a transgenic T cell receptor beta chain enhances collagen-induced arthritis. *J Exp Med* 176:381-388.
39. Osman, G. E., S. Cheunsuk, S. E. Allen, E. Chi, H. D. Liggitt, L. E. Hood, and W. C. Ladiges. 1998. Expression of a type II collagen-specific TCR transgene accelerates the onset of arthritis in mice. *Int Immunol* 10:1613-1622.
40. Goverman, J., A. Woods, L. Larson, L. P. Weiner, L. Hood, and D. M. Zaller. 1993. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell* 72:551-560.
41. O'Neill, S. K., M. J. Shlomchik, T. T. Glant, Y. Cao, P. D. Doodes, and A. Finnegan. 2005. Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis. *J Immunol* 174:3781-3788.
42. Hollo, K., T. T. Glant, M. Garzo, A. Finnegan, K. Mikecz, and E. Buzas. 2000. Complex pattern of Th1 and Th2 activation with a preferential increase of autoreactive Th1 cells in BALB/c mice with proteoglycan (aggrecan)-induced arthritis. *Clin Exp Immunol* 120:167-173.
43. Kaplan, C. D., S. K. O'Neill, T. Koreny, M. Czipri, and A. Finnegan. 2002. Development of inflammation in proteoglycan-induced arthritis is dependent on Fc gamma R regulation of the cytokine/chemokine environment. *J Immunol* 169:5851-5859.
44. Burmester, G. R., B. Stuhlmuller, G. Keyszer, and R. W. Kinne. 1997. Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? *Arthritis Rheum* 40:5-18.
45. Fox, D. A. 1997. The role of T cells in the immunopathogenesis of rheumatoid arthritis: new perspectives. *Arthritis Rheum* 40:598-609.
46. Peng, S. L. 2006. Fas (CD95)-related apoptosis and rheumatoid arthritis. *Rheumatology (Oxford)* 45:26-30.
47. Pope, R. M. 2002. Apoptosis as a therapeutic tool in rheumatoid arthritis. *Nat Rev Immunol* 2:527-535.
48. Goodnow, C. C., J. Sprent, B. Fazekas de St Groth, and C. G. Vinuesa. 2005. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature* 435:590-597.
49. Kronenberg, M., and A. Rudensky. 2005. Regulation of immunity by self-reactive T cells. *Nature* 435:598-604.
50. Feldmann, M., and L. Steinman. 2005. Design of effective immunotherapy for human autoimmunity. *Nature* 435:612-619.

CHAPTER 3

Cartilage Proteoglycan-Specific T Cells as Vectors of Immunomodulatory Biologicals in Chronic Proteoglycan-Induced Arthritis

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ABSTRACT

Systemic administration of agents that neutralize or antagonize T_h1-mediated pro-inflammatory responses has been demonstrated to ameliorate inflammation in chronic autoimmune disease. However, systemic administration of such immunosuppressive biologicals causes serious side effects and has only limited success. To minimize these side effects, autoantigen-specific lymphocytes have been proposed as a carrier to deliver immunosuppressive agents to sites of inflammation. Here we studied the effects of primary cartilage proteoglycan-specific $CD4^+$ T cells that were transduced using an efficient method of viral transduction with active genes encoding IL-1β receptor antagonist, soluble TNF-α receptor-Ig, IL-4 or IL-10 in chronic proteoglycan-induced arthritis in mice. This is the first study describing such gene therapy using primary $CD4^+$ T cells in a chronic arthritis. Moreover, the impact of proteoglycan-specific T_h1, T_h2 or naive T cells was studied. Although proteoglycan-TCR transgenic $CD4^+$ T cells can transfer arthritis to lymphopenic recipients, none of the proteoglycan-TCR transgenic T cell phenotypes that were tested induced worsening of arthritis in wild type hosts. Proteoglycan-specific T cells ameliorated arthritis when expressing the transduced IL-10 gene, and not when expressing the other transgenes/phenotypes. Although all of the tested biologicals can suppress in a wide range of different inflammatory disorders, especially IL-10 would therefore serve as a promising candidate to be used in cellular gene therapy for chronic arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease exhibiting chronic inflammation in the synovia of the joints that leads to progressive destruction of articular tissue. In RA-patients the balance between expression of pro-inflammatory cytokines and their inhibitors has shifted to that of an aggressive pro-inflammatory response. In addition, extensive studies in animal models indicate that tissue antigen (cartilage)-specific $CD4^+$ T cells are pivotal in the pathogenesis of arthritis (1, 2). Moreover, human studies have shown that T cells from patients with RA respond to cartilage antigens like proteoglycan (PG) (3-6). Thus, T cell immunity in RA seems dominated by a T_h1 response that may have escaped immune regulation by T_{reg} or counteracting T_h2 responses (7). Synovial inflammation is dominated by high concentrations of TNF-α and IL-1β (8) produced by synovial fibroblasts, mediating joint inflammation and destruction by production of catabolic metalloproteinases.

Biological agents neutralizing activity of pro-inflammatory cytokines such as IL-1 receptor antagonist (IL-1RA), anti-TNF-α antibody and soluble TNF-α receptors can exert considerable beneficial clinical effects in patients (9). In addition, treatment with cytokines like IL-4 and IL-10 that are known to down-regulate T_h1 responses has been proven beneficial in several models of RA (9, 10). However, alleviation of clinical disease outcome by systemic administration of such agents is accompanied by a high risk for adverse reactions. A major problem is the increased susceptibility to serious infection due to systemic immunosuppression (11). To avoid such unwanted effects, vectors that target immunosuppressive drugs to the site of the autoantigen-specific response may be a useful alternative for the treatment of autoimmune mediated disease.

Because $CD4^+$ T cells carry an antigen specific TCR, they have the ability to recognize antigens and interfere with certain immune responses in an antigen-specific manner. For this reason $CD4^+$ T cells that recognize tissue-specific autoantigens have been proposed as vectors that can be deployed to specifically target the pro-inflammatory autoimmune response with immunomodulatory agents (12, 13). This concept was first successfully demonstrated in experimental autoimmune encephalomyelitis, a model for multiple sclerosis, in which a myelin-specific T cell line transduced with a gene encoding nerve growth factor exerted anti-inflammatory effects (14). Promising possibilities of this so-called adoptive T-cell-based gene therapy have now become demonstrated for a wide range of different molecules in several models of autoimmunity and also of allergy (12, 13, 15).

In many studies either T cell hybridoma's or T cells with rather undefined antigen-specificities were used as carriers. The study presented here describes for the first time T-cell-based gene therapy with immunosuppressive proteins expressed by primary $CD4^+$ T cells with a defined specificity for cartilage-derived antigen in a chronic model of RA. We transduced proteoglycan-specific $CD4^+$ cells from a TCR-transgenic mouse (16, 17) with TNF-α receptor-Ig, IL-1 receptor antagonist, IL-4 or IL-10 and tested the immunomodulatory capacities of these $CD4^+$ cells in cartilage proteoglycan-induced arthritis (PGIA). The progressive inflammation in PGIA is caused by T_h1 immunity (10, 18, 19), depends on B cells (20) and bears similarities with clinical features and genetics of rheumatoid arthritis (RA) as reviewed in (1). In addition to transduction with modulatory genes, PG-specific T cells were forced to differentiate into either T_h1 or T_h2 cells *ex vivo* in the presence of immune deviating cytokines and then transferred to test their impact on PGIA. Although PG-specific T cells are arthritogenic (16, 17, 21) in lymphopenic hosts, naive or T_h1 PG-specific T cells, as well as the other phenotypes that were tested, did not add to severity of arthritis when transferred in

PGIA in wild type hosts. Surprisingly, most T cells expressing the different transgenes encoding well known immunosuppressive agents did not suppress arthritis. However, PG-specific T cells transduced with the active IL-10 gene significantly reduced both arthritis severity and arthritis incidence, indicating IL-10 as a very powerful immunosuppressive cytokine to be used in (adoptive gene) therapy.

MATERIALS AND METHODS

Mice and antigens

Retired breeder BALB/c mice (Charles River Laboratories, Maastricht, The Netherlands) were kept at the animal facility of the University of Utrecht; "Gemeenschappelijk Dierenlaboratorium" (GDL) under standard conditions in filtertopped cages. TCR-5/4E8-Tg BALB/c (PG-TCR Tg) mice (16, 17) were bred and kept at the GDL under specific pathogen free conditions. Human PG (hPG) and murine PG (mPG) were prepared as described elsewhere (22). All animal experiments were approved by the Animal Experimental Committee of the Veterinary Faculty of the University of Utrecht.

Generation of retroviral constructs

DNA sequences encoding IL-10, IL-4, TNFα Receptor Ig, and IL-1β receptor antagonist were generated as described below and cloned into the MSCV2.2 plasmid. mRNA was isolated from Balb/c cells and reversely transcribed to the cDNA that was used as template for all specific amplifications with PCR.

Murine IL-10 cDNA was amplified using primers F 5'-AGA TCT TTG CAG AAA AGA GAG CTC CA-3' and R 5'-GTC GAC TGG AGT CCA GCA GAC TCA AT-3', murine IL-4 cDNA was amplified using the primers F 5'-ACG GCA CAG AGC TAG TGA TG-3' and R 5'-GTC GAC AAG TTA AAG CAT GGT GGC TCA -3'. Murine IL-1βRA was amplified using primers F 5'-CTC GGG ATG GAA ATC TGC T-3' and 5'-GTC GAC TTA TTA CAG GCC TCG GCA GT-3', TNFR cDNA was amplified using primers F 5'-GCG GCC GCC AAT GGG GGA GTG AGG-3' and R 5'-TCT AGA GGG GTT TGT GAC ATT TGC AAG C-3'. The amplified TNFR cDNA was BAMHI/SalI restricted and cloned into pEDMIgG_{2a} mut+L to generate the TNFRIgG sequence. Amplified products were sequenced to confirm sequences.

Production of retrovirus

Ecotropic replication-deficient retrovirus was produced with a Phoenix-Eco packager cell line. Packager cells were cultured per $3x10^6$ cells in 10 ml complete DMEM (Gibco Life Technologies, Breda, The Netherlands) at 37°C. The next day the culture medium was refreshed and 500 µl of 0.25 M $CaCl_2$ containing 20 µg MSCV-plasmid + 5µg PCL-Eco plasmid was mixed with an equal volume HBS buffer pH 7.02 and added to the cells. At 20 hours after transfection supernatant was replaced with fresh medium. After 24 and 48 hours, supernatant containing the retrovirus was harvested, filtered with a 0.45 µM filter and stored frozen until use. Prior to T cell transductions, retroviral activity was tested on NIH3T3 cells using the protocol that was used for T cell transfection one day after seeding the 3T3 cells at a concentration of $3x10^4$/ml in supplemented DMEM.

Stimulation, retroviral transduction and transfer of transduced $CD4^+$ T cells

$CD4^+$ T cells were isolated from pooled spleens and lymph nodes of TCR-5/4E8-Tg BALB/c with anti-CD4 (L3T4) magnetic microbeads using the manufacturer's protocol (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and stimulated with magnetic M-450 Tosylactivated Dynabeads (Dynal Biotech ASA, Oslo, Norway) coated with anti-CD3 (145-2C11) and anti-CD28 (PV-1) mAbs in a 1:10 ratio. Anti-CD3/anti-CD28 mAb-coated beads were added to $1x10^6$ $CD4^+$ T cells (in a 2:1 ratio). After 48 hours of culturing, 750µl of the culture supernatant was replaced with 1 ml of retroviral supernatant supplemented with

8µg/ml hexadimethrine bromide (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for retroviral infection. Plates were then centrifuged at 930xG at 20°C for 2 hours. Subsequently, 1 ml of supernatant was replaced with fresh medium and cells were cultured for another 48 hours. Cells were removed from the stimulating beads and transduced cells were sorted by GFP expression with a FACSVantage SE (Becton Dickinson). Per recipient $1x10^6$ transduced $CD4^+$ cells were injected i.p. in PBS.

Differentiation of T_h1 and T_h2 cells

$CD4^+$ T cells from pooled spleens and lymph nodes of TCR-5/4E8-Tg BALB/c were isolated and activated as described for retroviral transduction. For differentiation to a T_h1-phenotype 10 ng/ml recombinant mouse IL-12p70 (BD Pharmingen) and 10 µg/ml anti-IL-4 mAb (11B11) were added to the culture. For differentiation to a T_h2-phenotype 20 ng/ml recombinant mouse IL-4 (BD Pharmingen) and 10 µg/ml anti-IFN-γ mAb (XMG1.2) were added to the culture. After four days of stimulation, cells were removed from the stimulating beads and prepared for phenotyping by flow cytometry or washed for transfer as described for transduced cells. After T_h1- or T_h2-differentiation protocols blastoid cells were gated as was done for sorting of transduced cells and sorted for transfer. Per recipient $1x10^6$ $CD4^+$ cells were injected i.p. in PBS.

Analysis of transgene expression

Anti-TNF-α activity of TNFαR-Ig was assessed using the WEHI-164 cytotoxicity bioassay as previously described (23). Alamar Blue was used to assess cell viability at OD 550/595. Activity of IL-1RA was assessed using a NF-κB-luciferase bioassay (24). IL-4 and IL-10 were measured using fluoresceinated microspheres coated with ELISA capture antibodies (BD Pharmingen) as described elsewhere (16) with a Luminex model 100 (Luminex, Austin, TX).

Flow cytometry

For flow cytometry of surface marker and GFP expression, cells were washed and stained with Allophycocyanin (APC) or Phycoerythrin (PE)-conjugated anti-CD4 mAb (BD Pharmingen), thoroughly washed with PBS/2% FCS and fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich Chemie BV). For analysis of intracellular IFN-γ and IL-4 cells were washed and stimulated prior to staining as described in Openshaw *et al.* (25) at 10^6/ml with 50 ng/ml PMA (Sigma-Aldrich Chemie BV) and 500 ng/ml ionomycin (Sigma-Aldrich Chemie BV) for 4 hours with Brefeldin A (Sigma-Aldrich Chemie BV) added at 10 µg/ml after the first 2 hours of stimulation. Before staining for intracellular cytokines cells were washed thoroughly, fixed in 4% PFA and washed again. Then cells were permeabilized with PBS/ 2% FCS/ 0.5% saponin and stained for intracellular cytokines with Fluorescein isothyocyanate (FITC)-conjugated anti-IL-4 mAb and PE-conjugated IFN- γ mAb (BD Pharmingen) in PBS/ 2% FCS/ 0.5% saponin (Sigma-Aldrich Chemie BV). After 3 washes cells were analyzed with a FacsCalibur flowcytometer (Becton Dickinson). Data were analyzed with Flow-Jo software.

Induction and assessment of arthritis

Arthritis was induced by i.p. injections of 2 mg hPG (crude extract) emulsified in 2 mg of the synthetic adjuvant dimethyl-dioctadecyl-ammoniumbromide (DDA) (Sigma) in PBS (total volume of 200 µl) on day 0 and day 21 as described elsewhere (16, 22). Paws were

examined three times per week in a blinded set-up to determine onset and severity of arthritis using a standard visual scoring system based on swelling and redness of the paws.

Statistics

Statistical analysis was done for comparison of T cell-recipients with the PBS-control group with a two-tailed *t*-test. *P* values less than 0.05 were considered significant. Unless stated otherwise, variation was shown as standard error of the means (SEM).

RESULTS

Generation of genetic constructs and transduction of CD4$^+$ cells

To generate genetically modified cartilage antigen-specific CD4$^+$ T cells, CD4$^+$ cells from mice expressing a PG-specific TCR were retrovirally transduced with different genes encoding immunomodulatory proteins. PG-specific CD4$^+$ cells were isolated from TCR-5/4E8-Tg BALB/c mice. In all experiments >90% of the CD4$^+$ cells express the Vβ4 chain, which is part of the PG-specific 5/4E8-Tg TCR. Wild type littermates express Vβ4-chains on only 6% of the CD4$^+$ T cells (16).

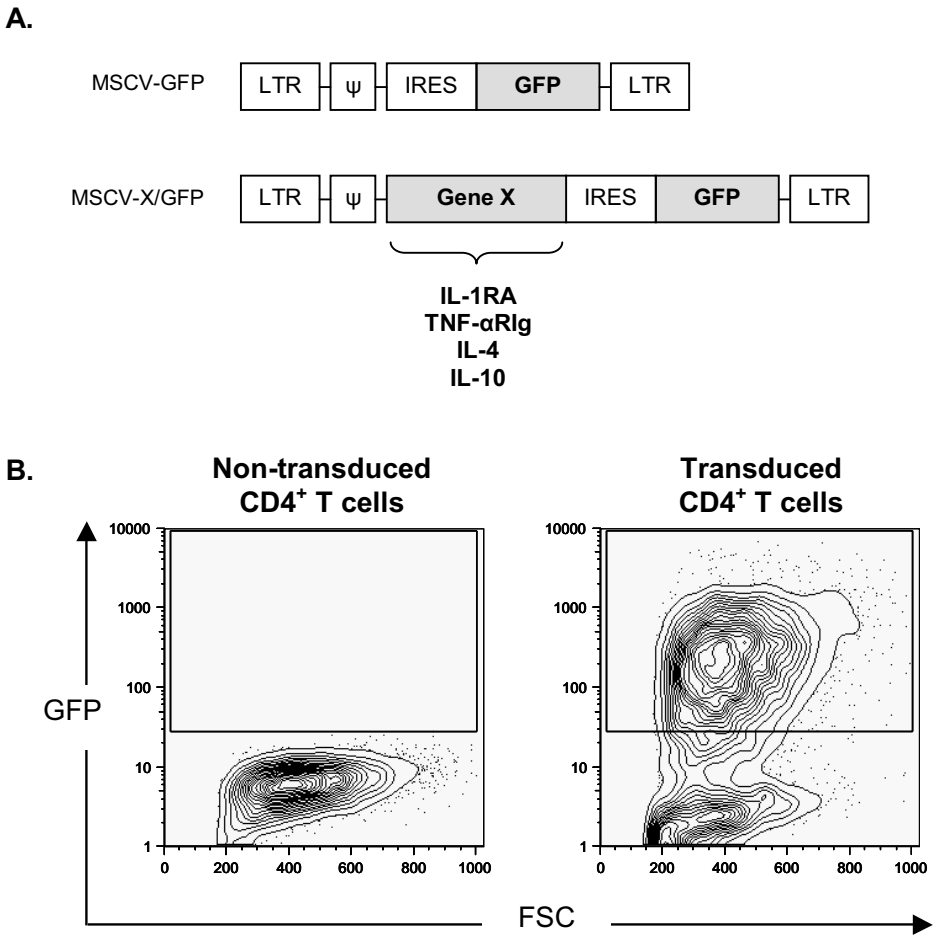

FIGURE 1. Retroviral constructs containing the different transgenes used to transduce CD4$^+$ T cells. (A) Genes encoding murine IL-1RA, TNF-αRIg that are known to block pro-inflammatory activity of IL-1β and TNF-α were inserted into the MSCV-GFP vector in front of the IRES sequence, enabling bicistronic translation of inserted genes and GFP. Alternatively, genes encoding murine IL-4 or IL-10, which are known to modulate T_h1-biology, were inserted into the MSCV-GFP vector. (B) CD4$^+$ cells from 5/4E8 TCR-Tg mice were activated with anti-CD3 + anti-CD28 mAbs and retrovirally transduced with the constructs described in figure 1A. Transduced cells were sorted by expression of GFP using the sort gates indicated. Left panel of figure B shows CD4$^+$ cells without transduction, right panel of figure B shows CD4$^+$ cells after transduction with MSCV-IL-10(GFP).

Constructs that were designed for T cell transduction were generated by PCR on murine cDNA and cloned into the murine stem cell virus (MSCV) plasmid. Ecotropic replication deficient retrovirus was produced from transiently plasmid-transfected Phoenix-

Eco producer cells that pack the designed construct into retroviral particles. The construct encodes the following sequence: LTR-cloning site-IRES-GFP-LTR (figure 1A). The LTR sequence enables stable genomic integration and contains promoter and enhancer activity for constant expression of cloned transgenes. Green fluorescent protein (GFP) allows for selection of transduced cells and is translated into a separate protein that is not conjugated to the immunomodulatory proteins due to the interribosomal entry site (IRES).

T cells must be in division to enable retroviral transduction (26) and were therefore activated *in vitro* to induce proliferation. To effectively induce T cell proliferation without the need for antigenic peptides and to circumvent the need to separate T cells from antigen presenting cells after stimulation and transduction we choose an APC-free T cell stimulation system. For this we used magnetic beads coated with anti-CD3 mAb and superagonistic anti-CD28 mAb as described earlier (27). Using this protocol numbers of T cells that were transduced reached up to 80% of the T cells in culture as analyzed by flow cytometry of GFP expression (figure 1B). $CD4^+$ purity after four days of culturing was usually >95%. GFP^+ cells were enriched especially in the blastoid fraction observed in the forward scatter/side scatter (FSC/SSC) plots. The sorted fractions used for transfer were >99.5% GFP^+. Altogether, these results show that stimulation via the CD3/TCR-complex plus CD28-mediated co-stimulation with mAbs is a very efficient means to stimulate T cells for retroviral transduction.

Table I. Transgene expression by transfected cells and transduction rates of TCR transgenic $CD4^+$ T-cells

Transgene from MSCV vector	Transgene expression (secreted protein)	% transduction (GFP^+) of $CD4^+$ cells	MFI of GFP in transduced $CD4^+$ cells
IL-1RA	20 ng/ml	86%	627
TNF-αRIg	147 ng/ml	55%	70
IL-4	16 ng/ml	78%	457
IL-10	11 ng/ml	73%	370
GFP (control)	-	80%	1216

Expression of transgenes

In order to show that proteins encoded by the constructed transgenes were secreted and biologically active, culture supernatant of transfected cells was analyzed for the presence of these proteins (table I). To analyze for the presence of functional IL-1RA, neutralizing IL-1RA activity was measured with a luciferase-reporter assay for IL-1RA activity. Active IL-1RA was found at a concentration of 20 ng/ml, indicating that biologically active IL-1RA was expressed from the MSCV-IL-1RA construct. Neutralizing activity of the constructed TNF-αRIg was analyzed with a TNF-α-sensitive WEHI-164 celline cultured with different concentrations of murine TNF-α inducing different levels of apoptosis of the WEHI-164 cells. Supernatant of MSCV-TNF-αRIg-GFP-transfected cells contained a blocking activity of 147 ng/ml murine TNF-α per ml compared with MSCV-GFP-transfection supernatant. Thus, the constructed TNF-αRIg encoded biologically active TNF-αRIg. To analyze expression of the cytokines IL-10 and IL-4, concentrations were measured in cell culture supernatant with a multiplex assay for quantification of protein expression. The concentrations of IL-4 and IL-10 secreted were about the same level (16 and 11 ng/ml) as IL-1RA. Altogether, these results show that all transgenes constructed were expressed and secreted.

T_h1/T_h2-differentiation

In order to stimulate differentiation of PG-specific T cells into T_h1 or T_h2, freshly isolated TCR-5/4E8-Tg $CD4^+$ cells were stimulated with anti-CD3 and anti-CD28 mAbs *in vitro*. For differentiation into T_h1 cells, $CD4^+$ cells were stimulated in the presence of IL-12p70 and anti-IL-4 mAb. To stimulate differentiation into T_h2 cells, PG-specific $CD4^+$ cells were stimulated in the presence of IL-4 and anti-IFN-γ mAb. After four days of stimulation, cells within the blastoid population, as selected by the FSC/SSC profile of these cells, were sorted and transferred in PGIA as described later on. This was done in order to select for the stimulated cells from the *in vitro* culture, thus to avoid transfer of undifferentiated cells. As shown in figure 2, numbers of $CD4^+$ cells expressing IFN-γ exceeded the number of IL-4 producers after anti-CD3/anti-CD28 stimulation in the absence of exogenous cytokines or cytokine-antibodies as analyzed at the cellular level by flow cytometry. The T_h1-skewing factors almost completely reduced this number of cells producing IL-4, while the number of IFN-γ-producers had doubled. In the presence of T_h2-differentiation factors the number of cells producing IFN-γ was reduced more than 50%, while the number of IL-4 producers had increased more than three fold.

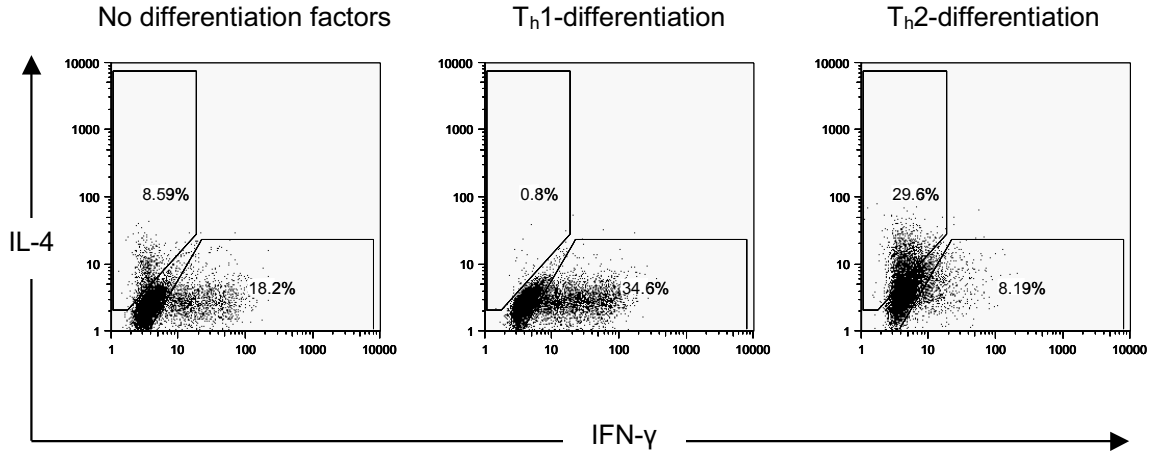

FIGURE 2. Differentiation of T_h1 and T_h2 cells. $CD4^+$ cells from 5/4E8 TCR-Tg mice were activated with anti-CD3 + anti-CD28 mAbs in the presence of anti-IL-4 mAb + recombinant murine IL-12p70 to stimulate differentiation to a T_h1-phenotype (middle panel), in the presence of anti-IFN-γ mAb + recombinant murine IL-4 to stimulate differentiation to a T_h2-phenotype (right panel) or in the absence of added differentiation factors (left panel). After four days of stimulation cells were analyzed for expression of IL-4 and IFN-γ upon stimulation by PMA and ionomycin. Numbers in the plots show the percentages of cells staining positive for IL-4 or IFN-γ within the $CD4^+$ population.

Pro-inflammatory effects of PG-specific T cells in PGIA

Arthritis is dominated by autoantigen-specific $CD4^+$ T cells which are characterized by a T_{h1} phenotype. The arthritogenic role for cartilage antigen-specific T cells was demonstrated also by an increased susceptibility for arthritis in transgenic mice that express a PG-specific TCR on their T cells (16). Therefore, we hypothesized that adding PG-specific $CD4^+$ T cells to the endogenous pool of cartilage antigen-specific $CD4^+$ T cells might exacerbate PGIA in wild type mice. This potential pro-inflammatory effect of PG-specific T cells in PGIA was assessed in two sets of experiments. In a first experiment $1x10^6$ naive PG-specific $CD4^+$ cells were transferred in PGIA. A group of mice receiving only PBS was used as untreated control.

Also, $CD4^+$ cells that were transduced with MSCV-GFP (T_{GFP}), thus without any gene encoding an immunosuppressive molecule, were transferred in PGIA. These T_{GFP} cells were used as a control for T cells that were transduced to express immunosuppressive proteins as described later on. In a second experiment, T_h1-differentiated and T_{GFP} cells were transferred to test for any additive pro-inflammatory, arthritogenic effect. On day 20, the day before the second arthritogenic PG-immunization, $1x10^6$ T cells were transferred to each mouse.

Six days after transfer, the earliest clinical symptoms of arthritis were observed. Neither $1x10^6$ naive PG-specific T cells (figure 3A, left panel) nor $1x10^6$ PG-specific T_{GFP} cells (figure 3A, right panel) induced exacerbation of arthritis severity during the course of disease and also the day of arthritis onset and the maximum arthritis severity observed during the experiment were not affected by these T cells. Moreover, PG-specific T_h1 cells did not worsen inflammation either as shown in a second experiment (figure 3B, left panel and table II). In addition, these naive T cells, T_{GFP} cells or T_h1 cells did not induce a higher incidence of arthritis (figure 4).

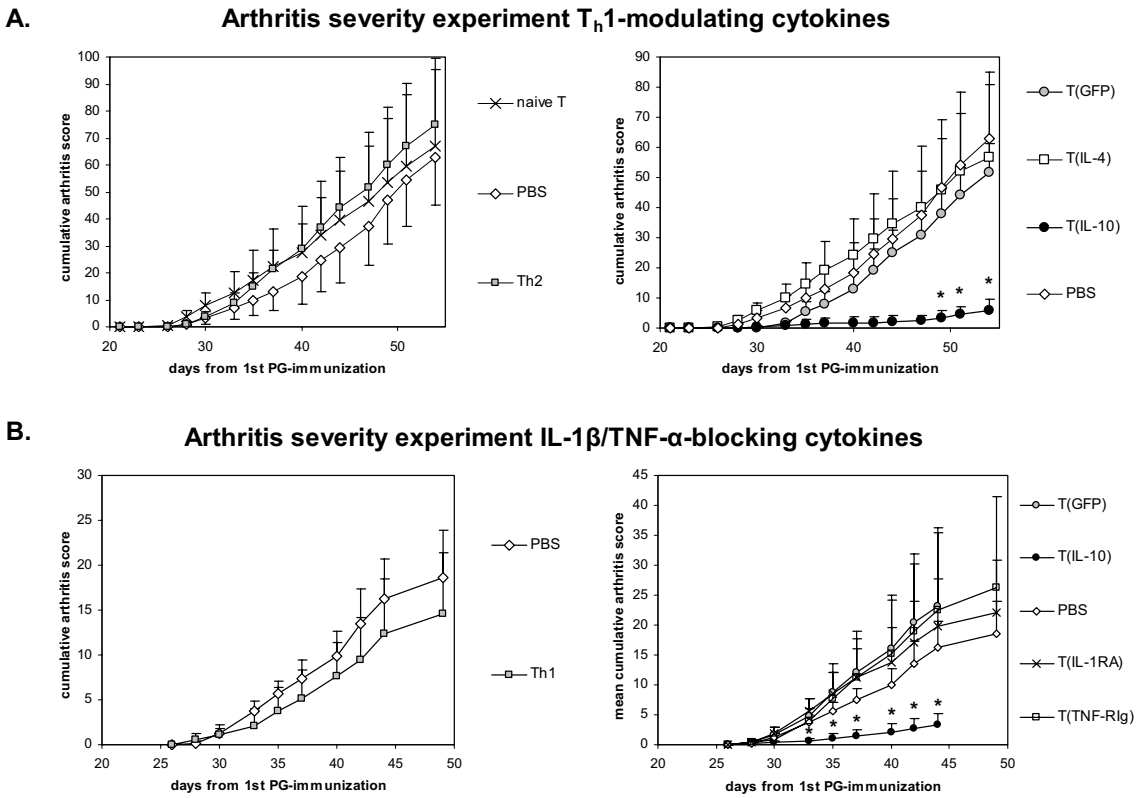

FIGURE 3. Effects of PG-specific $CD4^+$ T cells with different phenotypes on arthritis severity. (A) Mice were immunized with PG on day 0 and day 21 to induce arthritis and received either $1x10^6$ naïve $CD4^+$ cells (n=5), T_h2 cells (n=5) (left panel) or IL-4 transduced (n=5), IL-10 transduced (n=4), GFP-only transduced $CD4^+$ cells (n=4) from 5/4E8 TCR-Tg mice or PBS instead of cells (n=4) (right panel) on day 20. (B) In another experiment either $1x10^6$ T_h1 cells (n=5) (left panel) or IL-10 transduced (n=8), IL-1RA transduced (n=5), TNF-αRIg transduced (n=5), GFP-only transduced $CD4^+$ cells (n=6) from 5/4E8 TCR Tg mice or PBS instead of cells (n=5) were transferred. Arthritis severity was scored by redness and swelling of the joints and the mean cumulative arthritis score is shown. *p<0.05 for groups compared with the PBS control group.

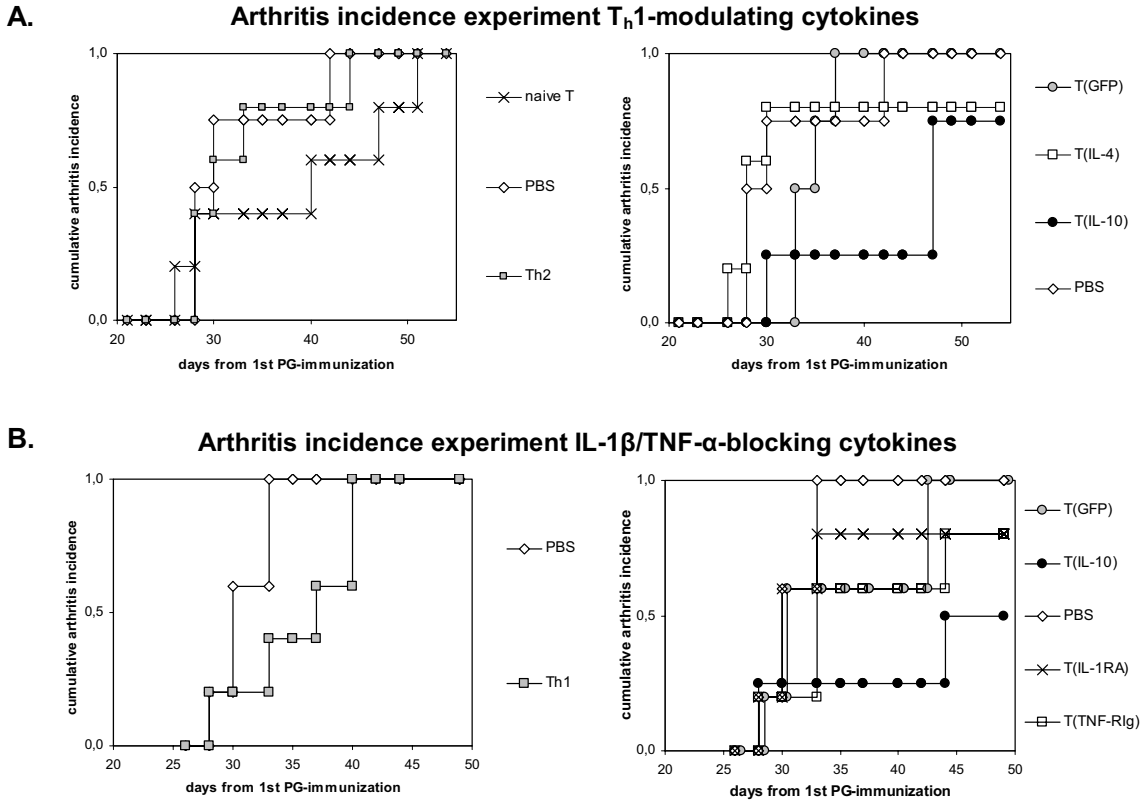

FIGURE 4. Effects of PG-specific CD4$^+$ T cells with different phenotypes on arthritis incidence. (A) Mice were treated as described in figure 3 and received 1x10^6 naïve CD4$^+$ cells (n=5), T_h2 cells (n=5), IL-4 transduced (n=5), IL-10 transduced (n=4), GFP-only transduced CD4$^+$ cells (n=4) from 5/4E8 TCR Tg mice or PBS instead of cells (n=4) on day 20. (B) In a second experiment 1x10^6 T_h1 cells (n=5), IL-10 transduced (n=8), IL-1RA transduced (n=5), TNF-αRIg transduced (n=5), GFP-only transduced CD4$^+$ cells (n=6) from 5/4E8 TCR-Tg mice or PBS instead of cells (n=5) were transferred as described in figure 1A. Arthritis severity was scored by redness and swelling of the joints and the cumulative arthritis incidence score is shown. Graphs are split in two for convenience of understanding.

Disease-suppressing effects of PG-specific transduced T cells and T_h2 cells in PGIA

The potential anti-inflammatory effect of PG-specific T cells that were either transduced to express immune modulatory agents or differentiated into a T_h2 phenotype, was assessed in PGIA similar to testing of the T_h1 and naive PG-specific T cells described above. PG-specific CD4$^+$ T cells were either transduced with IL-1RA, TNF-αRIg, IL-4 or differentiated to T_h2 cells *in vitro* and 1x10^6 of these T cells were transferred in PGIA. Although they were expected to counteract the arthritogenic T_h1 response, T_h2 cells or T cells transduced with the gene expressing the T_h2 characteristic cytokine IL-4 ($T_{IL\text{-}4}$ cells), did not suppress arthritis severity (figure 3A; left and right panel respectively). Also day of disease onset and maximum arthritis severity observed were not affected compared to the PBS-control group as found in experiment 1 (table II). Moreover, PG-specific T cells transduced with IL-1RA/GFP ($T_{IL\text{-}1RA}$ cells) or TNF-αRIg ($T_{TNF\text{-}\alpha RIg}$ cells) did not have a beneficial effect on the course of arthritis (figure 3B, right panel) or on the day of onset and maximum arthritis severity observed (table II) as shown in the second experiment. Moreover, no effect was found on the cumulative incidence of arthritis for all of these T cells (figure 4).

So far, none of these transferred populations of $1x10^6$ PG-specific T cells expressing anti-inflammatory molecules were able to suppress inflammation in PG-induced arthritis. However, when PG-specific T cells were transduced with the IL-10 gene (T_{IL-10} cells), these T cells significantly reduced arthritis severity during the course of disease in both experiments (right panels figure 3A and 3B) compared with the PBS-control. In addition, maximum arthritis severity observed was significantly lower after transfer of PG-specific T_{IL-10} cells (table II) and cumulative incidence was lower (figure 4, right panels) in mice that received T_{IL-10} cells. Although PG-specific T_{IL-10} cells tended to slightly delay the onset of disease, this effect on the day of arthritis onset was not significant. Altogether, these data indicate that IL-10 may be a very promising candidate to be expressed by antigen-specific T cells in order to suppress inflammation.

Table II. Arthritis onset, arthritis incidence and maximum arthritis severity

T_h1-modulating cytokines						
	PBS	**GFP**	**IL-10**	**IL-4**	**naive**	**T_h2**
Day of onset	11 (±3.4)	13.5 (±1.0)	20.3 (±4.9)	7.0 (±0.8)	17.4 (±5.0)	11.6 (±3.0)
Maximum severity	9.8 (±1.4)	8.4 (±0.9)	2.0 (±0.9)*	6.2 (±2.9)	9.4 (±2.7)	9.3 (±2.2)
Blocking cytokines						
	PBS	**GFP**	**IL-10**	**IL-1RA**	**TNF-αRIg**	**T_h1**
Day of onset	9.8 (±1.0)	13.4 (±3.12)	13.5 (±3.8)	9.3 (±1.0)	13.5 (±3.4)	14.6 (±2.3)
Maximum severity	4.0 (±1.1)	4.9 (±2.43)	0.8 (±0.4)*	4.0 (±1.4)	4.9 (±2.4)	3.4 (±1.2)

Day of arthritis onset (shown as mean of day from transfer ±SEM) and maximum severity (mean of maximum scores ±SEM) are shown. In an experiment in which cytokines modulating T_h1 cells (IL-4, IL-10) were tested, arthritis was analyzed up to day 54,. Another experiment, in which agents blocking pro-inflammatory IL-1β or TNF-α were tested, arthritis was analyzed up to day 44. PG-specific T cells, or PBS as a control, were injected i.p. on day 20. The second PG immunization was given on day 21 to induce arthritis. *, $p<0.05$.

DISCUSSION

Proteoglycan-induced arthritis is a chronic and progressive model of RA (1) and is characterized by a dominating T_h1 response (10, 18, 19, 21, 28). The generation of a TCR-specific mouse (16, 17) as a source for PG-specific $CD4^+$ T cells allows for studies on $CD4^+$ T cell-mediated functions in this arthritis model. In this study we examined the possibility to target inflammation in PGIA with *ex vivo* modulated PG-specific $CD4^+$ T cells. Because arthritogenic and PG-specific T cells can migrate not only to the lymphoid organs but also to inflamed joints (29), they may be suitable tools to deliver such anti-inflammatory agents at the site of inflammation. Application of (genetically) modified $CD4^+$ T cells expressing anti-inflammatory agents to successfully target autoimmune mediated inflammation was proposed and carried out previously (13). However, this is the first study which combined immunomodulatory molecules expressed by primary arthritogenic $CD4^+$ T cells, with a well defined specificity for a cartilage antigen, in a chronic and progressive arthritis driven by cartilage-specific immune responses.

To genetically modify T cells using a retroviral system we activated T cells with anti-CD3/anti-CD28 mAb-conjugated magnetic beads (27). Rheumatic joints contain clonally expanded T cell populations. This indicates that these cells are specifically involved in the inflammatory auto-antigen specific immune response and these T cells may therefore be used for adoptive cellular gene therapy. The use of anti-CD3/anti-CD28-stimulation by mAbs used for T cell activation is less time consuming than use of APC as has often been done since no APCs have to be sorted prior to and after T cell stimulation. This method turned out to be a very efficient method to stimulate T cells since transduction with retrovirus, which requires T cell division, resulted in up to 80% transduced T cells. Stimulation with anti-CD3/anti-CD28 mAbs resulted in a higher IFN-γ response over an IL-4 response, which is indicative of T_h1 cells as has been described before (27). However, this phenotype could be skewed to a more pronounced T_h1 phenotype or to a T_h2 phenotype when either IL-12p70 and anti-IL-4 mAb or IL-4 and anti-IFN-γ mAb were present during T cell activation, respectively. This indicates that further (co-)stimulatory molecules or cytokines expressed by APCs are not crucial for T cell differentiation of IL-4 or IFN- γ responses in the presence of these cytokines, although they may enhance this differentiation (30, 31).

Induction of PGIA requires a T_h1 response (10, 19) and can be achieved by transfer of PG-primed $CD4^+$ T cells to lymphopenic hosts (17, 21, 28). Moreover, TCR-Tg mice expressing a PG-specific TCR are more susceptible to PGIA (16). Therefore, we reasoned that transfer of PG-specific T_h1 cells to wild type recipients would exacerbate PGIA. However, $1x10^6$ PG-specific T_h1 cells did not promote disease development. Recently, it was shown that T_h1 cells, and also T_h2 cells, may block differentiation of $CD4^+$ T cells producing IL-17 (T_h17 cells), which is a newly identified T cell phenotype that is crucial for the induction of autoimmune disorders like arthritis (32). Therefore, it is possible that PG-specific T_{h1} cells could prevent differentiation or activity of pro-inflammatory T_h17 cells instead of exacerbating disease. No suppressive effect of T_h1 cells on disease was observed either. Therefore, suppression of an arthritogenic T_h17 response is unlikely. Moreover, naive PG-specific
PG-specific $CD4^+$ T cells that were transferred from highly arthritis susceptible TCR-Tg mice without prior *ex vivo* stimulation/ differentiation to wild type mice with PGIA, exerted no clinical effects. Therefore, a likely explanation may be that the number ($1x10^6$) of PG-specific T cells added to the already existing pool of arthritogenic lymphocytes is not enough to exacerbate arthritis in wild type recipients.

Neither PG-specific T_h2 cells nor PG-specific IL-4-transduced T cells suppressed arthritis, although T_h2 cells and their derived cytokines like IL-4 are well known to regulate T_h1 responses in inflammatory diseases like PGIA (10, 19, 33). This is in contrast with studies where OVA-specific T_h2 cells could suppress disease in an OVA-induced arthritis model (34) or where anti-inflammatory effects of collagen-specific T_{IL-4} cells have been demonstrated in collagen-induced arthritis (CIA) (35). Maybe the phenotype of such T cells in our studies was not strong enough or the number of transferred T cells not sufficient to counteract on an arthritogenic immune response. Whereas in our study $1x10^6$ primary $CD4^+$ T cells were used, in CIA $CD4^+$ T cell hybridoma's were used as a source of cartilage antigen-specific T cells. Such hybridoma's proliferate without antigenic-stimulation. Therefore, such hybridoma's constantly proliferate irrespective of their location and will therefore probably be present in larger numbers secreting higher amounts of cytokines. Discrepancy between our findings and previously documented findings may also be explained by differences between models. Although both CIA and PGIA are dominated by T_h1 cells, these models may be slightly differently regulated in terms of T_h1 and T_h2 immunity as the natural immunological backgrounds of the mice used for these models differ (1). In addition, a likely explanation may be found in the differences of $CD4^+$ T cells used in different studies.

IL-1β and TNF-α have prominent pro-inflammatory functions within the joint tissue and act downstream T_{h1} cell-mediated immunity (8). Blocking the biological activities of these cytokines with IL-1RA or TNF-αR respectively has been demonstrated a successful approach to ameliorate inflammation in a wide range of arthritis models and other models for autoimmune mediated inflammation (9). Agents blocking these cytokines have also been shown to suppress arthritis when expressed by transduced cartilage antigen-specific $CD4^+$ T cell hybridoma's (36) or synovial fibroblasts (37). However, in our study $1x10^6$ T_{IL-1RA} or $T_{TNF-\alpha RIg}$ cells did not suppress clinical symptoms of inflammation in PGIA, although IL-1RA and TNF-αRIg were expressed functionally. Again, these contrasting findings may be explained by the use of different models, the use of T cell hybridoma's or the number of T cells transferred.

IL-10 and TGF-β have been described as the major immunosuppressive cytokines required for different subsets of regulatory T cells (T_{reg} cells) to down regulate pro-inflammatory immune responses that lead to autoimmune disease, allergy or exaggerated responses to pathogens. Like IL-4, IL-10 has been shown to suppress PGIA when administered systemically (10). However, unlike PG-specific T_{IL-4} cells, PG-specific T_{IL-10} cells showed significant beneficial effects on PGIA. Because of all the anti-inflammatory agents tested IL-10 was the only one that suppressed arthritis when expressed by transduced PG-specific T cells, IL-10 may be a very promising to be used in T cell mediated intervention in arthritis. Not only transduction with the IL-10 gene, but also ways to promote IL-10 producing T_{reg} cells have been very promising in interventions in inflammatory diseases (38-40). Therefore, mechanisms that propagate the expression of IL-10 altering the autoimmune response that sustains inflammation may be more effective than blocking the resulting downstream effects such as expression of pro-inflammatory molecules that are amplified by this autoimmune response.

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REFERENCES

1. Glant, T. T., A. Finnegan, and K. Mikecz. 2003. Proteoglycan-induced arthritis: immune regulation, cellular mechanisms, and genetics. *Crit Rev Immunol* 23:199-250.
2. Brand, D. D., A. H. Kang, and E. F. Rosloniec. 2003. Immunopathogenesis of collagen arthritis. *Springer Semin Immunopathol* 25:3-18.
3. Goodstone, N. J., M. C. Doran, R. N. Hobbs, R. C. Butler, J. J. Dixey, and B. A. Ashton. 1996. Cellular immunity to cartilage aggrecan core protein in patients with rheumatoid arthritis and non-arthritic controls. *Ann Rheum Dis* 55:40-46.
4. Guerassimov, A., Y. Zhang, S. Banerjee, A. Cartman, J. Y. Leroux, L. C. Rosenberg, J. Esdaile, M. A. Fitzcharles, and A. R. Poole. 1998. Cellular immunity to the G1 domain of cartilage proteoglycan aggrecan is enhanced in patients with rheumatoid arthritis but only after removal of keratan sulfate. *Arthritis Rheum* 41:1019-1025.
5. Li, N. L., D. Q. Zhang, K. Y. Zhou, A. Cartman, J. Y. Leroux, A. R. Poole, and Y. P. Zhang. 2000. Isolation and characteristics of autoreactive T cells specific to aggrecan G1 domain from rheumatoid arthritis patients. *Cell Res* 10:39-49.
6. ter Steege, J., M. Vianen, J. van Bilsen, J. Bijlsma, F. Lafeber, and M. Wauben. 2003. Identification of self-epitopes recognized by T cells in rheumatoid arthritis demonstrates matrix metalloproteinases as a novel T cell target. *J Rheumatol* 30:1147-1156.
7. Skapenko, A., J. Leipe, P. E. Lipsky, and H. Schulze-Koops. 2005. The role of the T cell in autoimmune inflammation. *Arthritis Res Ther* 7 Suppl 2:S4-14.
8. McInnes, I. B., and G. Schett. 2007. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* 7:429-442.
9. van de Loo, F. A., R. L. Smeets, and W. B. van den Berg. 2004. Gene therapy in animal models of rheumatoid arthritis: are we ready for the patients? *Arthritis Res Ther* 6:183-196.
10. Finnegan, A., K. Mikecz, P. Tao, and T. T. Glant. 1999. Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. *J Immunol* 163:5383-5390.
11. Weisman, M. H. 2002. What are the risks of biologic therapy in rheumatoid arthritis? An update on safety. *J Rheumatol Suppl* 65:33-38.
12. Robbins, P. D., C. H. Evans, and Y. Chernajovsky. 2003. Gene therapy for arthritis. *Gene Ther* 10:902-911.
13. Tarner, I. H., A. J. Slavin, J. McBride, A. Levicnik, R. Smith, G. P. Nolan, C. H. Contag, and C. G. Fathman. 2003. Treatment of autoimmune disease by adoptive cellular gene therapy. *Ann N Y Acad Sci* 998:512-519.
14. Kramer, R., Y. Zhang, J. Gehrmann, R. Gold, H. Thoenen, and H. Wekerle. 1995. Gene transfer through the blood-nerve barrier: NGF-engineered neuritogenic T lymphocytes attenuate experimental autoimmune neuritis. *Nat Med* 1:1162-1166.
15. Oh, J. W., C. M. Seroogy, E. H. Meyer, O. Akbari, G. Berry, C. G. Fathman, R. H. Dekruyff, and D. T. Umetsu. 2002. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. *J Allergy Clin Immunol* 110:460-468.
16. Berlo, S. E., T. Guichelaar, C. B. Ten Brink, P. J. van Kooten, F. Hauet-Broeren, K. Ludanyi, W. van Eden, C. P. Broeren, and T. T. Glant. 2006. Increased arthritis susceptibility in cartilage proteoglycan-specific T cell receptor-transgenic mice. *Arthritis Rheum* 54:2423-2433.
17. Berlo, S. E., P. J. van Kooten, C. B. Ten Brink, F. Hauet-Broere, M. A. Oosterwegel, T. T. Glant, W. Van Eden, and C. P. Broeren. 2005. Naive transgenic T cells expressing cartilage proteoglycan-specific TCR induce arthritis upon in vivo activation. *J Autoimmun* 25:172-180.
18. Hollo, K., T. T. Glant, M. Garzo, A. Finnegan, K. Mikecz, and E. Buzas. 2000. Complex pattern of Th1 and Th2 activation with a preferential increase of autoreactive Th1 cells in BALB/c mice with proteoglycan (aggrecan)-induced arthritis. *Clin Exp Immunol* 120:167-173.
19. Kaplan, C., J. C. Valdez, R. Chandrasekaran, H. Eibel, K. Mikecz, T. T. Glant, and A. Finnegan. 2002. Th1 and Th2 cytokines regulate proteoglycan-specific autoantibody isotypes and arthritis. *Arthritis Res* 4:54-58.
20. O'Neill, S. K., M. J. Shlomchik, T. T. Glant, Y. Cao, P. D. Doodes, and A. Finnegan. 2005. Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis. *J Immunol* 174:3781-3788.
21. Buzas, E. I., F. R. Brennan, K. Mikecz, M. Garzo, G. Negroiu, K. Hollo, G. Cs-Szabo, E. Pintye, and T. T. Glant. 1995. A proteoglycan (aggrecan)-specific T cell hybridoma induces arthritis in BALB/c mice. *J Immunol* 155:2679-2687.
22. Hanyecz, A., S. E. Berlo, S. Szanto, C. P. Broeren, K. Mikecz, and T. T. Glant. 2004. Achievement of a synergistic adjuvant effect on arthritis induction by activation of innate immunity and forcing the immune response toward the Th1 phenotype. *Arthritis Rheum* 50:1665-1676.
23. Espevik, T., and J. Nissen-Meyer. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J Immunol Methods* 95:99-105.
24. Smeets, R. L., L. A. Joosten, O. J. Arntz, M. B. Bennink, N. Takahashi, H. Carlsen, M. U. Martin, W. B. van den Berg, and F. A. van de Loo. 2005. Soluble interleukin-1 receptor accessory protein ameliorates collagen-induced arthritis by a different mode of action from that of interleukin-1 receptor antagonist. *Arthritis Rheum* 52:2202-2211.
25. Openshaw, P., E. E. Murphy, N. A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J Exp Med* 182:1357-1367.
26. Costa, G. L., J. M. Benson, C. M. Seroogy, P. Achacoso, C. G. Fathman, and G. P. Nolan. 2000. Targeting rare populations of murine antigen-specific T lymphocytes by retroviral transduction for potential application in gene therapy for autoimmune disease. *J Immunol* 164:3581-3590.
27. Broeren, C. P., G. S. Gray, B. M. Carreno, and C. H. June. 2000. Costimulation light: activation of CD4+ T cells with CD80 or CD86 rather than anti-CD28 leads to a Th2 cytokine profile. *J Immunol* 165:6908-6914.
28. Bardos, T., K. Mikecz, A. Finnegan, J. Zhang, and T. T. Glant. 2002. T and B cell recovery in arthritis adoptively transferred to SCID mice: antigen-specific activation is required for restoration of autopathogenic CD4+ Th1 cells in a syngeneic system. *J Immunol* 168:6013-6021.
29. Mikecz, K., and T. T. Glant. 1994. Migration and homing of lymphocytes to lymphoid and synovial tissues in proteoglycan-induced murine arthritis. *Arthritis Rheum* 37:1395-1403.
30. Rogers, P. R., and M. Croft. 1999. Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. *J Immunol* 163:1205-1213.

31. Dong, C., and R. A. Flavell. 2001. Th1 and Th2 cells. *Curr Opin Hematol* 8:47-51.
32. Wynn, T. A. 2005. T(H)-17: a giant step from T(H)1 and T(H)2. *Nat Immunol* 6:1069-1070.
33. Finnegan, A., M. J. Grusby, C. D. Kaplan, S. K. O'Neill, H. Eibel, T. Koreny, M. Czipri, K. Mikecz, and J. Zhang. 2002. IL-4 and IL-12 regulate proteoglycan-induced arthritis through Stat-dependent mechanisms. *J Immunol* 169:3345-3352.
34. Maffia, P., J. M. Brewer, J. A. Gracie, A. Ianaro, B. P. Leung, P. J. Mitchell, K. M. Smith, I. B. McInnes, and P. Garside. 2004. Inducing experimental arthritis and breaking self-tolerance to joint-specific antigens with trackable, ovalbumin-specific T cells. *J Immunol* 173:151-156.
35. Tarner, I. H., A. Nakajima, C. M. Seroogy, J. Ermann, A. Levicnik, C. H. Contag, and C. G. Fathman. 2002. Retroviral gene therapy of collagen-induced arthritis by local delivery of IL-4. *Clin Immunol* 105:304-314.
36. Smith, R., I. H. Tarner, M. Hollenhorst, C. Lin, A. U. Levicnik, C. G. Fathman, and G. P. Nolan. 2003. Localized expression of an anti-TNF single-chain antibody prevents development of collagen-induced arthritis. *Gene Ther* 10:1248-1257.
37. Bandara, G., G. M. Mueller, J. Galea-Lauri, M. H. Tindal, H. I. Georgescu, M. K. Suchanek, G. L. Hung, J. C. Glorioso, P. D. Robbins, and C. H. Evans. 1993. Intraarticular expression of biologically active interleukin 1-receptor-antagonist protein by ex vivo gene transfer. *Proc Natl Acad Sci U S A* 90:10764-10768.
38. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737-742.
39. Bluestone, J. A. 2005. Regulatory T-cell therapy: is it ready for the clinic? *Nat Rev Immunol* 5:343-349.
40. Barrat, F. J., D. J. Cua, A. Boonstra, D. F. Richards, C. Crain, H. F. Savelkoul, R. de Waal-Malefyt, R. L. Coffman, C. M. Hawrylowicz, and A. O'Garra. 2002. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 195:603-616.

CHAPTER 4

Autoantigen-Specific IL-10-Transduced T Cells Suppress Chronic Arthritis by Promoting the Endogenous Regulatory IL-10 Response

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ABSTRACT

Deficient T cell regulation can be mechanistically associated with development of chronic autoimmune diseases. Therefore, combining the regulatory properties of IL-10 and the specificity of autoreactive $CD4^+$ T cells through adoptive cellular gene transfer of IL-10 via autoantigen-specific $CD4^+$ T cells seems an attractive approach to correct such deficient T cell regulation that avoids the risks of non-specific immunosuppressive drugs. Here, we studied how cartilage proteoglycan-specific $CD4^+$ T cells transduced with an active IL-10 gene ($T_{IL\text{-}10}$) may contribute to the amelioration of chronic and progressive proteoglycan-induced arthritis in BALB/c mice. TCR-transgenic proteoglycan-specific $T_{IL\text{-}10}$ cells ameliorated arthritis, whereas $T_{IL\text{-}10}$ cells with specificity for ovalbumin had no effect, showing the impact of antigen-specific targeting of inflammation. Furthermore, proteoglycan-specific $T_{IL\text{-}10}$ cells suppressed autoreactive pro-inflammatory T- and B cells, as $T_{IL\text{-}10}$ cells caused a reduced expression of IL-2, TNF-α and IL-17 and a diminished proteoglycan-specific IgG_{2a} antibody response. Moreover, proteoglycan-specific $T_{IL\text{-}10}$ cells promoted IL-10 expression in recipients but did not ameliorate arthritis in IL-10 deficient mice, indicating that $T_{IL\text{-}10}$ cells suppress inflammation by propagating the endogenous regulatory IL-10 response in treated recipients. This is the first demonstration that such targeted suppression of pro-inflammatory lymphocyte responses in chronic autoimmunity by IL-10-transduced T cells specific for a natural antigen can occur via the endogenous regulatory IL-10 response.

INTRODUCTION

Rheumatoid arthritis (RA) is a progressive autoimmune disease, characterized by chronic inflammation of the articular joints. The inflammation results in irreversible destruction of cartilage and bone by enzymes produced by macrophages and fibroblast-like cells that have invaded the inflamed synovium. Different types of cells that are involved in arthritis and in other autoimmune disorders have been exploited as a tool to study or treat disease (1-4). T cells are promising candidates for immunological interventions in autoimmunity because of their antigen-specificity and their ability to modulate other cells that are involved in autoimmune disease.

Although the actual triggers leading to a disease like RA are unknown, *in vitro* proliferation of T cells from RA-patients in response to several autoantigens (5, 6) and restriction of the synovial T cell repertoire to common clonotypes (7, 8) are indicative of an autoantigen driven T cell expansion in RA. Transfer and depletion studies using $CD4^+$ T cells and the use of T cell receptor-transgenic (TCR-Tg) animals in arthritis models have underscored that joint-antigen specific $CD4^+$ T cells with a T_h1-like phenotype mediate the induction and/or aggravation of arthritis (9-13).

In addition, B cells invade the inflamed synovium and are implicated in the pathogenesis of RA as producers of autoantibodies (14, 15) and as efficient antigen-presenting cells (14, 16, 17). Both autoreactive T and B cell responses are required to induce severe arthritis, suggesting that both lymphocyte subsets contribute to development of arthritis (9, 14).

A current hypothesis states that excessive development and/or function of (auto)aggressive $CD4^+$ T cells is controlled by regulatory T cells (T_{reg} cells) (18-22). For example, a recent study by Lohr *et al.* (23) has shown that T_{reg} cells can control development of the IL-17 producing T_h17 cells which are crucial for development of inflammation in several autoimmunity models (24). Several subsets of T_{reg} cells have been described to suppress inflammation. Of these subsets, naturally occurring $CD4^+CD25^+$ T cells, $CD4^+$ T_r1 cells and $CD4^+$ T_h3 cells are the best studied T_{reg} cells. Although their exact phenotypes and mechanisms of suppression are still not fully understood and may vary between different subsets, most T_{reg} cells have been described to require interleukin-10 (IL-10) for successful suppression as summarized by Bluestone *et al.*(1).

IL-10 plays an important role in the homeostatic regulation of the autoreactive T cell repertoire (25). In addition, IL-10 depletion and IL-10 treatment in murine arthritis models (26-29) have demonstrated the anti-inflammatory properties of IL-10 in arthritis. Moreover, reduced IL-10 expression by $CD4^+$ T cells is related to a higher frequency of T_h1 cells and more severe disease in RA (30). Extensive studies (reviewed by Moore *et al.* (31)) have demonstrated that IL-10 inhibits the production of pro-inflammatory cytokines and chemokines in activated monocytes/macrophages and inhibits proliferation of $CD4^+$ T cells by downregulation of APC function. Moreover, IL-10 drives the generation of a population of IL-10 producing T_r1 cells that suppress antigen-specific T cell responses and prevent colitis (32, 33). Thus, exploiting antigen-specific $IL\text{-}10^+$ T cells to propagate anti-inflammatory responses can be a promising method for therapy of autoimmune diseases.

Since peripheral blood of RA patients contains $CD4^+$ T cell populations that are cartilage antigen-specific (5, 34, 35), *ex vivo* induction of a regulatory phenotype in such antigen specific T cells may provide a tool for antigen-specific interventions in the chronic inflammation of RA. Therefore, we explored the potential mechanisms of targeting the

inflammatory autoimmune response in cartilage proteoglycan (PG)-induced arthritis (PGIA) with PG-specific $CD4^+$ T cells expressing IL-10 through retroviral transduction with an active IL-10 gene. PGIA is a chronic and progressive arthritis model induced by immunization with PG, representing many features of RA (36). A recently generated PG-specific TCR (PG-TCR)-Tg mouse served as donor of PG-specific $CD4^+$ T cells to be studied in this arthritis model (10, 11). We questioned if PG-specific $T_{IL\text{-}10}$ cells would suppress a chronic arthritis and whether these cells would modulate by propagating a protective immune response *in vivo*.

The results indicated that PG-specific $T_{IL\text{-}10}$ cells are suppressive *in vitro*, reduced the PG-specific inflammatory immune response *in vivo* and promoted the endogenous IL-10 response. The observed arthritis-suppressive effect of $T_{IL\text{-}10}$ cells depended on the presence of their PG-specific TCR. Interestingly, PG-specific $T_{IL\text{-}10}$ cells could not reduce the inflammatory response in IL-10 deficient recipients, indicating that autoantigen-specific $T_{IL\text{-}10}$ cells regulated chronic arthritis via induction of the endogenous IL-10 response.

MATERIALS AND METHODS

Mice and antigens

BALB/c mice (obtained from Charles River Laboratories, Maastricht, The Netherlands) and IL-10 knockout BALB/c mice (kind gift from Dr. A. van Oosterhout) were kept at the animal facility of the University of Utrecht; "Gemeenschappelijk Dierenlaboratorium" (GDL) under standard conditions. DO11.10 (OVA-TCR Tg) and TCR-5/4E8-Tg (PG-TCR Tg) (10, 11) were bred and kept at the GDL under specific pathogen free conditions. Human PG (hPG) and murine PG (mPG) were prepared as described elsewhere (11). All animal experiments were approved by the Animal Experimental Committee of the Veterinary Faculty of the University of Utrecht.

Constructs and production of retrovirus

Murine IL-10 cDNA was obtained using specific primers (5'-AGA TCT TTG CAG AAA AGA GAG CTC CA-3' and 5'-GTC GAC TGG AGT CCA GCA GAC TCA AT-3') and cloned into the MSCV2.2 plasmid (Fig. 1).

Ecotropic replication-deficient retrovirus was produced with a Phoenix-Eco packager cell line. Packager cells were cultured per $3x10^6$ cells in 10 ml DMEM (Gibco Life Technologies, Breda, The Netherlands) (+4500 mg/L glucose, +GlutaMAX I, -Pyruvate) supplemented with 10% heat inactivated FCS (Bodinco B.V., Alkmaar, The Netherlands), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco) in 100-mm tissue culture dishes (Nalge Nunc International, Roskilde, Denmark) and cultured at 37°C, 6% CO_2. The next day the culture medium was refreshed and 1-3 hours thereafter 500 µl 0.25 M $CaCl_2$ containing 20 µg MSCV-plasmid and 5µg PCL-Eco plasmid was mixed with an equal volume HBS buffer pH 7.02 (280 mM NaCl, 1.5 mM Na_2HPO_4, 12 mM glucose, 10 mM KCl, 50 mM HEPES) by bubbling and added to the cells. At 20 hours after transfection the supernatant was replaced with fresh medium. Within 24 hours thereafter, supernatant containing the retrovirus was harvested, filtered with a 0.45 µm filter, snap-frozen and stored frozen until use. Again fresh medium was added and virus was harvested the next day and pooled with the previous supernatant for infection.

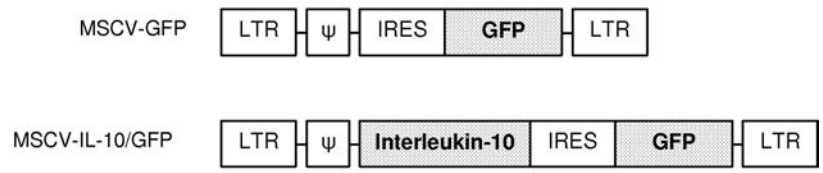

FIGURE 1. Retroviral constructs used for transduction of $CD4^+$ T cells. Murine IL-10 cDNA was inserted into the MSCV-GFP construct in front of the IRES sequence, enabling bicistronic translation of IL-10 and GFP genes.

Stimulation, retroviral transduction and transfer of $CD4^+$ T cells

$CD4^+$ T cells of pooled spleens and lymph nodes of DO11.10 or TCR-5/4E8-Tg were isolated with anti-CD4 (L3T4) magnetic microbeads (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and stimulated with magnetic M-450 Tosylactivated Dynabeads (Dynal Biotech ASA, Oslo, Norway) coated with anti-CD3 (145-2C11) and anti-CD28 (PV-1) mAbs (own production) in a 1:10 ratio. Anti-CD3/anti-CD28 coated beads were added to $1x10^6$ $CD4^+$ T cells (in a 2:1 ratio) in 1 ml supplemented DMEM in 24-well flat bottom plates (Corning Incorporated, Corning, NY) and cultured at 37°C, 6% CO_2. After 48 hours 750µl of the culture supernatant was replaced with 1 ml of retroviral supernatant supplemented with 8µg/ml hexadimethrine bromide (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Plates were then centrifuged at 930xG at 20°C for 2 hours. Subsequently, 1 ml of supernatant was replaced with fresh medium and cells were cultured for another 48 hours. Cells were removed from the stimulating beads and transduced cells (normally 60-80% prior to sorting) were sorted by GFP expression with a FACSVantage SE (Becton Dickinson). Acceptor mice received $1x10^6$ sorted IL-10/GFP-transduced or GFP-transduced $CD4^+$ cells, injected i.v. or i.p. in PBS.

Induction and assessment of arthritis

Arthritis was induced by i.p. injections of 2 mg hPG emulsified in 2 mg of the synthetic adjuvant dimethyl-dioctadecyl-ammoniumbromide (DDA) (Sigma) in PBS (total volume of 200 µl) on day 0 and day 21 as described elsewhere (11, 37). Paws were examined three times per week in a blinded set-up to determine onset and severity of arthritis using a standard visual scoring system based on swelling and redness of the paws (11, 37). Limbs were dissected, fixed in 10% buffered formalin, decalcified in 0.5 M EDTA and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin and examined for histopathology of ankle joints.

***In vitro* suppression assay**

$CD4^+$ responder cells were isolated from pooled spleen and lymph node (LN) cells of DO11.10 mice by negative selection with Dynabeads (Dynal) using an excess of anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-MHC class-II (M5/114) and anti-CD8 (YTS169) mAbs and were subsequently labeled with CFSE (Molecular Probes, Leiden, The Netherlands) as described elsewhere (38). At day 0 bone marrow cells from tibia and femurs were seeded at $2x10^6$ per 100 mm suspension dish (Corning) in 10 ml supplemented IMDM (Gibco) with 20 ng/ml rGM-CSF (Cytocen, Utrecht) to generate dendritic cells (DCs). At day 3, 10 ml supplemented IMDM with 20 ng/ml rGM-CSF was added. At day 6, 10 ng/ml rGM-CSF was added. At day 8 the non-adherent cells were harvested, washed and used. The suppression assays were done in 96-well flat bottom plates (Corning) with $2x10^5$ CFSE-labeled DO11.10 $CD4^+$ cells and $2.5x10^4$ bone marrow-derived BALB/c DCs cultured in the presence of 0.32 µg/ml $OVA_{323-339}$ peptide in 200 µl during 4 days. To analyze the suppressive activity of culture supernatant, 50 µl of the final culture medium consisted of this conditioned supernatant. To test the suppressive activity of transduced PG-specific T cells, indicated numbers of transduced cells and 0.30 µg/ml hPG_{70-84} peptide were added to the culture.

***Ex vivo* antigenic stimulation of splenocytes**

Single-cell suspensions of spleens of hPG-immunized mice that had received IL-10/GFP-transduced or GFP-transduced TCR-5/4E8-Tg $CD4^+$ cells were cultured in 96-well flat bottom plates (Corning) at $2x10^5$ cells per well, in the presence or absence of hPG (10 µg protein/ml) in supplemented IMDM (Gibco) for 72 hours.

Flow cytometry

Single-cell suspensions of spleen cells were cultured at $1\text{-}2x10^6$ cells/ml supplemented IMDM (Gibco) with 50 ng/ml PMA (Sigma) plus 500 ng/ml ionomycin (Sigma) during 4-5 hours for intracellular cytokine staining. Brefeldin A (Sigma) was added at 10µg/ml after the first two hours. Cells were washed, fixed in 4% PFA/PBS for 10 minutes and washed again. After permeabilization in PBS/2% FCS/0.5% saponin (Sigma), cells were washed and stained with anti-IL-10-PE (BD Biosciences Pharmingen, Woerden, The Netherlands) mAb and washed twice, all in the presence of 0.5% saponin. Cells were then washed and stained with anti-CD4-APC or anti-CD4-PerCP mAbs (BD Pharmingen). After extensive washing, cells were analyzed on a FACS Calibur (Becton Dickinson). Analysis of migration of GFP^+ $CD4^+$ cells with flow cytometry was done with unstimulated single-cell suspensions, stained with anti-CD4-APC without permeabilization of the cell membrane. For analysis of CFSE-suppression assays cells were stained with anti-CD4-PerCP mAb in combination with biotinylated KJ1.26 mAb (Caltag Laboratories, Uden, The Netherlands) and streptavidin-APC (BD Pharmingen). Results were analyzed with Cellquest software (Becton Dickinson).

Cytokine (protein) quantification

IL-2 was measured with fluoresceinated microspheres coated with ELISA capture antibodies (BD Pharmingen) as described elsewhere (11). In brief, coated beads were added to 50 µl of culture supernatant. After overnight incubation at 4°C, microspheres were washed and incubated with biotinylated detection antibodies (BD Pharmingen) for 15 min. Subsequently, streptavidin-PE was added to the microspheres, which were incubated for another 15 min and then measured with a Luminex model 100 (Luminex, Austin, TX). IL-10 and TNF-α in culture supernatant were measured with a comparable detection system using Lincoplex beads (LINCO Research, Inc., St. Charles, Missouri) according to the manufacturer's protocol.

cDNA synthesis and quantitative real-time(RT)-PCR for analysis of cytokine- and GFP expression

Total mRNA was extracted with the RNeasy kit (Qiagen Benelux B.V., Venlo, The Netherlands) and treated with DNase (Qiagen) using the manufacturer's protocol. Subsequently, RNA was reversely (RT) transcribed into cDNA using the iScript^{TM}cDNA Synthesis Kit (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Quantitative real-time PCR was performed in a total volume of 25 µl using iQ^{TM} SYBR Green® Supermix (Bio-Rad). 0.25 µM of primers specific for IL-10 (5'-GGT TGC CAA GCC TTA TCG GA-3' and 5'-ACC TGC TCC ACT GCC TTG CT-3'), IL-17a (5'-GCT CCA GAA GCC CCT CAG A-3' and 5'- AGC TTT CCC TCC GCA TTG A-3'), HPRT (5'-CTG GTG AAA AGG ACC TCT CG-3' and 5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3') and GFP (5'-GCA GTG CTT CAG CCG CTA-3' and 5'-AAG AAG ATG GTG CGC TCC TG-3'). PCR (3 min at 95°C and 40 cycles of 10 s at 95°C and 45 s at 59,5°C or, for GFP, 50 cycles of 10 s at 95°C and 45 s at

60,2°C) and real-time detection was done with a Bio-Rad MyiQ iCycler (Bio-Rad). Expression was normalized to the detected Ct values of HPRT for each sample.

Quantification of antigen specific serum antibodies

Serum mPG- and hPG-specific antibodies were measured by ELISA as described elsewhere (37). In brief, 96-well ELISA plates (Corning) were coated overnight with hPG or mPG in PBS. Free binding sites were blocked with 1% fat-free milk (Bio-Rad) in PBS. Sera were added at increasing dilutions in 1% fat-free milk in PBS. PG-specific IgG_1 and IgG_{2a} were determined using peroxidase-conjugated rat mAb to IgG_1 or IgG_{2a} (BD Pharmingen). Serum antibody levels were calculated as OD relative to the OD measured for the corresponding isotypes of a standard of pooled sera from arthritic mice.

Statistics

Data are expressed as mean ± standard error of the means (SEM) unless stated otherwise and statistical evaluation was done with nonparametric Mann-Whitney's U test (two-tailed) or with one-way ANOVA when more than two test groups were compared. $P<0.05$ was considered significant.

RESULTS

Retroviral transduction with the IL-10 gene is efficient and results in non-anergic CD4$^+$ T cells producing a high amount of IL-10

To generate functionally modulated cartilage antigen-specific CD4$^+$ T cells *ex vivo,* PG-specific CD4$^+$ T cells were stably transduced with the murine IL-10 gene using murine stem cell virus (MSCV). GFP was used as a marker to select transduced cells. To obtain a high transduction rate, PG-specific CD4$^+$ T cells isolated from naive PG-TCR Tg mice were stimulated with anti-CD3/anti-CD28 coated beads prior to infection with MSCV-IL10/GFP (Fig. 1). As a control CD4$^+$ T cells were transduced with the GFP gene alone (T_{GFP}) by MSCV-GFP. Due to an internal ribosomal entry site (IRES), the IL-10 gene and the GFP gene are translated into separate proteins.

CD3/CD28-stimulation of PG-specific CD4$^+$ T cells typically resulted in an increased number (1.5-2 times increase of number of starting population at two days post transfection) of CD4$^+$ cells of which 60-80% were transduced as analyzed for GFP expression by flow cytometry. After transduction with MSCV-IL-10/GFP, CD4$^+$ T cells showed a substantial increase in IL-10 expression compared to MSCV-GFP transduced CD4$^+$ T cells (Table I). This increase was observed at both mRNA level by quantitative real-time PCR and protein level measured in culture supernatants of transduced cells two days after transduction. At this moment no difference was found in IFN-γ, IL-2 or IL-4 concentration between culture supernatants of T_{IL-10} cells and T_{GFP} cells (data not shown). When T_{IL-10} cells were restimulated with anti-CD3 and irradiated APCs (data not shown) or with anti-CD3/anti-CD28 coated beads (Table I), these cells were fully able to proliferate as compared to T_{GFP} cells.

Table I. Phenotypes of TCR transgenic CD4$^+$ T cells transduced with IL-10/GFP or GFP

TCR	Construct	MFI GFP	IL-10[1]	IL-10[2]	Proliferation
PG-TCR	**IL-10/GFP**	644	62.0	31,999	14,896
PG-TCR	**GFP**	1,817	1.3	193	11,463
OVA-TCR	**IL-10/GFP**	630	50.0	38,651	ND
OVA-TCR	**GFP**	1,382	1.6	783	ND

Mean fluorescence intensity (MFI) was determined for the GFP$^+$ population by flow cytometry. [1]mRNA expression relative to HPRT expression in GFP$^+$ (sorted) cells as analyzed by quantitative real-time PCR two days after retroviral transduction. [2]Secretion of IL-10 (pg/ml) as measured in culture supernatant two days after transduction (60-70% of cells were GFP$^+$). Proliferation of GFP-sorted transduced T cells, shown as Δcpm, was measured by ^{3}H-Thymidine incorporation in response to anti-CD3/anti-CD28-stimulation. ND, not analyzed.

Transfer of PG-specific T_{IL-10} cells ameliorates arthritis

To examine if PG-specific T_{IL-10} cells could suppress chronic arthritis, PG-specific T_{IL-10} cells were transferred in the PGIA model. Arthritis was induced by two immunizations with PG in the synthetic adjuvant DDA with an interval of three weeks. One day before the second PG immunization $1x10^6$ PG-specific T_{IL-10} cells were transferred to acceptor mice. As a control, $1x10^6$ PG-specific T_{GFP} cells were transferred. Another control group received PBS instead of transduced T cells.

Mice that received PG-specific T_{GFP} cells or PBS developed a chronic arthritis while recipients of PG-specific T_{IL-10} cells developed a significantly reduced form of arthritis (Fig. 2A). In addition, maximum arthritis severity and cumulative incidence were dampened by the PG-specific T_{IL-10} cells (Table II). Amelioration of arthritis in PG-specific T_{IL-10} cell recipients

was confirmed by histological examination of ankle joint-sections showing less cellular infiltration in the ankle joints of the PG-specific T_{IL-10} cell recipients (Fig. 2C), compared to PG-specific T_{GFP} cell recipients (Fig. 2B).

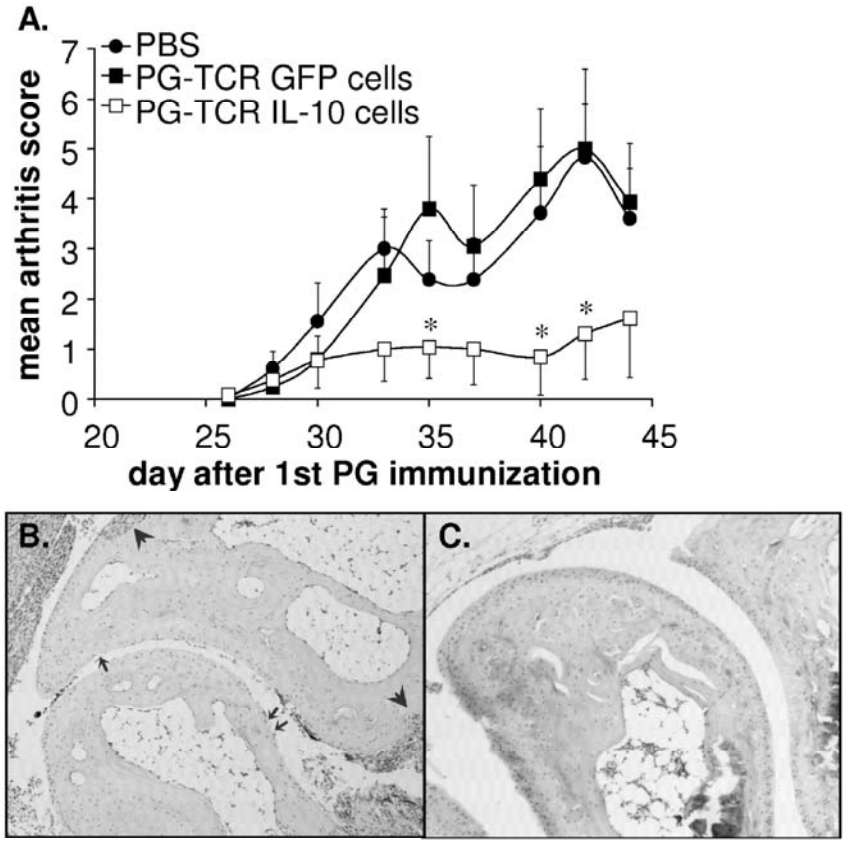

FIGURE 2. PG-specific CD4$^+$ T cells retrovirally transduced to produce IL-10 ameliorate arthritis. (A) Mice were immunized with PG on day 0 and day 21 and received 1x10^6 PG-specific T_{IL-10} cells (□, n=12), PG-specific T_{GFP} cells (■, n=10) or PBS (●, n=9) on the day before the second PG immunization. Data represent pooled data of two experiments. (B, C) Five weeks after transfer of (B) PG-specific T_{GFP} and (C) PG-specific T_{IL-10} in PGIA histological examination of hematoxylin and eosin stained sections of ankle joints was performed. Large arrowheads indicate infiltrating cells, small arrowheads indicate cartilage damage. *, $p<0.05$ for difference between recipients of PG-specific T_{GFP} cells and PG-specific T_{IL-10} cells.

Table II. Arthritis onset, arthritis incidence and maximum arthritis severity in mice receiving PG-TCR T_{GFP} cells, PG-TCR T_{IL-10} cells, OVA-TCR T_{IL-10} cells or PBS

	PBS	PG-TCR GFP	PG-TCR IL-10	OVA-TCR IL-10
Day of onset	31.3 (±1.5)	33.0 (±1.1)	31.1 (±0.9)	33.9 (±1.9)
Incidence	9/9 (100%)	24/26 (92%)	18/30 (60%)	11/12 (92%)
Maximum severity	5.3 (±1.2)	6.3 (±1.0)	3.2 (±0.8)[1]	5.8 (±1.6)

Day of arthritis onset (mean ± SEM), arthritis incidence (cumulative until day 44) and maximum severity (mean of maximum scores ±SEM) are shown for pooled data of four experiments analyzed for 44 days upon the primary PG immunization. Transduced T cells or PBS as a control were transferred on day 20. The second PG immunization was given on day 21 to induce arthritis. [1], $P<0.05$ for difference with PG-TCR T_{GFP} cell-recipient group and with OVA-TCR T_{IL-10} cell-recipient group.

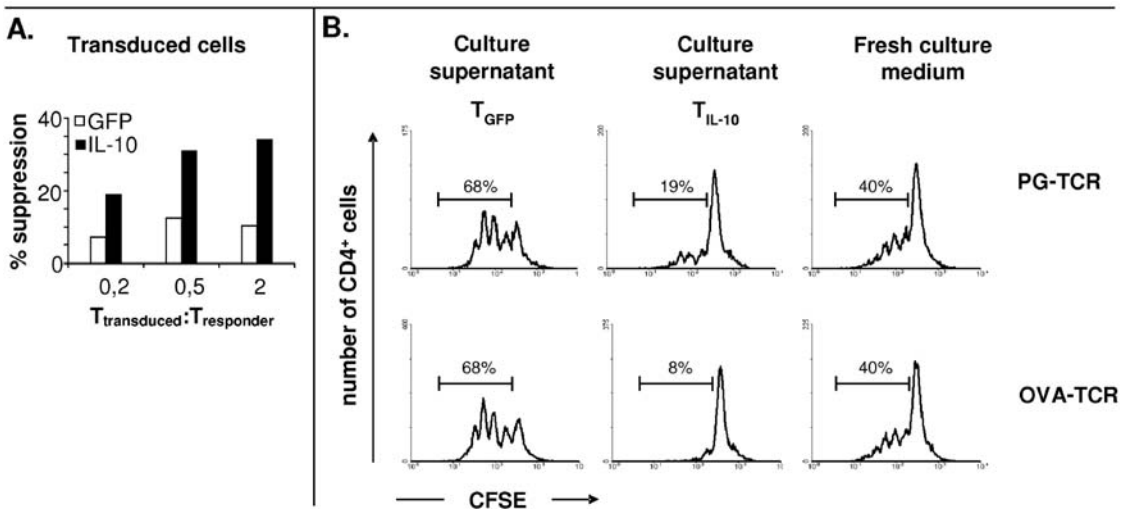

FIGURE 3. CD4$^+$ T cells retrovirally transduced with the murine IL-10 gene suppress proliferation of freshly isolated CD4$^+$ T cells *in vitro*. (A) CFSE-labeled CD4$^+$ T_{resp} cells were stimulated by peptide-loaded DC in the presence of different numbers of T_{IL-10} cells (■) or T_{GFP} cells (□) as indicated. Suppression is plotted as % suppression compared to proliferation in the absence of transduced T cells. (B) CFSE-labeled OVA-specific CD4$^+$ T_{resp} cells were stimulated by $OVA_{323-339}$-loaded DC in the presence of supernatants taken from cultured IL-10-transduced or GFP-transduced (approximately 70% of population transduced) PG-specific CD4$^+$ T-cells (upper panel) or OVA-specific CD4$^+$ T-cells (lower panel). Numbers indicate % of cells in dividing population.

T_{IL-10} cells suppress proliferation of CD4$^+$ T cells

One of the regulatory functions of IL-10 is suppression of proliferation of effector CD4$^+$ T cells in their response to antigenic stimulation. Therefore, we tested if T_{IL-10} cells could suppress proliferation of CD4$^+$ T cells responding to antigen presented by bone marrow derived DCs *in vitro*. For this purpose, CFSE-labeled OVA-specific CD4$^+$ T cells from OVA-TCR Tg mice were used as a model for responder CD4$^+$ T (T_{resp}) cells and stimulated in the presence of PG-specific T_{IL-10} cells or PG-specific T_{GFP} control cells. DCs loaded simultaneously with $OVA_{323-339}$ peptide and hPG_{70-84} peptide (the arthritogenic epitope that is recognized by PG-specific cells) were used to stimulate both the OVA-specific T_{resp} population and the transduced PG-specific (T_{IL-10} or T_{GFP}) cells simultaneously. Varying numbers of PG-specific T_{IL-10} cells or PG-specific T_{GFP} cells were added to this culture, with a T_{GFP}:T_{resp} or T_{IL-10}:T_{resp} ratio ranging from 2:1, 0.5:1 to 0.2:1. Within the population of T_{resp} cells the percentage of cells that went into division was determined from their CFSE profiles and plotted in figure 3A as percentage suppression relative to the proliferation in the absence of T_{IL-10} or T_{GFP} cells. Proliferation of T_{resp} cells was clearly suppressed in the presence of T_{IL-10} cells as compared to proliferation in the presence of T_{GFP} (control) cells. This suppression was more pronounced when higher numbers of T_{IL-10} cells were added to the T_{resp} cells.

To check if suppression by T_{IL-10} cells was mediated by secreted factors, a culture system was set up with $OVA_{323-339}$-loaded DC to stimulate CFSE labeled OVA-specific T_{resp} cells in the presence of supernatant from cultured T_{GFP} or T_{IL-10} cells. Division of these CFSE$^+$ T cells was used as a read out for the presence of such secreted factors. Thus, conditioned medium of cultured T_{IL-10} or T_{GFP} cells taken 48 hours after transduction was added to the DC-T_{resp} culture to check for the presence of secreted suppressive factors (Fig. 3B). Fresh culture medium was used as control. Conditioned medium of T_{IL-10} cells suppressed

proliferation of $CD4^+$ T_{resp} cells as shown by reduced CFSE dilution (Fig. 3B) compared to conditioned medium of T_{GFP} cells or fresh culture medium. This is shown for supernant taken from both PG-specific (upper panel) and OVA-specific (lower panel) T_{IL-10} cells, showing that the OVA-specific T_{IL-10} cells that are used as a control for antigen-specificity for suppression *in vivo* (see next paragraph) secrete functional IL-10 to the same extend as PG-specific T_{IL-10} cells. When IL-10 in supernatant taken from cultured T_{IL-10} cells was neutralized with anti-IL-10 mAb (JES-2A5), the rate of proliferation of CFSE-labeled T_{resp} cells increased with 86% (data not shown). Blocking IL-10 function in conditioned medium taken from cultured T_{GFP} cells yielded an increase of proliferation of only 2%. This indicates that IL-10 secreted by T_{IL-10} cells is the main secreted factor responsible for suppression.

T_{IL-10} cells require recognition of the cartilage antigen PG *in vivo* to ameliorate arthritis

Subsequently, we asked whether T_{IL-10} cells require recognition of PG to exert their anti-inflammatory properties. To this end, T_{IL-10} cells expressing a transgenic TCR specific for OVA (OVA-TCR) were transferred in PGIA as described in the previous section. Unlike PG-specific T_{IL-10} cells, OVA-specific T_{IL-10} cells could not reduce severity of arthritis (Table II). In addition, OVA-specific T_{IL-10} cells did not reduce the incidence of arthritis whereas PG-specific T_{IL-10} cells did. Although a difference was found for arthritis suppressive capacity *in vivo*, OVA-specific T_{IL-10} cells showed a similar phenotype as PG-specific T_{IL-10} cells when analyzed for IL-10 expression (Table I) and suppressive activity *in vitro* (Fig. 3B) two days post transduction. This indicates that differences found for regulation *in vivo* may be ascribed to the different specificities of TCRs.

The need of their specificity for cartilage antigens to regulate arthritis suggests that T_{IL-10} cells may act locally and thus migrate to where the antigen is processed and presented. To address whether transferred PG-specific T_{IL-10} cells and OVA-specific T_{IL-10} cells migrate differentially, GFP expression was analyzed in spleen, joint-draining lymph nodes (LN), joints and in cervical LN and non-lymphoid tissue. Detection of GFP^+ T cells in the lymphoid organs was done by flow cytometry for GFP^+ cells within the $CD4^+$ population. In joints and pancreas this was done by RT-PCR for mRNA expression of GFP. In table III the ratio of $CD4^+GFP^+$ cells within the joint-draining LN over the $CD4^+GFP^+$ cells in spleen or cervical LN is shown to depict preferential migration of the GFP^+ T_{IL-10} cells. Up to two weeks after transfer, both OVA-specific and PG-specific T_{IL-10} cells were found in all lymphoid organs analyzed (up to 0.66% of the total $CD4^+$ population), indicating that, irrespective of the specificity of the TCR, the T_{IL-10} cells migrate throughout the whole lymphoid compartment. However, at two weeks after transfer the number of PG-specific T_{IL-10} cells tended, compared to OVA-specific T_{IL-10} cells, to preferentially be sustained in joint-draining LN and spleen as shown by a higher ratio of GFP^+ cells in joint-draining LNs over cervical LNs and a lower ratio of these cells in draining LNs over spleen. However, at four weeks after transfer no GFP^+CD4^+ cells could be distinguished from background (=0,002% in arthritic mice receiving no GFP^+ cells) in these lymphoid organs anymore. In addition, table III shows that although GFP expression was found in joints of some animals that received OVA-specific T_{IL-10} cells (50% of all animals analyzed), in most animals that received PG-specific T_{IL-10} cells (83% of all animals analyzed) GFP expression was found in the joints. Moreover, GFP expression was not found in pancreatic tissue (data not shown), indicating that T_{IL-10} cells preferentially migrate to the inflamed tissue (joints).

Table III. Migration of PG-TCR T_{IL-10} cells and OVA-TCR T_{IL-10} cells at different time-points after transfer in PGIA

	1 week		2 weeks			4 weeks		
	draining LN/ spleen[1]	*draining LN/ cervical LN*[1]	*draining LN/ spleen*[1]	*draining LN/ cervical LN*[1]	*Joints*[2]	*draining LN/ spleen*[1]	*draining LN/ cervical LN*[1]	*Joints*[2]
PG-TCR T_{IL-10}	0.24 (±0.02)	0.63 (±0.26)	0.21 (±0.04)	1.11 (±0.52)	2/3 (66%)	none detected	none detected	3/3 (100%)
OVA-TCR T_{IL-10}	0.22 (±0.03)	0.88 (±0.06)	0.61 (±0.18)	0.76 (±0.24)	2/4 (50%)	none detected	none detected	2/4 (50%)

The presence of transferred PG-TCR T_{IL-10} and OVA-TCR T_{IL-10} cells in different organs was determined by their expression of their transduced GFP-gene at 1, 2 and 4 weeks after transfer in PGIA. [1]Ratio of transferred T cells within the $CD4^+$ population of joint-draining lymph nodes to spleens or cervical lymph nodes as analyzed by flow cytometry for GFP expression (mean of 3-5 animals per group ±SEM). [2]Presence of transferred T cells in joints was determined by RT-PCR for GFP mRNA expression. Number of positive animals out of the number of animals tested is shown.

PG-specific T_{IL-10} cells suppress the pro-inflammatory cytokine response *in vivo*

$CD4^+$ effector T cells (T_{eff}) contribute to the pathogenesis of arthritis. *In vitro* data show that T_{IL-10} cells suppress proliferation of $CD4^+$ T cells. This suggests that T_{IL-10} cells might reduce the activation of the PG-specific T_{eff} cells in arthritis. Since activated T cells produce IL-2, we measured the PG-specific IL-2 response to determine the effect of PG-specific T_{IL-10} cells on PG-specific T_{eff} activation. Therefore, spleen cells were taken from animals two weeks after transfer of PG-specific T_{IL-10} cells or PG-specific T_{GFP} control cells in PGIA, stimulated with or without hPG *ex vivo* and secreted IL-2 was measured subsequently. Figure 4A shows that the hPG specific IL-2 response was significantly lower in the group that had received PG-specific T_{IL-10} cells compared to the PG-specific T_{GFP} cell recipients. In addition, reduced antigen-specific expression of the pro-inflammatory cytokine TNF-α in splenocytes from T_{IL-10} cell recipients paralleled the observed protection by PG-specific T_{IL-10} cells (Fig. 4B).

Recently, the pro-inflammatory cytokine IL-17 has been described as a pathogenic T cell derived cytokine in autoimmune inflammatory disorders. Therefore, we wondered whether T_{IL-10} cells could suppress the IL-17 response *in vivo*. To this end, we quantified IL-17 (IL-17a) expression by real-time PCR in spleen cells taken at four weeks after transfer of PG-specific T_{IL-10} cells or PG-specific T_{GFP} control cells in PGIA (Fig. 4C). After transfer of PG-specific T_{IL-10} cells, IL-17 mRNA expression was significantly reduced compared to the PG-specific T_{GFP} recipient group.

Together with the anti-proliferative effects, this cytokine profile suggests that regulation of growth and/or activation of pro-inflammatory effector $CD4^+$ T cells may be part of the protective effect by PG-specific T_{IL-10} cells in PGIA.

PG-specific T_{IL-10} cells propagate the IL-10 response *in vivo*

Since IL-10 has been shown to propagate the expression of IL-10 *in vitro* (32, 39), we wondered whether the transferred PG-specific T_{IL-10} cells would cause a substantial increase in the IL-10 response *in vivo* during PGIA. To this end, IL-10 expression in spleen cells of recipient mice was analyzed two weeks after transfer of PG-specific T_{IL-10} cells or PG-specific T_{GFP} (control) cells in PGIA. Spleen cells were cultured *ex vivo* with hPG or medium as a control and IL-10 in culture supernatants was quantified (Fig. 4D). Culture supernatants of

cultured spleen cells of PG-specific T_{IL-10} cell recipients contained higher concentrations of PG-specific IL-10 than that of PG-specific T_{GFP} cell recipients. Furthermore, spontaneous IL-10 production by unstimulated spleen cells (medium control) was also slightly enhanced in the PG-specific T_{IL-10} cell recipients (data not shown). In addition, quantitative real-time PCR with unstimulated spleen cells taken directly after isolation showed a significant increase of IL-10 expression in the PG-specific T_{IL-10} cell recipients (Fig. 4D). Flow cytometry to determine numbers of IL-10-producing splenocytes of mice that received PG-specific T_{IL-10} cells showed an increase in the number of IL-10^+ cells in the $CD4^-$ population (1.3% ±0.31 in T_{IL-10}-recipients and 0.9% ±0.09 in T_{GFP}-recipients). An increase was also found in the $CD4^+$ population (2.2% ±0.30 in T_{IL-10}-recipients and 1.9% ±0.43 in T_{GFP}-recipients) compared with PG-specific T_{GFP}-recipients. As described previously, no GFP^+ (from transferred T_{IL-10}) cells could be detected by flow cytometry at this time point anymore, indicating that the increased IL-10^+ population consisted of endogenous cells. Together, these data indicate that the PG-specific T_{IL-10} cells enhance the IL-10 response *in vivo*.

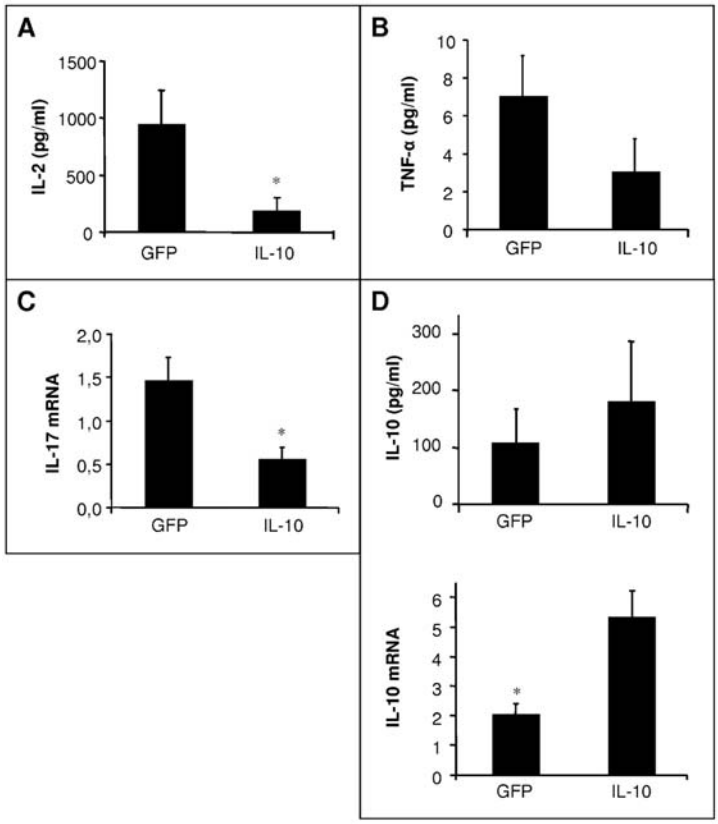

FIGURE 4. PG-specific T_{IL-10} $CD4^+$ T cells propagate an anti-inflammatory cytokine response *in vivo*. Spleen cells were isolated after transfer of PG-specific T_{IL-10} or PG-specific T_{GFP} cells in PGIA as described for fig. 2 and were either cultured *in vitro* (A, B, D upper graph) or used for RNA isolation directly (C, D lower graph). Protein concentrations were measured in supernatants after 72 h. culture in the presence or absence of hPG, two weeks after transfer. (A) IL-2, (B) TNF-α and (D) IL-10 is plotted as the amount (pg/ml) of protein hPG-specifically produced and is representative for two experiments (C) IL-17 and (D) IL-10 mRNA expression was quantified by quantitative PCR on reversely transcribed mRNA, four weeks or two weeks after transfer respectively. Expression of mRNA is normalized to HPRT-mRNA expression ($x10^3$). All data are expressed as means ± SEM (n=5 per group).
*, $p<0.05$.

PG-specific T_{IL-10} cells suppress the antigen specific IgG_{2a} antibody response

A PG-specific B cell response is required to cause severe PGIA and depends on the interaction between B cells and T cells (14, 40). To examine whether PG-specific T_{IL-10} cells would suppress the PG-specific B cell response, PG-specific IgG_1 and IgG_{2a} antibody responses were analyzed. Therefore, sera were taken from mice four weeks after they had received PG-specific T_{IL-10} cells or PG-specific T_{GFP} control cells in PGIA and hPG-specific antibodies of the IgG_1 and IgG_{2a} isotypes were analyzed by ELISA. Figure 5 shows that the hPG specific IgG_{2a} response was significantly suppressed by the PG-specific T_{IL-10} cells

compared to T_{GFP} cells. A similar trend was found for the anti-murine (m)PG-specific IgG_{2a} response; 364.1 (±171.5, n=6) in T_{GFP} cell recipients and 130.1 (± 40.3, n=7) in $T_{IL\text{-}10}$ cell recipients. Collectively, these data demonstrate that PG-specific $T_{IL\text{-}10}$ cells act on the PG-specific B cell response by inhibiting PG-specific IgG_{2a} antibody production.

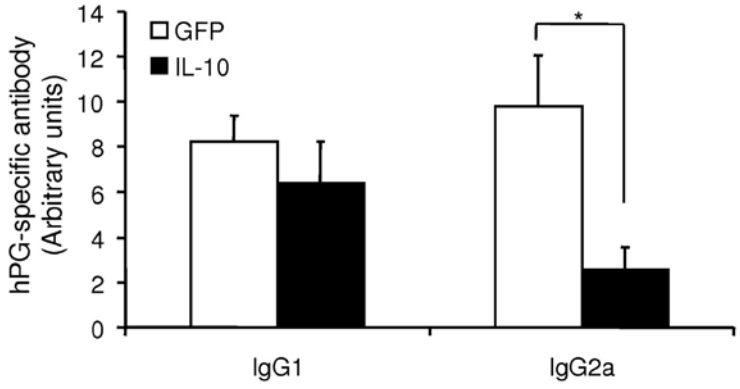

FIGURE 5. PG-specific $CD4^+$ $T_{IL\text{-}10}$ cells induce a shift in PG-specific B cell immunity towards a reduced IgG_{2a} response *in vivo*. hPG specific antibodies of the IgG_1- and IgG_{2a} isotype were measured in sera by ELISA at 4 weeks after transfer of PG-specific $CD4^+$ $T_{IL\text{-}10}$ cells (■) or T_{GFP} cells (□) to PG immunized mice as described for fig. 2. Concentrations are shown as units relative to a standard of pooled sera of arthritic mice ± SEM (n=5 mice per group). The results are representative for two experiments. *, $p<0.05$ for the hPG-specific IgG_{2a} response in PG-specific $T_{IL\text{-}10}$ cell recipients compared with PG-specific T_{GFP} cell recipients.

PG-specific $T_{IL\text{-}10}$ cells suppress inflammation via the endogenous IL-10 response

IL-10 has been shown to promote expression of IL-10 not only in several cell types, but IL-10 also has been shown to induce regulatory capacities in APCs (39) and in $CD4^+$ cells (32, 41) during their activation, as a mechanism for infectious tolerance (42). The boosted IL-10 response that was observed in animals which had received PG-specific $T_{IL\text{-}10}$ cells suggests that $T_{IL\text{-}10}$ cells stimulate a protective endogenous IL-10 response. To test if the PG-specific $T_{IL\text{-}10}$ cells indeed need to boost the endogenous IL-10 response of the recipient mice to accomplish suppression of the arthritic immune response we transferred PG-specific $T_{IL\text{-}10}$ cells in PGIA in IL-10 deficient BALB/c mice. PG-specific $T_{IL\text{-}10}$ cells or PG-specific T_{GFP} control cells were transferred to IL-10 deficient BALB/c mice one day before the second PG immunization of the PGIA induction protocol. Transfers to IL-10 deficient animals were done in parallel with transfers of the same T cell populations to wild type recipients that were used as a positive control. Although PG-specific $T_{IL\text{-}10}$ cells ameliorated arthritis in these wild type recipients, as shown by data integrated in table II, no difference in the arthritis score could be observed compared to the PG-specific T_{GFP} cell recipient control group at any time point in IL-10 deficient mice (Fig. 6), showing that PG-specific $T_{IL\text{-}10}$ cells could not suppress arthritis in IL-10 deficient mice. Taken together, these data indicate that PG-specific $T_{IL\text{-}10}$ cells regulate the arthritic immune response via propagation of the endogenous IL-10 response *in vivo*.

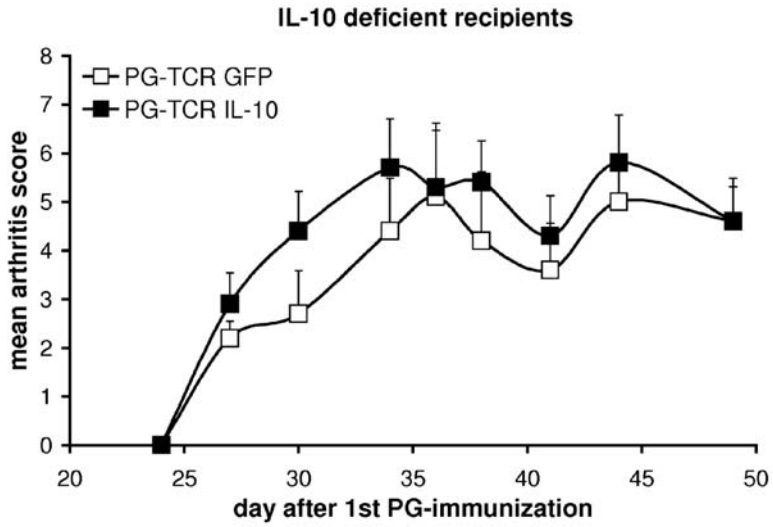

FIGURE 6. PG-specific CD4$^+$ T_{IL-10} cells do not suppress arthritis in IL-10 deficient recipients. Mice were immunized with PG and received 1x10^6 PG-specific T_{IL-10} cells (■) or PG-specific T_{GFP} (□) cells on the day before the boosting PG immunization as described for fig. 2. The graph shows the mean arthritis severity (score) per group (n=5 per group) per day ± SEM and is representative for two separate experiments. No significant differences were observed at any time point.

DISCUSSION

Inflammatory autoimmune disease may result from a disturbed homeostatic balance between autoaggressive T_{eff} cells and autoreactive T_{reg} cells. Although several immunoregulatory mechanisms have been described, numerous studies indicate IL-10 being crucial for several populations of T_{reg} cells to maintain this balance (31). The requirement for an IL-10 response to control excessive autoreactive immune responses in arthritis is underscored by studies showing that reduced numbers of $CD4^+$ T cells producing IL-10 in rheumatoid arthritis patients (30) and a genetic predisposition to low IL-10 production in juvenile idiopathic arthritis seemed to correlate with enhanced disease severity (43). Despite these facts, treatment of existing autoimmune inflammatory diseases like arthritis with systemic IL-10 administration has not been particularly successful so far (44). Most likely, for IL-10 to have a regulatory effect, targeting of this cytokine to sites of relevant cell-cell interaction is essential. In addition, the *in vivo* administration of *ex vivo* expanded non-antigen specific T_{reg} cells, although seemingly attractive, is probably risky as it may lead to uncontrolled immune suppression. For these reasons we endeavored to combine antigen directed targeting and delivery of IL-10 by the use of antigen-specific IL-10-transduced T cells. In the present study we show that administration of IL-10-transduced cartilage PG-specific $CD4^+$ T cells can ameliorate chronic PGIA by amplifying a regulatory endogenous IL-10 response.

The $CD4^+$ T cells used in this study are specific for the arthritogenic immunodominant T cell epitope of cartilage PG and have been shown previously to induce the T_h1-dominated PG-induced arthritis when activated *in vivo* (10). Although stimulation of $CD4^+$ T cells via CD3 plus CD28 that was used for efficient retroviral transduction has been shown to induce a T_h1-like phenotype (45), $1x10^6$ CD3/CD28-stimulated PG-specific T cells significantly suppressed clinical arthritis when transduced with an active IL-10 gene. In addition, analysis for the presence of transduced T cells by flow cytometry and RT-PCR after transfer *in vivo* indicated that rather low numbers of transduced T cells are effective in suppressing disease. This disease suppressive effect was paralleled by a reduced PG-specific production of both IL-2 and TNF-α, indicating that PG-specific T_{IL-10} cells specifically control the inflammatory autoimmune response. In addition, the finding that OVA-specific T_{IL-10} cells did not suppress arthritis indicated that IL-10 produced by T_{IL-10} cells was targeted to the PG-specific inflammatory response.

Adoptive transfer studies with transduced antigen-specific T cells in arthritis (46, 47) indicated requirement for autoantigen-specificity of transduced T cells to exert their suppressive functions locally at the site of inflammation. PG-specific T_{IL-10} cells migrated to joints and draining lymph nodes. This suggests that these cells may interact with pro-inflammatory cells at locations where cartilage antigens are presented. Although OVA-specific T_{IL-10} cells could be found in the inflamed joints and joint-draining LNs, presumable as part of a steady-state influx of cells into the site of inflammation, migration of PG-specific T_{IL-10} cells was found in these organs at an increased rate. Moreover, PG-specific T_{IL-10} cells could not be found in irrelevant non-lymphoid tissue (pancreas) indicating that PG-specific T_{IL-10} cells preferentially migrated to the inflamed joint-tissue. Furthermore, the IL-10 concentration in blood was below detection level in protected animals (data not shown), suggesting that the systemic level of IL-10 was not substantially increased. Though systemic administration of IL-10 is known to suppress PGIA (27), the findings in this study indicate that PG-specific T_{IL-10} cells target IL-10 to the actual site of the autoimmune response rather than through a systemic IL-10 response. Recognition of the antigen is required for adequate

regulation and only the ability to home to the inflamed organ is not sufficient to regulate inflammation. Altogether, these results suggest that autoantigen-specific $CD4^+$ T_{IL-10} cells depend on recognition of their cognate autoantigen to sustain interactions of these T cells at locations where they counteract inflammatory cells.

Moreover, it is now becoming clear that a distinct T cell population, T_h17 cells (24, 48), contributes to (auto)inflammatory responses. This population is characterized as a source of the pro-inflammatory IL-17, which has been described to be a crucial cytokine for development of autoimmunity and destruction of cartilage in arthritis (49). The reduced IL-17a response we observed in the protected animals during the chronic phase of arthritis would therefore be in line with the idea of IL-17 as a pro-inflammatory cytokine in arthritis and suggests that $CD4^+$ T cells that produce IL-10 dampen the pro-inflammatory IL-17 response.

Early generation of T_h17 cells, by stimulation in the presence of TGF-β and IL-6, has been shown to be restricted by IL-2 (50). However, other recent studies have demonstrated that IL-2 will ultimately lead to expansion of the T_h17 cell population (51, 52). Therefore, the reduction of IL-17 in our study has to be noted in the context of suppression of the PG-specific IL-2 response by $CD4^+$ T_{IL-10} cells. In our study *in situ* IL-17 mRNA expression in a mature stage of disease was reduced by T_{IL-10} cells, indicating that the reduced PG-specific IL-2 response does not sustain development of T_h17 cells. These data would rather indicate the opposite; reduction of hPG-specific IL-2 in the arthritic immune response may help to reduce the expansion of T_h17 cells, which would be in line with the recent studies mentioned above (51, 52) showing final expansion of T_h17 cells by IL-2. The suppression of IL-2 and IL-17 we found may therefore be placed in the context of IL-10 in the cytokine milieu and further study is needed to help elucidating differentiation and growth of T_h17 cells. In addition to IL-17 mRNA expression *in situ*, PG-specific secretion of IL-17 by splenocytes was analyzed at four weeks after transfer of PG-specific T_{IL-10} or T_{GFP} cells in PGIA. However, splenocytes stimulated with PG did not produce IL-17 amounts that were significantly different from the unstimulated (medium) controls (data not shown), which is indicative of a rather small population of PG-specific T_h17 cells.

Since B cells and antibodies are essential for the pathogenesis of (PG-induced) arthritis and $CD4^+$ T cells are determinants of antigen specific antibody responses, we studied how T_{IL-10} cells would influence autoantibody production. T_{IL-10} cells reduced the human- and mouse PG-specific IgG_{2a} response. This is in line with the effect of T_{IL-10} cells in reducing T_h1 responses, as IgG_{2a} is considered a T_h1-induced isotype (53) and PG-specific IgG_{2a} autoantibodies correlate with severity of T_h1-mediated PGIA (27, 40, 54).

It has been speculated that IgG_{2a} autoantibodies may elicit a pathogenic effect through Fcγ receptor III (FcγRIII)-mediated mechanisms (40, 55) indispensable for development of PGIA (56, 57). During the effector phase of inflammation, FcγR-immune complex interaction is supposedly required for the expression of pro-inflammatory cytokines and β-chemokines in ankle joints to stimulate the influx of lymphocytes, macrophages and neutrophils into the joint (57). Therefore, the reduced PG-specific IgG_{2a} response in PG-specific T_{IL-10} recipients may indicate that T_{IL-10} cells control the pro-inflammatory B cell response by preventing interaction of harmful autoreactive T_h1 cells with B cells.

Besides suppressing the inflammatory response, another notable feature of IL-10 is its property to promote IL-10 expression and concurrent immunosuppressive features in $CD4^+$ T cells (32) and DCs (39). Therefore, the immune modulatory potency of IL-10 produced by T_{IL-10} cells may not just be the inhibition of pro-inflammatory mediators, such as

TNF-α and IL-17, but may be found even more in the amplification of IL-10 expression and concomitant transfer of regulatory qualities. Indeed, expression of IL-10 was elevated in T_{IL-10} cell recipients not only at the mRNA level and cytokine level, but also relative numbers of cells producing IL-10 *in situ* were elevated as analyzed by flow cytometry, which was most pronounced within the $CD4^-$ population. Moreover, since within the $IL\text{-}10^+$ cell population no transduced cells were detected, these data indicate that the elevated IL-10 level measured in T_{IL-10} cell-recipients was not solely produced by the transferred T_{IL-10} cells, but, at least in part, by endogenous cells of the recipient. Furthermore, the finding that PG-specific T_{IL-10} cells did not protect IL-10 deficient recipients from arthritis indicated that propagation of the endogenous regulatory IL-10 response by these T_{IL-10} cells was indeed required to generate regulation of arthritis. Considering their antigen specific interaction with T_{IL-10} cells, APCs such as DCs or antigen specific B cells are good candidates in which T_{IL-10} cells might antigen specifically propagate IL-10 expression. These cells have, in turn, shown to propagate the IL-10 producing regulatory T_r1 population (39) and to suppress the generation of a pathogenic T_{eff} cell response (3, 39).

In summary, this study shows that IL-10-transduced $CD4^+$ T cells may control the chronic autoimmune response in arthritis, and that their specificity for a cartilage antigen is essential. Besides controlling the autoantigen-specific pro-inflammatory cytokine response, a suppressive effect was found at the level of B cell immunity. Moreover, it was shown for the first time that one of the crucial mechanisms by which such T_{IL-10} cells control inflammation in arthritis is the spreading of expression of IL-10 and concomitant regulatory properties to the endogenous immune response. Therefore, autoantigen-specific T_{IL-10} cells may restore immune homeostasis by suppressing the pro-inflammatory response and promoting the regulatory endogenous IL-10 response.

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REFERENCES

1. Bluestone, J. A. 2005. Regulatory T-cell therapy: is it ready for the clinic? *Nat Rev Immunol* 5:343-349.
2. Tarner, I. H., A. J. Slavin, J. McBride, A. Levicnik, R. Smith, G. P. Nolan, C. H. Contag, and C. G. Fathman. 2003. Treatment of autoimmune disease by adoptive cellular gene therapy. *Ann N Y Acad Sci* 998:512-519.
3. Fillatreau, S., C. H. Sweenie, M. J. McGeachy, D. Gray, and S. M. Anderton. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 3:944-950.
4. Rabinovich, G. A., G. Daly, H. Dreja, H. Tailor, C. M. Riera, J. Hirabayashi, and Y. Chernajovsky. 1999. Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med* 190:385-398.
5. Li, N. L., D. Q. Zhang, K. Y. Zhou, A. Cartman, J. Y. Leroux, A. R. Poole, and Y. P. Zhang. 2000. Isolation and characteristics of autoreactive T cells specific to aggrecan G1 domain from rheumatoid arthritis patients. *Cell Res* 10:39-49.
6. ter Steege, J., M. Vianen, J. van Bilsen, J. Bijlsma, F. Lafeber, and M. Wauben. 2003. Identification of self-epitopes recognized by T cells in rheumatoid arthritis demonstrates matrix metalloproteinases as a novel T cell target. *J Rheumatol* 30:1147-1156.
7. Stamenkovic, I., M. Stegagno, K. A. Wright, S. M. Krane, E. P. Amento, R. B. Colvin, R. J. Duquesnoy, and J. T. Kurnick. 1988. Clonal dominance among T-lymphocyte infiltrates in arthritis. *Proc Natl Acad Sci U S A* 85:1179-1183.
8. Kobari, Y., Y. Misaki, K. Setoguchi, W. Zhao, Y. Komagata, K. Kawahata, Y. Iwakura, and K. Yamamoto. 2004. T cells accumulating in the inflamed joints of a spontaneous murine model of rheumatoid arthritis become restricted to common clonotypes during disease progression. *Int Immunol* 16:131-138.
9. Bardos, T., K. Mikecz, A. Finnegan, J. Zhang, and T. T. Glant. 2002. T and B cell recovery in arthritis adoptively transferred to SCID mice: antigen-specific activation is required for restoration of autopathogenic CD4+ Th1 cells in a syngeneic system. *J Immunol* 168:6013-6021.
10. Berlo, S. E., P. J. van Kooten, C. B. Ten Brink, F. Hauet-Broere, M. A. Oosterwegel, T. T. Glant, W. Van Eden, and C. P. Broeren. 2005. Naive transgenic T cells expressing cartilage proteoglycan-specific TCR induce arthritis upon in vivo activation. *J Autoimmun* 25:172-180.
11. Berlo, S. E., T. Guichelaar, C. B. Ten Brink, P. J. van Kooten, F. Hauet-Broere, K. Ludanyi, W. van Eden, C. P. Broeren, and T. T. Glant. 2006. Increased arthritis susceptibility in cartilage proteoglycan-specific T cell receptor-transgenic mice. *Arthritis Rheum* 54:2423-2433.
12. Maffia, P., J. M. Brewer, J. A. Gracie, A. Ianaro, B. P. Leung, P. J. Mitchell, K. M. Smith, I. B. McInnes, and P. Garside. 2004. Inducing experimental arthritis and breaking self-tolerance to joint-specific antigens with trackable, ovalbumin-specific T cells. *J Immunol* 173:151-156.
13. Osman, G. E., S. Cheunsuk, S. E. Allen, E. Chi, H. D. Liggitt, L. E. Hood, and W. C. Ladiges. 1998. Expression of a type II collagen-specific TCR transgene accelerates the onset of arthritis in mice. *Int Immunol* 10:1613-1622.
14. O'Neill, S. K., M. J. Shlomchik, T. T. Glant, Y. Cao, P. D. Doodes, and A. Finnegan. 2005. Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis. *J Immunol* 174:3781-3788.
15. Chaiamnuay, S., and S. L. Bridges, Jr. 2005. The role of B cells and autoantibodies in rheumatoid arthritis. *Pathophysiology* 12:203-216.
16. Brennan, F. R., K. Mikecz, E. I. Buzas, D. Ragasa, G. Cs-Szabo, G. Negroiu, and T. T. Glant. 1995. Antigen-specific B cells present cartilage proteoglycan (aggrecan) to an autoreactive T cell hybridoma derived from a mouse with proteoglycan-induced arthritis. *Clin Exp Immunol* 101:414-421.
17. Takemura, S., P. A. Klimiuk, A. Braun, J. J. Goronzy, and C. M. Weyand. 2001. T cell activation in rheumatoid synovium is B cell dependent. *J Immunol* 167:4710-4718.
18. Mason, D., and F. Powrie. 1998. Control of immune pathology by regulatory T cells. *Curr Opin Immunol* 10:649-655.
19. Mills, K. H., and P. McGuirk. 2004. Antigen-specific regulatory T cells--their induction and role in infection. *Semin Immunol* 16:107-117.
20. von Herrath, M. G., and L. C. Harrison. 2003. Antigen-induced regulatory T cells in autoimmunity. *Nat Rev Immunol* 3:223-232.
21. O'Garra, A., and P. Vieira. 2004. Regulatory T cells and mechanisms of immune system control. *Nat Med* 10:801-805.
22. Maloy, K. J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat Immunol* 2:816-822.
23. Lohr, J., B. Knoechel, J. J. Wang, A. V. Villarino, and A. K. Abbas. 2006. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *J Exp Med* 203:2785-2791.
24. Dong, C. 2006. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. *Nat Rev Immunol* 6:329-333.
25. Anderson, A. C., J. Reddy, R. Nazareno, R. A. Sobel, L. B. Nicholson, and V. K. Kuchroo. 2004. IL-10 plays an important role in the homeostatic regulation of the autoreactive repertoire in naive mice. *J Immunol* 173:828-834.
26. Finnegan, A., C. D. Kaplan, Y. Cao, H. Eibel, T. T. Glant, and J. Zhang. 2003. Collagen-induced arthritis is exacerbated in IL-10-deficient mice. *Arthritis Res Ther* 5:R18-24.
27. Finnegan, A., K. Mikecz, P. Tao, and T. T. Glant. 1999. Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. *J Immunol* 163:5383-5390.
28. Fellowes, R., C. J. Etheridge, S. Coade, R. G. Cooper, L. Stewart, A. D. Miller, and P. Woo. 2000. Amelioration of established collagen induced arthritis by systemic IL-10 gene delivery. *Gene Ther* 7:967-977.
29. Kasama, T., R. M. Strieter, N. W. Lukacs, P. M. Lincoln, M. D. Burdick, and S. L. Kunkel. 1995. Interleukin-10 expression and chemokine regulation during the evolution of murine type II collagen-induced arthritis. *J Clin Invest* 95:2868-2876.
30. Yudoh, K., H. Matsuno, F. Nakazawa, T. Yonezawa, and T. Kimura. 2000. Reduced expression of the regulatory CD4+ T cell subset is related to Th1/Th2 balance and disease severity in rheumatoid arthritis. *Arthritis Rheum* 43:617-627.
31. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.
32. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737-742.
33. Asseman, C., and F. Powrie. 1998. Interleukin 10 is a growth factor for a population of regulatory T cells. *Gut* 42:157-158.

34. Goodstone, N. J., M. C. Doran, R. N. Hobbs, R. C. Butler, J. J. Dixey, and B. A. Ashton. 1996. Cellular immunity to cartilage aggrecan core protein in patients with rheumatoid arthritis and non-arthritic controls. *Ann Rheum Dis* 55:40-46.
35. Guerassimov, A., Y. Zhang, S. Banerjee, A. Cartman, J. Y. Leroux, L. C. Rosenberg, J. Esdaile, M. A. Fitzcharles, and A. R. Poole. 1998. Cellular immunity to the G1 domain of cartilage proteoglycan aggrecan is enhanced in patients with rheumatoid arthritis but only after removal of keratan sulfate. *Arthritis Rheum* 41:1019-1025.
36. Glant, T. T., A. Finnegan, and K. Mikecz. 2003. Proteoglycan-induced arthritis: immune regulation, cellular mechanisms, and genetics. *Crit Rev Immunol* 23:199-250.
37. Hanyecz, A., S. E. Berlo, S. Szanto, C. P. Broeren, K. Mikecz, and T. T. Glant. 2004. Achievement of a synergistic adjuvant effect on arthritis induction by activation of innate immunity and forcing the immune response toward the Th1 phenotype. *Arthritis Rheum* 50:1665-1676.
38. Hauet-Broere, F., W. W. Unger, J. Garssen, M. A. Hoijer, G. Kraal, and J. N. Samsom. 2003. Functional CD25- and CD25+ mucosal regulatory T cells are induced in gut-draining lymphoid tissue within 48 h after oral antigen application. *Eur J Immunol* 33:2801-2810.
39. Wakkach, A., N. Fournier, V. Brun, J. P. Breittmayer, F. Cottrez, and H. Groux. 2003. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* 18:605-617.
40. Kaplan, C., J. C. Valdez, R. Chandrasekaran, H. Eibel, K. Mikecz, T. T. Glant, and A. Finnegan. 2002. Th1 and Th2 cytokines regulate proteoglycan-specific autoantibody isotypes and arthritis. *Arthritis Res* 4:54-58.
41. Zheng, S. G., J. H. Wang, J. D. Gray, H. Soucier, and D. A. Horwitz. 2004. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol* 172:5213-5221.
42. Cobbold, S., and H. Waldmann. 1998. Infectious tolerance. *Curr Opin Immunol* 10:518-524.
43. Crawley, E., S. Kon, and P. Woo. 2001. Hereditary predisposition to low interleukin-10 production in children with extended oligoarticular juvenile idiopathic arthritis. *Rheumatology (Oxford)* 40:574-578.
44. Asadullah, K., W. Sterry, and H. D. Volk. 2003. Interleukin-10 therapy--review of a new approach. *Pharmacol Rev* 55:241-269.
45. Broeren, C. P., G. S. Gray, B. M. Carreno, and C. H. June. 2000. Costimulation light: activation of CD4+ T cells with CD80 or CD86 rather than anti-CD28 leads to a Th2 cytokine profile. *J Immunol* 165:6908-6914.
46. Nakajima, A., C. M. Seroogy, M. R. Sandora, I. H. Tarner, G. L. Costa, C. Taylor-Edwards, M. H. Bachmann, C. H. Contag, and C. G. Fathman. 2001. Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. *J Clin Invest* 107:1293-1301.
47. Setoguchi, K., Y. Misaki, Y. Araki, K. Fujio, K. Kawahata, T. Kitamura, and K. Yamamoto. 2000. Antigen-specific T cells transduced with IL-10 ameliorate experimentally induced arthritis without impairing the systemic immune response to the antigen. *J Immunol* 165:5980-5986.
48. Wynn, T. A. 2005. T(H)-17: a giant step from T(H)1 and T(H)2. *Nat Immunol* 6:1069-1070.
49. Lubberts, E., M. I. Koenders, and W. B. van den Berg. 2005. The role of T-cell interleukin-17 in conducting destructive arthritis: lessons from animal models. *Arthritis Res Ther* 7:29-37.
50. Laurence, A., C. M. Tato, T. S. Davidson, Y. Kanno, Z. Chen, Z. Yao, R. B. Blank, F. Meylan, R. Siegel, L. Hennighausen, E. M. Shevach, and J. O'Shea J. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26:371-381.
51. Acosta-Rodriguez, E. V., G. Napolitani, A. Lanzavecchia, and F. Sallusto. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8:942-949.
52. Amadi-Obi, A., C. R. Yu, X. Liu, R. M. Mahdi, G. L. Clarke, R. B. Nussenblatt, I. Gery, Y. S. Lee, and C. E. Egwuagu. 2007. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. *Nat Med* 13:711-718.
53. Liew, F. Y. 2002. T(H)1 and T(H)2 cells: a historical perspective. *Nat Rev Immunol* 2:55-60.
54. Hollo, K., T. T. Glant, M. Garzo, A. Finnegan, K. Mikecz, and E. Buzas. 2000. Complex pattern of Th1 and Th2 activation with a preferential increase of autoreactive Th1 cells in BALB/c mice with proteoglycan (aggrecan)-induced arthritis. *Clin Exp Immunol* 120:167-173.
55. Gerber, J. S., and D. M. Mosser. 2001. Stimulatory and inhibitory signals originating from the macrophage Fcgamma receptors. *Microbes Infect* 3:131-139.
56. Kaplan, C. D., Y. Cao, J. S. Verbeek, M. Tunyogi-Csapo, and A. Finnegan. 2005. Development of proteoglycan-induced arthritis is critically dependent on Fcgamma receptor type III expression. *Arthritis Rheum* 52:1612-1619.
57. Kaplan, C. D., S. K. O'Neill, T. Koreny, M. Czipri, and A. Finnegan. 2002. Development of inflammation in proteoglycan-induced arthritis is dependent on Fc gamma R regulation of the cytokine/chemokine environment. *J Immunol* 169:5851-5859.

CHAPTER 5

IL-10-producing T_{reg} cells promote IL-10 expression by B cells in arthritis; a novel pathway to induction of regulatory cells in vivo?

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ABSTRACT

T_{reg} and B_{reg} cells are crucial immune regulators, preventing excessive pro-inflammatory responses that can lead to autoimmune disease or excessive inflammatory immune responses to pathogens. Regulation by these cells is characteristically mediated by their production of IL-10, which can suppress T_h1 and T_h2 responses. Moreover, IL-10 can promote growth of regulatory IL-10^+ T cells *in vitro*. Although T_h1 and T_h2 cells have been shown to modulate differentiation of the B cell cytokine and antibody response during B-T cell interactions, regulation by IL-10^+ T_{reg} cells of B cell differentiation *in vivo* is unclear. Therefore, we addressed in a T_h1 and B cell mediated mouse model for rheumatoid arthritis, cartilage proteoglycan-induced arthritis, how proteoglycan-specific IL-10^+ $CD4^+$ T_{reg} cells modulate differentiation of B cells and T cells. T_{reg} cells producing interleukin-10 neither down-regulate IFN-γ and IL-2 expression by B and $CD4^+$ T cells nor suppress proliferation of proteoglycan-specific T cells in their early response in arthritis. However, IL-10^+ T_{reg} cells increase populations of B and T cells producing IL-10 but do not induce TGF-β and Foxp3 expression. Together, these results suggest that T_{reg} cells producing IL-10 regulate autoimmune differentiation in inflammation mainly in sustaining B and T cells producing IL-10.

INTRODUCTION

In the past decade there has been an explosion of interest in the phenotype and immunosuppressive functions of regulatory CD4$^+$ T (T_{reg}) cell subsets in active regulation (1, 2) of harmful antigen-driven immune responses. Recently, also crucial regulatory activities of B cells in antigen-driven inflammatory disorders have been identified (3-8). These findings suggest that active regulation of antigen-driven inflammation is part of a complex mechanism of immune homeostasis maintaining well balanced differentiation of adequate but non-excessive responses of B cells and T cells.

Interleukin-10 (IL-10) production has been shown to be an important characteristic of the phenotype and regulatory actions of T_{reg} cells (2). Beside suppressing immune activation, IL-10 promotes development of a population of IL-10-expressing CD4$^+$ T_{reg} cells *in vitro* (9). Moreover, systemic over-expression of IL-10 induces CD4$^+$CD25$^+$ cells *in vivo* (10). However, the role of IL-10 for B cell function seems more diverse. IL-10 has long been known as a growth and differentiation factor for B cells *in vitro* as it stimulates B cell proliferation and antibody class switching (11, 12). In addition, some B cells have been described to have regulatory properties (B_{reg} cells), and IL-10 expression is characteristic of and required for the regulatory effects of these B_{reg} cells (3-8, 13). However, studies on effects of IL-10 on activation and differentiation of B cells in the local context in which IL-10 is provided by CD4$^+$ T cells *in vivo* remains rather undefined.

It is evident that B cells and T cells direct each other's expansion and differentiation during B-T cell interaction (14). This interaction is needed to generate effective immune responses to infections as well as to induce or sustain several inflammatory autoimmune responses (14-17). During such interaction T_h cells can direct differentiation of effector B (B_e) cells (18), which can in turn, influence differentiation of T_h1 or T_h2 cells. However, lymphocyte interactions leading to regulatory phenotypes are still rather unexplored. Recently, B-T cell interaction for regulatory responses was demonstrated in a study by Mann *et al*. (19) showing that B cells regulate CD4$^+$CD25$^+$ T_{reg} cells and IL-10 expression. Regarding these observations it is tempting to argue that IL-10-producing (IL-10$^+$) T_{reg} cells may interact with B cells to induce polarization towards IL-10 production, which is characteristic for B_{reg} cells. In this study we asked whether IL-10$^+$ T_{reg} cells direct differentiation of the cytokine responses of B cells and T cells in an inflammatory setting at the cellular level. To assess differentiation in an inflammatory setting we studied lymphocytes in proteoglycan-induced arthritis (PGIA). This is a progressive model in mice for study on rheumatoid arthritis (20) and is mediated by both B and T_h1 cells responding to cartilage proteoglycan (16, 20, 21).

Recently, we have shown that cartilage antigen-specific IL-10 producing CD4$^+$ T (IL-10$^+$ T_{reg}) cells ameliorate arthritis by propagating the endogenous regulatory IL-10 response, while reducing the antigen-specific IgG_{2a} response by B cells (chapter 4). Therefore, we hypothesized that these IL-10$^+$ T_{reg} cells promote IL-10 expression in B cells and naïve cartilage antigen-specific CD4$^+$ T cells. To this end we studied the influence of cartilage proteoglycan (PG)-specific IL-10$^+$ T_{reg} cells on B and CD4$^+$ T cell-differentiation in PGIA. At different time points after T_{reg} transfer in PGIA, endogenous B cells, endogenous CD4$^+$ T cells and the transferred IL-10$^+$ T_{reg} cells were isolated and analyzed for differentiation of their cytokine responses. In addition, CFSE-labeled naive arthritogenic PG-specific CD4$^+$ T cells were co-transferred with the IL-10$^+$ T_{reg} cells, and analyzed in order to determine the effect of IL-10$^+$ T_{reg} cells on proliferation and differentiation of PG-specific T cells *in vivo*.

MATERIALS AND METHODS

Mice and antigens

BALB/c retired breeder mice (obtained from Charles River Laboratories, Maastricht, The Netherlands) were kept at the animal facility of the University of Utrecht; "Gemeenschappelijk Dierenlaboratorium" (GDL) under standard conditions in filtertopped cages. TCR-5/4E8-Tg BALB/c mice (22, 23) were bred and kept at the GDL under specific pathogen free conditions. Human PG (hPG) was prepared as described earlier (22). All animal experiments were approved by the Animal Experimental Committee of the Veterinary Faculty of the University of Utrecht.

Construction of plasmids and production of retrovirus

Murine IL-10 cDNA was obtained using specific primers (5'-AGA TCT TTG CAG AAA AGA GAG CTC CA-3' and 5'-GTC GAC TGG AGT CCA GCA GAC TCA AT-3') and cloned into the MSCV2.2 plasmid encoding the selection marker GFP.

Ecotropic replication-deficient retrovirus was produced with a Phoenix-Eco packager cell line cultured in supplemented DMEM (Gibco Life Technologies, Breda, The Netherlands) containing 10% heat inactivated FCS (Bodinco B.V., Alkmaar, The Netherlands) at 37°C. For transfection, 500 µl of 0.25 M $CaCl_2$ containing 20 µg MSCV-plasmid and 5µg PCL-Eco plasmid was mixed thoroughly with an equal volume HBS buffer pH 7.02 and added to the cells. At 20 hours after transfection the supernatant was replaced with fresh medium. Within 24 hours thereafter, supernatant containing the retrovirus was harvested, filtered with a 45 µM filter, snap-frozen and stored frozen until use. Again fresh medium was added, virus was harvested the next day and pooled with the previous supernatant for infection.

Generation of IL-10$^+$ T_{reg} cells

Single-cell suspensions were prepared from pooled spleen and lymph nodes of TCR-5/4E8-Tg BALB/c mice using cell strainers. $CD4^+$ T cells from single cell suspensions were isolated with anti-CD4 (L3T4) magnetic microbeads (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and stimulated with magnetic M-450 Tosylactivated Dynabeads (Dynal Biotech ASA, Oslo, Norway) coated with anti-CD3 (145-2C11) and anti-CD28 (PV-1) mAbs in supplemented DMEM. After 48 hours cells were transduced with retrovirus-containing supernatant supplemented with 8µg/ml hexadimethrine bromide (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Plates were centrifuged at 730xG at 20°C for two hours for retroviral transduction. Subsequently, supernatant was replaced with fresh medium and cells were cultured for another 48 hours. Cells were removed from the stimulating beads and transduced cells (normally 60-80% prior to sorting) were sorted by GFP expression with a FACSVantage SE (Becton Dickinson). Acceptor mice received $2x10^6$ sorted IL-10/GFP-transduced $CD4^+$ cells or, as a control, GFP-transduced $CD4^+$ cells, injected *iv.* in PBS on day 20.

Preparation and labeling of CD4$^+$ responder T cells

$CD4^+$ T cells from single-cell fractions of pooled spleens and lymph nodes of TCR-5/4E8-Tg BALB/c mice were enriched by negative selection with Dynabeads (Dynal) using anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-MHC class II (M5/114) and anti-CD8 (YTS169) mAbs as hybridoma supernatants and were subsequently labeled with CFSE (Molecular Probes,

Leiden, The Netherlands) as described elsewhere (24). Per mouse 8x10^6 CFSE$^+$ cells were transferred in PBS *i.v.* on day 20.

Induction of arthritis

Arthritis was induced in retired breeder BALB/c mice by *i.p*. injections with 400µg of purified hPG emulsified in 2 mg of the synthetic adjuvant dimethyl-dioctadecyl-ammoniumbromide (DDA) (Sigma) in PBS (total volume of 200 µl) on day 0 and day 21 as described elsewhere (25, 26).

Flow cytometry and sorting of CD19$^+$ and CD4$^+$ cells from treated mice

Unstimulated spleen- or lymph node single cell populations from acceptor mice were isolated at day two, four or six after the arthritogenic second PG-immunization and stained with anti-CD19-PE, anti-CD4-APC or anti-CD8-APC (BD Pharmingen) in PBS/ 2% FCS/ 5% NMS and either analyzed with a FACS Calibur (Becton Dickinson) or sorted with a FACS Vantage (Becton Dickinson). For cell sorting GFP$^+$ and CFSE$^+$ cells were distinguished using a 550nm long pass filter.

For intracellular cytokine staining single-cell suspensions of spleen cells were cultured for six hours at 2x10^6 cells/ml supplemented IMDM (Gibco) with 50 ng/ml PMA (Sigma) plus 500 ng/ml ionomycin (Sigma) in the presence of 10µg/ml Brefeldin A (Sigma) during the final four hours. Cells were stained with anti-CD19-PE and anti-CD4-PerCP mAbs (BD Pharmingen) and non-specific binding was blocked by 5% NMS. After washing, cells were fixed, permeabilized and stained anti-IL-10-APC, anti-IL-2-APC or anti-IFN-γ-biotin mAb (BD Pharmingen) using Permeabilization/fixation buffers (BD Pharmingen) according to the manufacturer's instructions. Anti-IFN-γ-biotin stained cells were stained with streptavidin-APC and washed in permeabilization buffer (BD Pharmingen). Cells were analyzed with a FACS Calibur and results were analyzed with FlowJo software.

cDNA synthesis and quantitative real-time (RT)-PCR for cytokine- and GFP expression

Total mRNA was extracted with the RNeasy kit (Qiagen Benelux B.V., Venlo, The Netherlands) and treated with DNase (Qiagen) using the manufacturers protocol. Subsequently, RNA was transcribed into cDNA using the iScript^{TM}cDNA Synthesis Kit (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Quantitative real-time PCR was performed in a total volume of 25 µl using iQ^{TM} SYBR Green$^{®}$ Supermix (Bio-Rad). Primers specific for IL-10 (5'-GGT TGC CAA GCC TTA TCG GA-3' and 5'-ACC TGC TCC ACT GCC TTG CT-3'), HPRT (5'-CTG GTG AAA AGG ACC TCT CG-3' and 5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3'), Foxp3 (5'-CCC AGG AAA GAC AGC AAC CTT-3' and 5'-TTC TCA CAA CCA GGC CAC TTG-3'), TGF-β (5'-GCC CTG TAT TCC GTC TCC TCC TTG-3' and 5'-CGT AAC CGG CTG CTG ACC-3'), IFN-γ (5'-TCA AGT GGC ATA GAT GTG GAA GAA-3' and 5'-TGG CTC TGC AGG ATT TTC ATG-3'), IL-2 (5'-TGA GCA GGA TGG AGA ATT ACA GG-3' and 5'-GTC CAA GTT CAT CTT CTA GGC AC-3') and GFP (5'-AGA ACG GCA TCA AGG TGA AC-3' and 5'-TGC TCA GGT AGT GGT TGT CG-3') were used at a concentration of 0.25 µM. PCR (3 min at 95°C, 40 cycles of 10 s at 95°C and 45 s at 59.5°C) and real-time detection was done with a Bio-Rad MyiQ iCycler (Bio-Rad). Expression of IL-10 was normalized to the detected Ct values of HPRT for each sample.

Statistics

Data are expressed as mean ± standard error of the mean (SEM). Statistical evaluation was done with a *t*-test (two-tailed). *P* values below 0.05 were considered significant.

RESULTS

IL-10$^+$ T_{reg} cells do not suppress proliferation of antigen-specific T cells in arthritis

A well known characteristic of T_{reg} cells is their capacity to suppress proliferation of T cells responding in their vicinity through IL-10. Suppression of the number of autoreactive helper T cells may be a mechanism to ameliorate autoimmune inflammation. Therefore, the capacity of PG-specific IL-10$^+$ T_{reg} cells to suppress the proliferative response of autoreactive (PG-specific) CD4$^+$ T cells in arthritis was tested *in vivo* by co-transferring CFSE-labeled (CFSE$^+$) PG-specific CD4$^+$ T cells together with PG-specific IL-10$^+$ T_{reg} cells in PG-induced arthritis on the day before the arthritogenic second PG-immunization (group 2, Fig. 1). IL-10$^+$ T_{reg} cells were generated *ex vivo* through transduction with an active IL-10 gene. As a control, PG-specific CD4$^+$ T cells transduced with only a GFP-gene (no IL-10) were co-transferred with the PG-specific CFSE$^+$CD4$^+$ T cells (group 1, Fig. 1). On day two, four and six after the second PG-immunization, CFSE$^+$CD4$^+$ cells in spleen and joint-draining lymph nodes were analyzed for proliferation by dilution of CFSE. Dividing CFSE$^+$ PG-specific CD4$^+$ T cells were clearly visible within four days after PG-immunization in both spleen as well as the joint-draining lymph nodes (Fig. 2A). The number of dividing cells in the PG-specific CFSE$^+$CD4$^+$ T cell population in the IL-10$^+$ T_{reg} cell-recipients did, however, not significantly differ from the number of dividing PG-specific CFSE$^+$CD4$^+$ T cells in the control group at any time point analyzed within the first six days after PG-immunization.

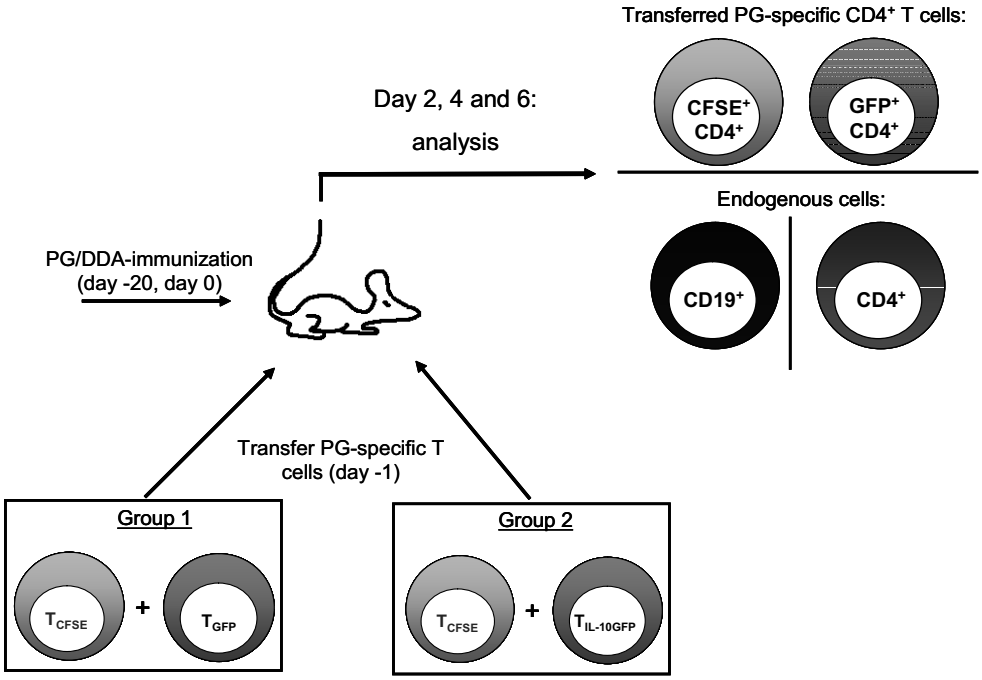

FIGURE 1. Co-transfer of PG-specific IL-10$^+$ T_{reg} cells or controls with CFSE-labeled PG-specific T cells in PG-induced arthritis. To induce arthritis BALB/c mice were immunized with proteoglycan (PG) twice with an interval of 20 days. On the day before the arthritogenic second PG-immunization either 2x10^6 IL-10$^+$ T_{reg} cells (IL-10GFP-transduced; group 2) or 2x10^6 PG-specific control (GFP-only-transduced; group 1) CD4$^+$ T cells were transferred together with 7.5x10^6 CFSE-labeled PG-specific CD4$^+$ T cells. B cells (CD19$^+$), CFSE-labeled PG-specific CD4$^+$ T cells (CD4$^+$CFSE$^+$), PG-specific IL-10$^+$ T_{reg} cells (CD4$^+$IL-10GFP$^+$), PG-specific control T cells (CD4$^+$GFP$^+$) or endogenous CD4$^+$ T cells (CD4$^+$(IL-10)GFP$^-$CFSE$^-$) cells were analyzed by flow cytometry or sorted by FACS for analysis.

To analyze the survival of the IL-10$^+$ T_{reg} cell population *in vivo*, the number of IL-10$^+$ T_{reg} cells (group 2) or control CD4$^+$ T cells (group 1) was determined by flow cytometry for the number of GFP expressing CD4$^+$ cells. The number of PG-specific IL-10$^+$ T_{reg} cells did not differ from the number of control T cells at any time point analyzed in both spleen and joint-draining lymph nodes (Fig. 2B).

Together, these data suggest that PG-specific IL-10$^+$ T_{reg} cells do not regulate autoimmune inflammation by blocking early proliferation of autoantigen-specific T cells and that IL-10 expressed by autoantigen-specific IL-10$^+$ T_{reg} cells does not inhibit proliferation of these T_{reg} cells *in vivo*.

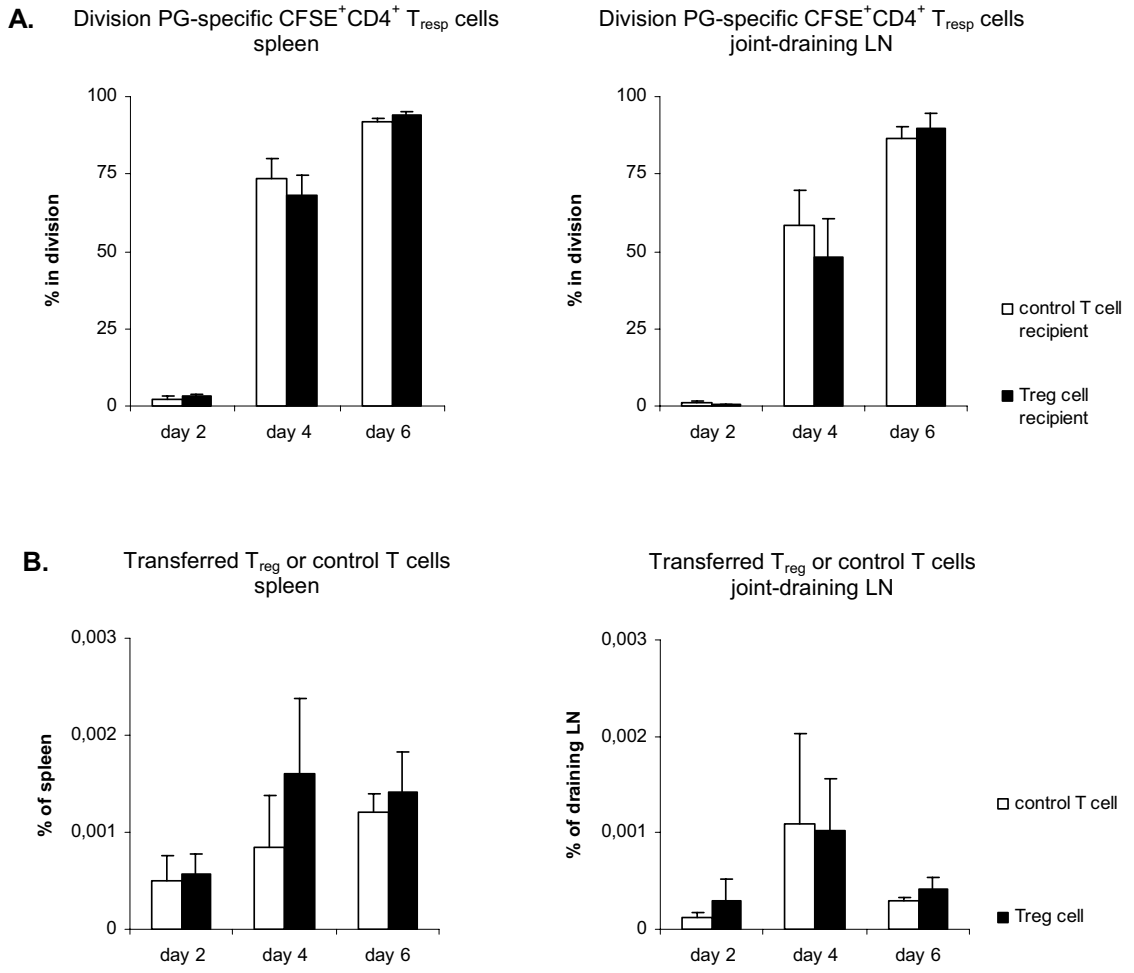

FIGURE 2. PG-specific IL-10$^+$ T_{reg} cells do not suppress division of PG-specific CD4+ T cells. Flow cytometry of numbers (%) of CD4$^+$ T cells that were transferred as described in figure 1 at two, four or six days after the arthritogenic PG-immunization. Upper graphs show analysis of the number of cells in division within the CFSE$^+$CD4$^+$ T cell population in spleen (A, left panel) and joint-draining LN (A, right panel) at different time points after the second PG-immunization. Lower panels show numbers (%) of IL-10$^+$ T_{reg} cells or control T cells detected by flow cytometry within spleen (B, left panel) or joint-draining LN (B, right panel). Black bars (■) show means of IL-10$^+$ T_{reg}-recipients (n=3), white bars (□) show controls (n=3). Error bars represent SEM.

IL-10$^+$ T_{reg} cells do not down-regulate IL-2 and IFN-γ in CD4$^+$ T cells during the early immune response

Reduced IFN-γ and IL-2 production by T cells has been associated with downregulation of T_h1-mediated autoimmune disorders like arthritis. To test whether IL-10$^+$ T_{reg} cells alter differentiation of PG-specific T cells towards IL-2 and IFN-γ producing cells in arthritis, we transferred PG-specific IL-10$^+$ T_{reg} cells or control T cells together with PG-specific CFSE$^+$CD4$^+$ T cells in PG-induced arthritis as described in the previous section (Fig. 1). The transferred PG-specific CFSE$^+$CD4$^+$ T cells in spleen were analyzed by flow cytometry for their IL-2 and IFN-γ production on day two, four and six after the arthritogenic PG-immunization (Fig. 3A). In addition, differentiation of intracellular IFN-γ and IL-2 production within the recipient's endogenous CD4$^+$ T cell population was analyzed by flow cytometry (Fig. 3B).

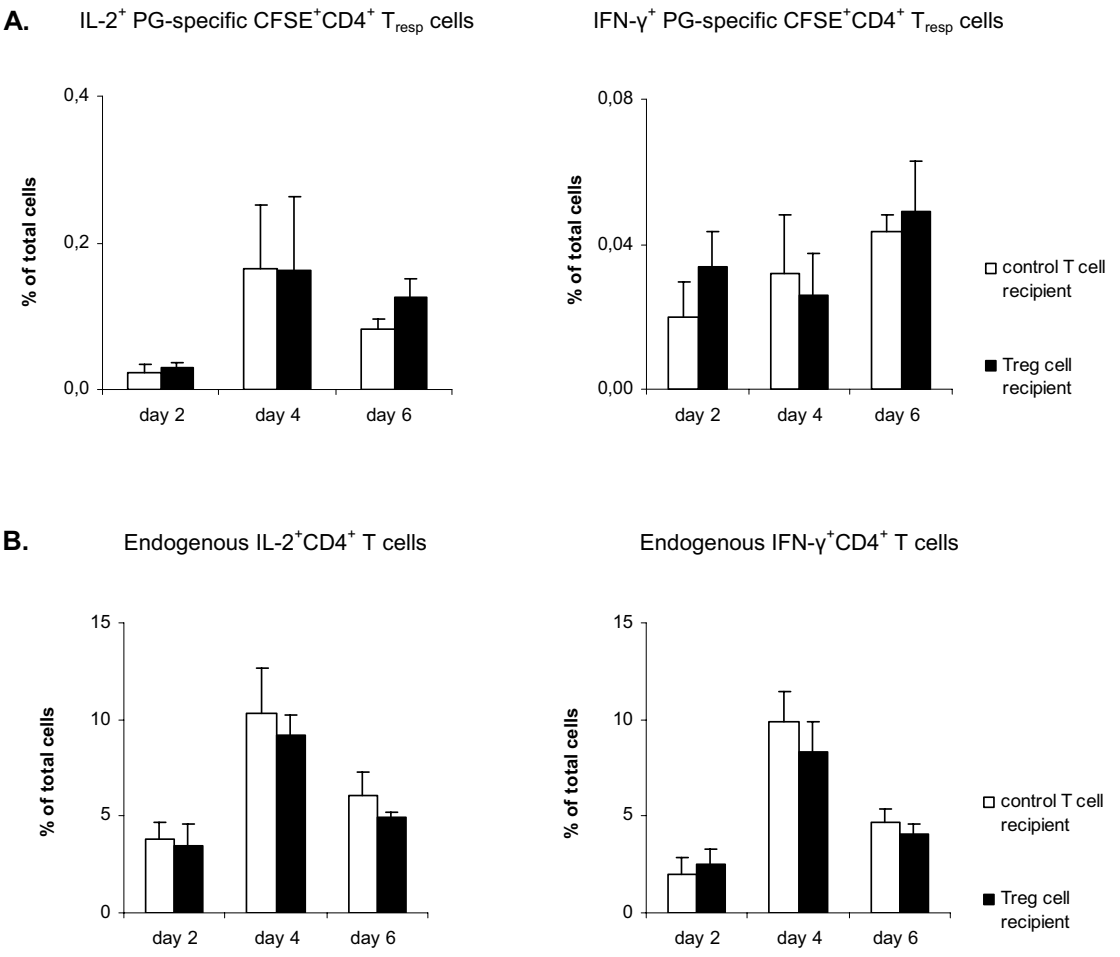

FIGURE 3. PG-specific IL-10$^+$ T_{reg} cells do not regulate differentiation of CD4$^+$ T cells to IL-2 or IFN-γ producers in the early arthritogenic response. Mice were treated as described in figure 1. On the different time points after the arthritogenic PG-immunization indicated PG-specific CFSE$^+$ T cells were analyzed by flow cytometry for expression of IL-2 (A, left panel) and IFN-γ expression (A, right panel). In addition, endogenous CD4$^+$ T cells from spleen were analyzed for intracellular IL-2 (B, left panel) and IFN-γ (B, right panel) by flow cytometry. Data are represented as means of the number (%) of cytokine$^+$CD4$^+$ cells in spleens of IL-10$^+$ T_{reg}-recipients (■) (n=3) or controls (□) (n=3). Error bars represent SEM.

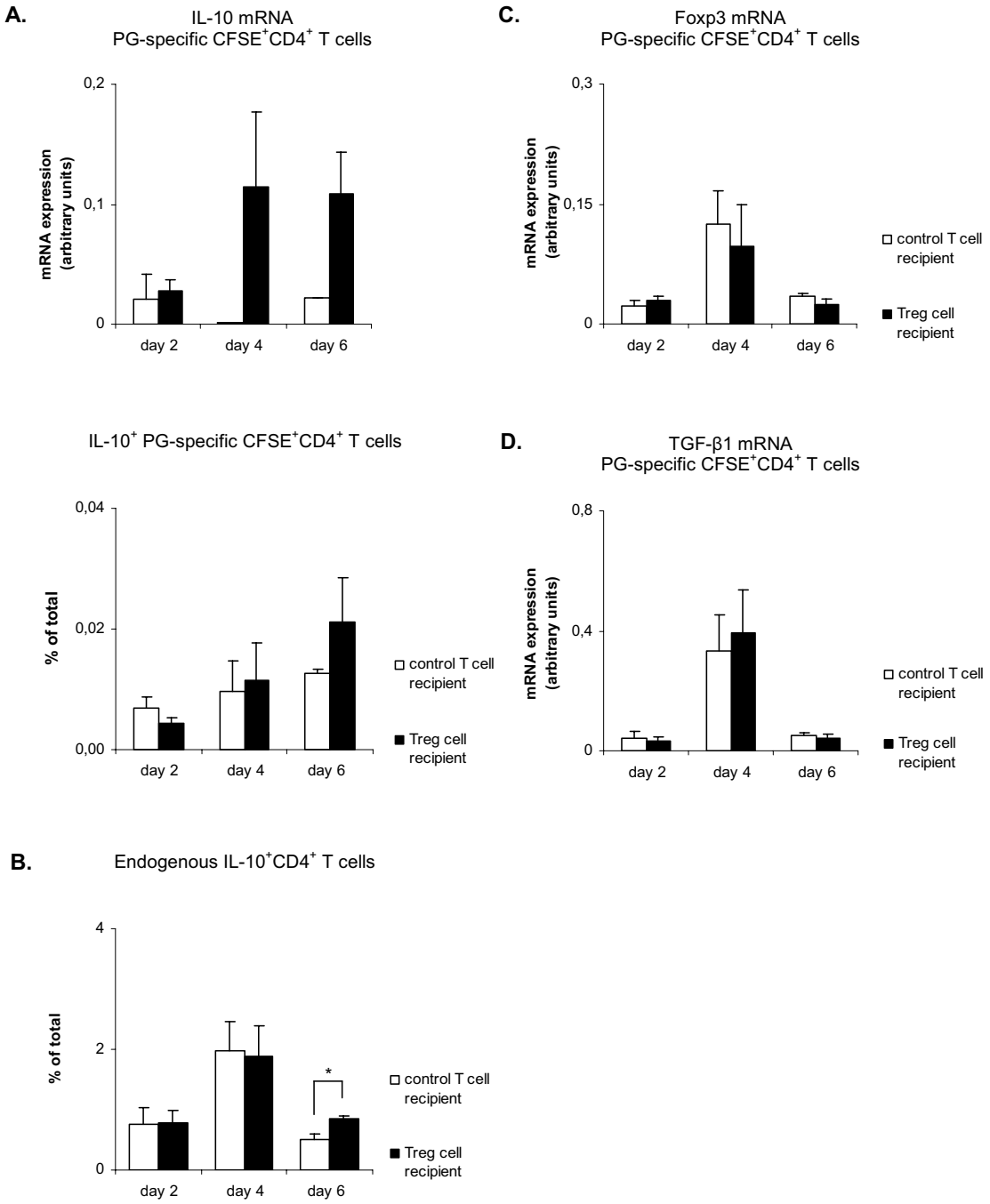

FIGURE 4. PG-specific IL-10$^+$ T_{reg} cells do not induce expression of TGF-β and Foxp3, but propagate IL-10 expression in CD4$^+$ T cells. On the different days after the arthritogenic PG-immunization, PG-specific CFSE$^+$ T cells transferred as described in figure 1 were sorted from spleen with FACS and analyzed for expression of IL-10 (A, upper panel), Foxp3 (C) and TGF-β1 (D) by quantitative RT-PCR. IL-10 expression in spleen T cells was also quantified by flow cytometry of the number of IL-10$^+$CD4$^+$CFSE$^+$ (transferred PG-specific) cells (A, lower panel) and the number of endogenous (CFSE$^-$) IL-10$^+$CD4$^+$ cells (B). Black bars (■) show means of values in IL-10$^+$ T_{reg}-recipients (n=3), white bars (□) show means of controls (n=3). Error bars represent SEM. *p<0.05.

The data show that within the first six days after the PG-immunization, IL-10$^+$ T_{reg} cells did not substantially affect the number of IL-2$^+$ or IFN-γ$^+$ PG-specific CFSE$^+$ (Fig. 3A) or endogenous CD4$^+$ T cells compared with the control group (Fig. 3B). This suggests that IL-10$^+$ T_{reg} cells do not regulate primarily by suppressing arthritogenic IFN-γ and IL-2 production in (PG-specific) T cells.

IL-10$^+$ T_{reg} cells promote the production of IL-10 but not TGF-β or Foxp3 in T cells

IL-10 has been described to play an important role in expansion of several regulatory cell types. For example, IL-10 can induce differentiation and expansion of IL-10$^+$ regulatory T_R1 cells *in vitro* (9) and IL-10 is involved in the induction of TGF-β$^+$ regulatory T_h3 cells (27). In addition, IL-10 participates together with IL-2 and TGF-β in the induction of Foxp3$^+$ CD25$^+$CD4$^+$ T_{reg} cells *in vitro* (28). Therefore, we asked if IL-10$^+$ T_{reg} cells could induce expression of IL-10, TFG-β and Foxp3 in the early response of CD4$^+$ T cells in an arthritogenic environment *in vivo*. To address this question, we co-transferred PG-specific IL-10$^+$ T_{reg} cells or controls together with PG-specific CFSE$^+$CD4$^+$ T cells in PG-induced arthritis as described in figure 1. For *in situ* expression of IL-10, TGF-β1 and Foxp3 in PG-specific CFSE$^+$ T cells, these T cells were sorted from spleens of IL-10$^+$ T_{reg}-recipients or controls and expression of IL-10, Foxp3 and TGF-β1 was analyzed by quantitative RT-PCR.

The PG-specific CFSE$^+$ T cell population showed a substantial increase in IL-10 mRNA expression *in situ* in those animals that had received IL-10$^+$ T_{reg} cells as compared with the control group at six days after arthritogenic PG-immunization (Fig. 4A, upper panel), indicating propagation of IL-10 in the PG-specific T cells by the IL-10$^+$ T_{reg} cells.

In addition to the quantitative RT-PCR data, propagation of IL-10 expression in PG-specific CD4$^+$ T cells and in endogenous CD4$^+$ T cells was analyzed by flow cytometry of intracellular IL-10 production within transferred PG-specific CFSE$^+$CD4$^+$ T cells (Fig. 4A, lower panel) and endogenous GFP$^-$CFSE$^-$CD4$^+$ T cells (Fig. 4B). A substantial increase in the number of PG-specific (CFSE$^+$) IL-10$^+$ T cells was observed (Fig. 4A) in hosts that had received IL-10$^+$ T_{reg} cells. In addition, a significant elevation in the number of endogenous IL-10$^+$ T cells was found in this group compared to the control group at six days after the arthritogenic PG-immunization (Fig. 4B).

IL-10$^+$ T_{reg} cells did not affect expression of Foxp3 (Fig. 4C) or TGF-β1 (Fig. 4D) at the transcriptional level in sorted CFSE$^+$ PG-specific CD4$^+$ T cells. Also induction of TGF-β1 or Foxp3 was not found in the IL-10$^+$ T_{reg} cells, compared with control T cells at any of these time points (data no shown).

Altogether, these data indicate that autoantigen-specific IL-10$^+$ T_{reg} cells regulate on the autoantigen specific T cell response by propagating IL-10$^+$ CD4$^+$ T cells and not by inducing TGF-β$^+$ and/or Foxp3$^+$ T cells.

PG-specific IL-10$^+$ T_{reg} cells sustain B cell activation in PG-induced arthritis

IL-2 and IFN-γ are two major cytokines characterizing effector B cell differentiation in the presence of T_h1 or T_h2 cells (18). To find out if IL-10$^+$ T_{reg} cells suppress B cell differentiation in T_h1-mediated PGIA, the IL-2 and IFN-γ responses were analyzed in the CD19$^+$ spleen population after transfer of PG-specific IL-10$^+$ T_{reg} cells or control T cells as described in figure 1. Intracellular staining for IL-2$^+$CD19$^+$ cells and IFN-γ$^+$CD19$^+$ cells showed that IL-10$^+$ T_{reg} cells did not alter the number of IFN-γ$^+$ B cells compared with the control group (Fig. 5A, right panel). However, a significant rise in the number of IL-2$^+$CD19$^+$ cells was found cells in

IL-10$^+$ T_{reg} cell-recipients compared with controls at six days after arthritogenic PG-immunization (Fig. 5A, left panel), left panel. At the same time of six days, we found increased numbers of CD19$^+$ cells in spleens of mice that had received IL-10$^+$ T_{reg} cells (Fig. 5B) compared with controls.

These data indicate that IL-10$^+$ T_{reg} cells do not regulate inflammation in arthritis by suppressing polarization of the B cells to IL-2 or IFN-γ producers, but rather sustain the presence of higher numbers of B cells producing IL-2.

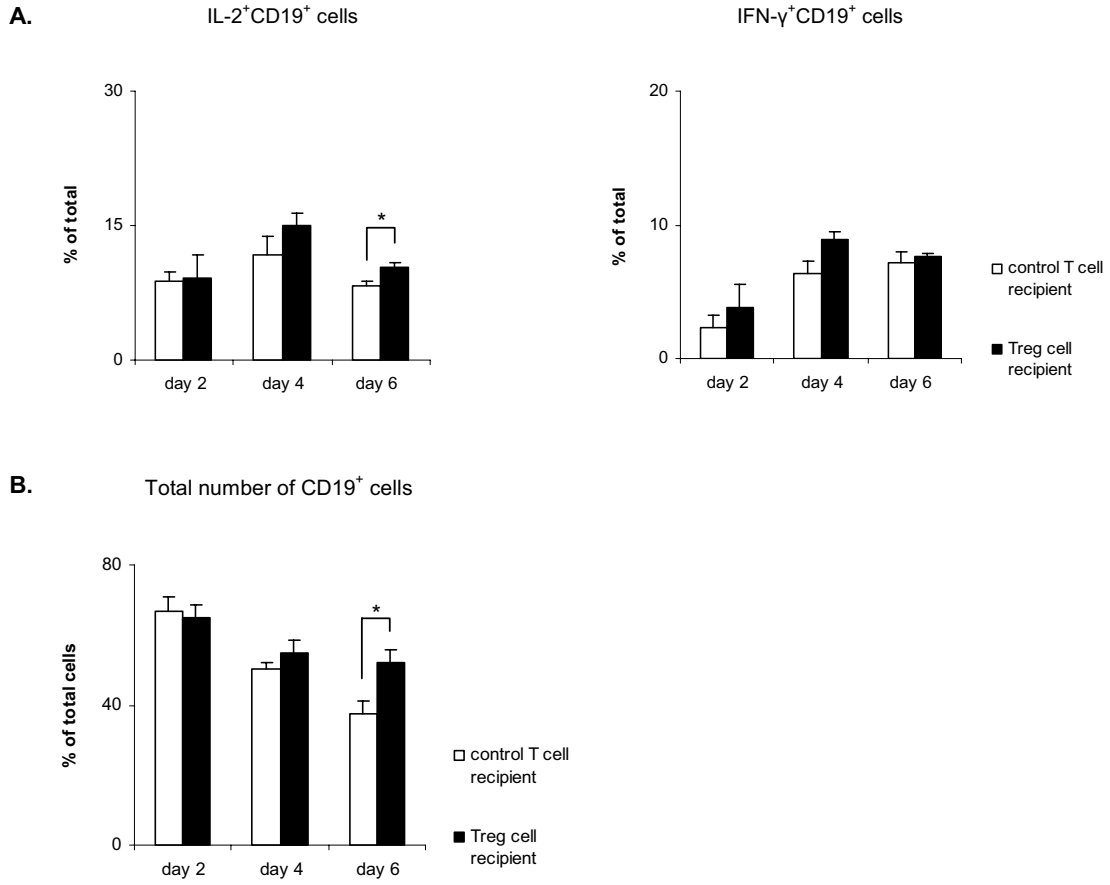

FIGURE 5. PG-specific IL-10$^+$ T_{reg} cells do not inhibit differentiation of the IL-2 and IFN-γ response in B cells. After transfer of T cells as described in figure 1, numbers (%) of IL-2$^+$CD19$^+$ cells (A, left panel) and IFN-γ$^+$CD19$^+$ cells in spleen were determined with flow cytometry. Also numbers (%) of CD19$^+$ cells in spleens were analyzed with flow cytometry. Black bars (■) show means of counted numbers in IL-10$^+$ T_{reg}-recipients (n=3), white bars (□) show controls (n=3). Error bars represent SEM. *p<0.05.

IL-10$^+$ T cells promote growth of IL-10$^+$ B cells

We hypothesized that IL-10$^+$ T_{reg} cells may propagate the IL-10 response in B cells in arthritis. Therefore, IL-10 expression by B cells was analyzed after transfer of PG-specific IL-10$^+$ T_{reg} cells or PG-specific control T cells as described in figure 1. At different time points after transfer IL-10 expression by B cells *in situ* was analyzed by direct quantitative RT-PCR on CD19$^+$ cells sorted from spleen. In addition, differentiation of the B cell population was analyzed by flow cytometry for intracellular IL-10 in CD19$^+$ cells (Fig. 6). Both quantitative RT-PCR (left panel) and flow cytometry (right panel) showed a substantial rise in the expression of IL-10 in B cells four days after transfer of IL-10$^+$ T_{reg} cells.

In summary, these data suggest that IL-10$^+$ T_{reg} cells do not regulate an early inflammatory response by suppressing the IL-2 or IFN-γ lymphocyte response but rather by propagating the number of IL-10$^+$ B and T cells in an otherwise arthritogenic immune response.

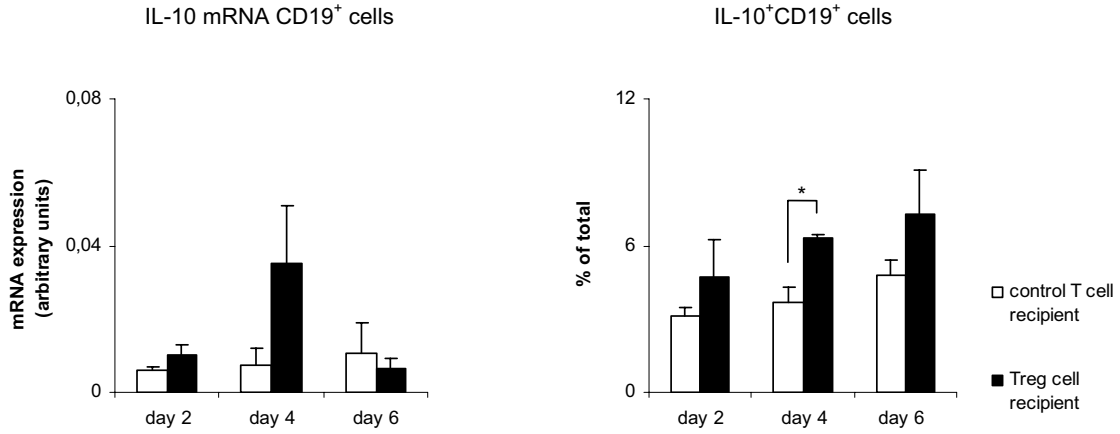

FIGURE 6. PG-specific IL-10$^+$ T_{reg} cells promote differentiation of the IL-10 response in B cells. After transfer of T cells as described in figure 1 differentiation of the IL-10 response in splenic B cells was analyzed by quantitative RT-PCR on sorted CD19$^+$ cells (left panel) and by flow cytometry for the number of IL-10+CD19$^+$ cells (right panel). Black bars (■) show means found in IL-10$^+$ T_{reg}-recipients (n=3), white bars (□) show mean values of controls (n=3). Error bars represent SEM. *p<0.05.

DISCUSSION

The phenotype of lymphocytes is a critical determinant for either maintenance of immune tolerance or induction of inflammation. The mode of stimulation (29-31) and the cytokine milieu (32) of lymphocytes are major factors that contribute to the final outcome of lymphocyte differentiation. Since B cells can specifically interact with T_h cells recognizing antigens presented by B cells, both cell types can cooperatively determine the differentiation of antigen-specific responses (18, 33).

IL-10 has often been demonstrated to be crucial for prevention or down regulation of IFN-γ and IL-2 expressed in T_h1-responses that dominate in many inflammatory autoimmune disorders. IL-10 is produced and required for active regulation by different subsets of T_{reg} cells (2), B_{reg} cells (3) but also, as part of an autocrine negative feedback mechanism, by T_h1 cells (34). Recent studies have focused on identifying mechanisms by which $CD4^+$ T_{reg} cells regulate T_h1 or T_h2 effector responses through IL-10 and other regulatory molecules (35). However, little is known on how $CD4^+$ T_{reg} cells expressing IL-10 regulate B cell cytokine responses *in vivo*. Especially, studies on the induction of IL-10 in B cells by T cells are scarce. In this study we therefore addressed if regulatory T cells that produce IL-10 could modulate the B cell cytokine response during the early inflammatory autoimmune response in arthritis. Furthermore, also the effect of IL-10^+ T_{reg} cells on the IL-10 response of T cells *in vivo* is rather undefined. For this we tested the modulating effects of IL-10-transduced cartilage PG-specific $CD4^+$ T cells (IL-10^+ T_{reg} cells) in PGIA. Such antigen-specific IL-10^+ T_{reg} cells have previously been shown to suppress inflammation in this B and T cell mediated arthritis model (chapter 3&4), and also in OVA-induced arthritis and allergy (36, 37) and in non-obese diabetic mice (38). In addition to studying effects on the B cell response, we questioned in which direction IL-10^+ T_{reg} cells would differentiate the $CD4^+$ T cell response *in vivo*.

We analyzed cytokine responses of B and T cells at different time points during the first week after arthritogenic PG-immunization. B-T interactions and resulting lymphocyte responses *in vivo* are evident within four days after immunization (15, 39). Moreover, differentiation of the cytokine response of T and B cells during their interaction is induced within three to four days (18, 39). Overt proliferation of PG-specific $CD4^+$ T cells was found within four days after PG-immunization. However, PG-specific IL-10^+ T_{reg} cells did not significantly suppress the proliferation of naive PG-specific T cells compared with the control groups within the first six days after transfer. This indicates that IL-10^+ T_{reg} cells do not suppress the autoimmune response that leads to inflammation by just reducing the number of responding autoreactive T cells during the early phase of the immune response. Moreover, the absence of effects on antigen-specific T cell proliferation in the early phase of the arthritogenic response has been described earlier in a study showing that antigen-specific IL-10^+ T_{reg} cells suppressed antigen-induced arthritis without affecting the proliferation of antigen-specific T cells (36). However, IL-10^+ T cells have been shown to suppress T cell proliferation by secretion of IL-10 *in vitro*. This contrasting finding may be explained by the differences between the *in vitro* and *in vivo* situations like localization of cell-cell interactions, stimulatory properties of different types of APCs and pro-inflammatory micro-environment, which are more diverse and dynamic in the *in vivo* situation. Furthermore, timing, which contributes to the outcome of immune interactions (40), may differ between studies.

Besides exploring their effects on T cell proliferation, we assessed whether IL-10^+ T_{reg} cells affected the early cytokine response of T cells *in vivo* since IL-10 has been described to

suppress IFN-γ and IL-2 expression in T cells that sustain inflammation (45). IL-2 is a proliferative factor that can stimulate growth of T_h1 cells producing IFN-γ and B cells. Moreover, IL-2 is also important for regulation by Foxp3$^+$ T_{reg} cells and may be involved in modulation of pro-inflammatory T_h17 cell responses (41-44). PG-specific IL-10$^+$ T_{reg} cells did not suppress the number of IL-2 producing cells in either naive PG-specific CD4$^+$ T population or the endogenous CD4$^+$ T cell population, which would be in line with the finding that IL-10$^+$ T_{reg} cells did not significantly suppress early T cell proliferation *in vivo*. Moreover, PG-specific IL-10$^+$ T_{reg} cells did not suppress the numbers of IFN-γ$^+$CD4$^+$ T cells in the endogenous and transferred PG-specific cell populations. Together, these data indicate that IL-10$^+$ T_{reg} cells do not per se regulate autoimmune mediated inflammation by inhibiting activation of an early T_h1 response.

Since IL-10$^+$ T_{reg} cells did not suppress early IL-2 and IFN-γ expression in CD4$^+$ T cells, we reasoned that not suppression of these particular cytokines, but rather stimulation of regulatory cytokines in CD4$^+$ T cells may be part of immune regulation by IL-10$^+$ T_{reg} cells. In concert with IL-2 and TGF-β, IL-10 has been shown to induce of expression of Foxp3$^+$ T cells (28), which is, together with expression of TGF-β, a hallmark of the phenotype of CD4$^+$CD25$^+$ natural T_{reg} cells (2, 46). However, in our study IL-10$^+$ T_{reg} cells did not induce an increase in Foxp3 or TGF-β expression in CD4$^+$ T cells. This indicates that IL-10 expression by T cells does not promote expansion of cells with a natural T_{reg} phenotype (Foxp3$^+$) or T_h3 phenotype (TGF-β$^+$) *in vivo*. Previous studies, however, have shown that IL-10 is involved in the induction of these T_{reg} phenotypes *in vitro* (9, 27). Conflicting results may be explained by the fact that *in vitro*, only a limited number of differentiation factors were present to induce Foxp3$^+$ T_{reg} cells. In the *in vivo* setting in our study, however, IL-10 was expressed by antigen-specific T cells in a more physiologic and richer milieu. Furthermore, our data indicate that T cells can gain anti-inflammatory functions without elevated Foxp3 expression. This is supported by earlier studies (47), showing that although Foxp3 is an important factor for development and function of some T_{reg} cells, it is not a prerequisite for regulation by all T_{reg} cells. In addition, suppression without up-regulation of TGF-β expression may be explained by the capacity of IL-10 to induce expression of TGF-β receptor type II (48), rendering cells more sensitive to TGF-β mediated regulation, while expression of TGF-β, which is widely expressed in different tissues, does not necessarily has to be elevated.

Another mechanism that may amplify regulatory activity in T cells, especially by IL-10, is the propagation of IL-10 expressing T cells. Despite their production of IFN-γ, these IL-10-induced regulatory T cells (T_R1 cells) abundantly express IL-10 and suppress the inflammation of colitis (9). In our study we found that IL-10$^+$ T_{reg} cells sustained the number of IL-10$^+$CD4$^+$ cells in both co-transferred PG-specific CD4$^+$ T cell and endogenous CD4$^+$ T cell populations. Altogether, these data show that in the CD4$^+$ T cell response in the early phase of arthritis uniquely IL-10 expression is modulated by IL-10$^+$ T_{reg} cells, which may be a major part of the mechanism by which IL-10$^+$ T_{reg} cells maintain immune homeostasis to suppress harmful tissue-specific inflammation.

B cells are crucial for the induction and perpetuation of arthritis as APC for T cells and as producers of pro-inflammatory autoantibodies (16). On the other hand, B cells expressing IL-10 have been show to exert immunoregulatory functions crucial for suppression of autoimmune-mediated inflammation (4-6, 8). Previously, we have shown that PG-specific IL-10$^+$ T_{reg} cells suppress the production of PG-specific IgG_{2a} antibodies (chapter 4). Regarding close cellular interactions between antigen-specific B and T_h cells and their capacity to

reciprocally influence T_h and B cell cytokine responses (15-18, 49), we addressed the effect of IL-10^+ T_{reg} cells on B cell differentiation as shown by their cytokine responses.

At six days after the second PG-immunization the number of B cells was elevated in mice that had received PG-specific IL-10^+ T_{reg} cells as compared with animals that had received control T cells. Moreover, the number of IFN-γ^+ B cells was not affected by IL-10^+ T_{reg} cells, but the number of IL-2^+ B cells appeared higher compared to controls. *In vitro* studies have indicated that IL-10 can induce B cell proliferation mediated by IL-2 (11, 12). Therefore, our latter finding suggests that IL-10 from T cells may promote growth of B cells that interact with the T cells through propagation of IL-2 expression in the B cell population during the early response.

IL-10^+ T_{reg} cells stimulated IL-10 production in the B cell population during the early phase of PGIA. This indicates that in parallel with induction of IL-4 in B cells by T_h2 cells that produce IL-4 or the induction of IFN-γ in B cells by T_h1 cells that produce IFN-γ (18), T cells producing IL-10 favor expression of IL-10 in B cells. Since IL-10 production is crucial for regulatory functions of B_{reg} cells (4-6), our results suggest that T_{reg} cells that produce IL-10 may perpetuate their regulatory functions through induction of IL-10^+ B_{reg} cells during their interaction with B cells responding to (auto)antigens.

Thus, we have shown that PG-specific IL-10^+ T_{reg} cells stimulate IL-10 expression not only in the $CD4^+$ T cell population but also in the B cell population during the early phase of PGIA. These cells did so without substantially inhibiting T cell proliferation or differentiation of T cell cytokine responses that are thought to correlate with activation of T_h1 cells. Moreover they did not up-regulate expression of Foxp3 or TGF-β in $CD4^+$ T cells. In a previous study we have shown that the anti-inflammatory action PG-specific IL-10^+ T_{reg} cells in PG-induced arthritis critically depends on the amplification of the endogenous IL-10 response of the host (chapter 4). Therefore, amplification of the IL-10 response in interacting T and B cells, rendering them regulatory cells, may be part of the mechanism that is responsible for antigen-specific regulation by IL-10^+ T_{reg} cells. Further studies should decipher how these induced IL-10^+ T and B cells act on immune responses to help translate such underlying regulatory mechanisms to curative therapies. moreover, propagation of the expression of immunosuppressive IL-10 in T cells as well as B cells may be taken into account in development of therapies that target B (27) or T cells (50) to treat autoimmune-mediated inflammatory disease.

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REFERENCES

1. Shevach, E. M. 2006. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* 25:195-201.
2. Bluestone, J. A. 2005. Regulatory T-cell therapy: is it ready for the clinic? *Nat Rev Immunol* 5:343-349.
3. Mizoguchi, A., and A. K. Bhan. 2006. A case for regulatory B cells. *J Immunol* 176:705-710.
4. Mizoguchi, A., E. Mizoguchi, H. Takedatsu, R. S. Blumberg, and A. K. Bhan. 2002. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* 16:219-230.
5. Fillatreau, S., C. H. Sweenie, M. J. McGeachy, D. Gray, and S. M. Anderton. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 3:944-950.
6. Mauri, C., D. Gray, N. Mushtaq, and M. Londei. 2003. Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* 197:489-501.
7. Sun, C. M., E. Deriaud, C. Leclerc, and R. Lo-Man. 2005. Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. *Immunity* 22:467-477.
8. Lund, F. E., B. A. Garvy, T. D. Randall, and D. P. Harris. 2005. Regulatory roles for cytokine-producing B cells in infection and autoimmune disease. *Curr Dir Autoimmun* 8:25-54.
9. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737-742.
10. Goudy, K. S., B. R. Burkhardt, C. Wasserfall, S. Song, M. L. Campbell-Thompson, T. Brusko, M. A. Powers, M. J. Clare-Salzler, E. S. Sobel, T. M. Ellis, T. R. Flotte, and M. A. Atkinson. 2003. Systemic overexpression of IL-10 induces CD4+CD25+ cell populations in vivo and ameliorates type 1 diabetes in nonobese diabetic mice in a dose-dependent fashion. *J Immunol* 171:2270-2278.
11. Rousset, F., E. Garcia, T. Defrance, C. Peronne, N. Vezzio, D. H. Hsu, R. Kastelein, K. W. Moore, and J. Banchereau. 1992. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci U S A* 89:1890-1893.
12. Fluckiger, A. C., P. Garrone, I. Durand, J. P. Galizzi, and J. Banchereau. 1993. Interleukin 10 (IL-10) upregulates functional high affinity IL-2 receptors on normal and leukemic B lymphocytes. *J Exp Med* 178:1473-1481.
13. Gray, M., K. Miles, D. Salter, D. Gray, and J. Savill. 2007. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci U S A* 104:14080-14085.
14. Crawford, A., M. Macleod, T. Schumacher, L. Corlett, and D. Gray. 2006. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. *J Immunol* 176:3498-3506.
15. Yan, J., B. P. Harvey, R. J. Gee, M. J. Shlomchik, and M. J. Mamula. 2006. B cells drive early T cell autoimmunity in vivo prior to dendritic cell-mediated autoantigen presentation. *J Immunol* 177:4481-4487.
16. O'Neill, S. K., M. J. Shlomchik, T. T. Glant, Y. Cao, P. D. Doodes, and A. Finnegan. 2005. Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis. *J Immunol* 174:3781-3788.
17. Guay, H. M., J. Larkin, 3rd, C. C. Picca, L. Panarey, and A. J. Caton. 2007. Spontaneous autoreactive memory B cell formation driven by a high frequency of autoreactive CD4+ T cells. *J Immunol* 178:4793-4802.
18. Harris, D. P., L. Haynes, P. C. Sayles, D. K. Duso, S. M. Eaton, N. M. Lepak, L. L. Johnson, S. L. Swain, and F. E. Lund. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol* 1:475-482.
19. Mann, M. K., K. Maresz, L. P. Shriver, Y. Tan, and B. N. Dittel. 2007. B cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. *J Immunol* 178:3447-3456.
20. Glant, T. T., A. Finnegan, and K. Mikecz. 2003. Proteoglycan-induced arthritis: immune regulation, cellular mechanisms, and genetics. *Crit Rev Immunol* 23:199-250.
21. Bardos, T., K. Mikecz, A. Finnegan, J. Zhang, and T. T. Glant. 2002. T and B cell recovery in arthritis adoptively transferred to SCID mice: antigen-specific activation is required for restoration of autopathogenic CD4+ Th1 cells in a syngeneic system. *J Immunol* 168:6013-6021.
22. Berlo, S. E., T. Guichelaar, C. B. Ten Brink, P. J. van Kooten, F. Hauet-Broere, K. Ludanyi, W. van Eden, C. P. Broeren, and T. T. Glant. 2006. Increased arthritis susceptibility in cartilage proteoglycan-specific T cell receptor-transgenic mice. *Arthritis Rheum* 54:2423-2433.
23. Berlo, S. E., P. J. van Kooten, C. B. Ten Brink, F. Hauet-Broere, M. A. Oosterwegel, T. T. Glant, W. Van Eden, and C. P. Broeren. 2005. Naive transgenic T cells expressing cartilage proteoglycan-specific TCR induce arthritis upon in vivo activation. *J Autoimmun* 25:172-180.
24. Hauet-Broere, F., W. W. Unger, J. Garssen, M. A. Hoijer, G. Kraal, and J. N. Samsom. 2003. Functional CD25- and CD25+ mucosal regulatory T cells are induced in gut-draining lymphoid tissue within 48 h after oral antigen application. *Eur J Immunol* 33:2801-2810.
25. Hanyecz, A., S. E. Berlo, S. Szanto, C. P. Broeren, K. Mikecz, and T. T. Glant. 2004. Achievement of a synergistic adjuvant effect on arthritis induction by activation of innate immunity and forcing the immune response toward the Th1 phenotype. *Arthritis Rheum* 50:1665-1676.
26. Berlo, S. E., T. Guichelaar, C. B. Ten Brink, P. J. van Kooten, F. Hauet-Broeren, K. Ludanyi, W. van Eden, C. P. Broeren, and T. T. Glant. 2006. Increased arthritis susceptibility in cartilage proteoglycan-specific T cell receptor-transgenic mice. *Arthritis Rheum* 54:2423-2433.
27. Weiner, H. L. 2001. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* 182:207-214.
28. Zheng, S. G., J. H. Wang, J. D. Gray, H. Soucier, and D. A. Horwitz. 2004. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol* 172:5213-5221.
29. Anderson, P. O., B. A. Manzo, A. Sundstedt, S. Minaee, A. Symonds, S. Khalid, M. E. Rodriguez-Cabezas, K. Nicolson, S. Li, D. C. Wraith, and P. Wang. 2006. Persistent antigenic stimulation alters the transcription program in T cells, resulting in antigen-specific tolerance. *Eur J Immunol* 36:1374-1385.
30. van Berkel, M. E., and M. A. Oosterwegel. 2006. CD28 and ICOS: similar or separate costimulators of T cells? *Immunol Lett* 105:115-122.
31. Rogers, P. R., and M. Croft. 1999. Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. *J Immunol* 163:1205-1213.
32. Liew, F. Y. 2002. T(H)1 and T(H)2 cells: a historical perspective. *Nat Rev Immunol* 2:55-60.

33. Edwards, J. C., and G. Cambridge. 2006. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. *Nat Rev Immunol* 6:394-403.
34. O'Garra, A., and P. Vieira. 2007. T(H)1 cells control themselves by producing interleukin-10. *Nat Rev Immunol* 7:425-428.
35. O'Garra, A., and P. Vieira. 2004. Regulatory T cells and mechanisms of immune system control. *Nat Med* 10:801-805.
36. Setoguchi, K., Y. Misaki, Y. Araki, K. Fujio, K. Kawahata, T. Kitamura, and K. Yamamoto. 2000. Antigen-specific T cells transduced with IL-10 ameliorate experimentally induced arthritis without impairing the systemic immune response to the antigen. *J Immunol* 165:5980-5986.
37. Oh, J. W., C. M. Seroogy, E. H. Meyer, O. Akbari, G. Berry, C. G. Fathman, R. H. Dekruyff, and D. T. Umetsu. 2002. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. *J Allergy Clin Immunol* 110:460-468.
38. Moritani, M., K. Yoshimoto, S. Ii, M. Kondo, H. Iwahana, T. Yamaoka, T. Sano, N. Nakano, H. Kikutani, and M. Itakura. 1996. Prevention of adoptively transferred diabetes in nonobese diabetic mice with IL-10-transduced islet-specific Th1 lymphocytes. A gene therapy model for autoimmune diabetes. *J Clin Invest* 98:1851-1859.
39. Toellner, K. M., S. A. Luther, D. M. Sze, R. K. Choy, D. R. Taylor, I. C. MacLennan, and H. Acha-Orbea. 1998. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. *J Exp Med* 187:1193-1204.
40. Sojka, D. K., A. Hughson, T. L. Sukiennicki, and D. J. Fowell. 2005. Early kinetic window of target T cell susceptibility to CD25+ regulatory T cell activity. *J Immunol* 175:7274-7280.
41. Laurence, A., and J. O'Shea J. 2007. T(H)-17 differentiation: of mice and men. *Nat Immunol* 8:903-905.
42. Stockinger, B. 2007. Good for Goose, but not for Gander: IL-2 interferes with Th17 differentiation. *Immunity* 26:278-279.
43. Fehervari, Z., T. Yamaguchi, and S. Sakaguchi. 2006. The dichotomous role of IL-2: tolerance versus immunity. *Trends Immunol* 27:109-111.
44. Waldmann, T. A. 2006. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* 6:595-601.
45. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.
46. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.
47. Vieira, P. L., J. R. Christensen, S. Minaee, E. J. O'Neill, F. J. Barrat, A. Boonstra, T. Barthlott, B. Stockinger, D. C. Wraith, and A. O'Garra. 2004. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J Immunol* 172:5986-5993.
48. Cottrez, F., and H. Groux. 2001. Regulation of TGF-beta response during T cell activation is modulated by IL-10. *J Immunol* 167:773-778.
49. Roth, R., T. Nakamura, and M. J. Mamula. 1996. B7 costimulation and autoantigen specificity enable B cells to activate autoreactive T cells. *J Immunol* 157:2924-2931.
50. Chatenoud, L., and J. A. Bluestone. 2007. CD3-specific antibodies: a portal to the treatment of autoimmunity. *Nat Rev Immunol* 7:622-632.

CHAPTER 6

Summarizing Discussion

Under homeostatic conditions a fine-tuned balance between pro-inflammatory and anti-inflammatory immune responses exists to defend the host against unwanted invaders, while avoiding damage to the host that would occur by immune responses becoming too aggressive. Disturbance of this physiological immune homeostasis may provoke the generation of aggressive immune responses directed to tissue antigens that lead to autoimmune disorders (1, 2), for example responses to joint-antigens in rheumatoid arthritis (RA). Therapies used for treatment of inflammation in autoimmune disorders aim to suppress aggressive immune responses by administration of (biological) drugs that neutralize or counteract pro-inflammatory activity of cells. Although they can relieve symptoms in patients, systemically applied drugs increase risk for serious infections (3). Therefore, current research explores alternative approaches for therapy that aim to restrict immune intervention to restoring tissue antigen-specific immune homeostasis.

Autoreactive $CD4^+$ T cells perform important functions in both pathogenesis and regulation of tissue inflammation in autoimmune disorders, depending on their phenotype (4). Autoantigen-specific $CD4^+$ T cells with an (*ex vivo* induced) benign phenotype have therefore been proposed to intervene in the pro-inflammatory autoantigen-specific immune response in favor of immunoregulatory responses to restore immune homeostasis (5-7). The studies described in this thesis were designed to explore such antigen-specific intervention in experimentally induced autoimmune arthritis with *ex vivo* manipulated autoantigen-specific T cells. The following paragraphs will put the major findings in perspective.

Proteoglycan-specific $CD4^+$ T cells can promote arthritis

Therapies that aim at depletion or manipulation of T and B cells in patients have demonstrated the arthritogenic potential of lymphocytes (8, 9). Moreover, T cell- (10-12) and B cell responses (13) with specificity for PG have been found in RA patients, indicating PG as a candidate autoantigen involved in the induction or perpetuation of arthritis. In BALB/c mice, immunization with cartilage PG induces a chronic arthritis. This proteoglycan (PG)-induced arthritis (PGIA) shares clinical and histopathological features with RA (14, 15). In addition, autoimmunity in PGIA depends on $CD4^+$ T cells (16, 17) and is also mediated by B cells and their antigen-specific antibodies (18). Therefore, PGIA is a representative model to study immune pathology of RA.

In *chapter 2* we have described the generation and characterization of a transgenic mouse containing a $CD4^+$ T cell population that is highly enriched for cells expressing a functional T cell receptor (TCR) that is specific (19) for the immunodominant arthritogenic T-cell epitope (16, 20-22) of PG (which is the $I\text{-}A^d$-restricted 5/4E8 epitope; peptide $PG_{70\text{-}84}$). The genetic code of this transgenic TCR was derived from the arthritogenic 5/4E8 T-cell hybridoma (16). PG-specific $CD4^+$ T cells from 5/4E8 TCR-Tg mice had high arthritogenic potential, when compared with $CD4^+$ T cells from wild type donors, as shown by a higher arthritis-susceptibility of these transgenic mice and more effective adoptive transfer of arthritis to SCID-recipients by transgenic spleen cells. In addition, constitutive expression of the PG-specific TCR by T cells partially bypassed the requirement for adjuvant in the induction of arthritis. Furthermore, spontaneous arthritis, thus without immunization, was sometimes found and underlines cross reactivity between human $PG_{70\text{-}84}$ and the homologous self (mouse) $PG_{70\text{-}84}$ as a result of molecular similarity as was found for the arthritogenic TCR-donating 5/4E8 hybridoma (16, 19). Although these data stress the importance of cartilage-specificity of $CD4^+$ T cells for induction of arthritis, recognition of

antigen is not the sole trigger for T cells to induce arthritis. In addition to antigen-recognition, triggering via T cell co-stimulatory molecules like inducible co-stimulator (ICOS), for example, is crucial for induction of arthritis as was demonstrated by the finding that mice deficient for ICOS did not develop collagen-induced arthritis (23) or PGIA (data not shown). Moreover, cytokines present during antigenic stimulation of T cells affect immune responses that are mediated by these T cells, as will be explained in the next paragraphs.

In conclusion, this TCR-transgenic mouse serves as an excellent donor for a homologous population of potentially arthritogenic antigen-specific $CD4^+$ T cells to be used to study antigen-specific $CD4^+$ T cell mediated intervention and $CD4^+$ T cell behavior in chronic arthritis in the PGIA model.

Immune balance in arthritis shifts towards T_h1-like immunity

Studies in RA patients and healthy controls have shown that increased IFN-γ production or high numbers of IFN-γ^+ $CD4^+$ T cells relative to cells expressing IL-4 or IL-10 is associated with disease (24-27). Moreover, in human studies T_h1 cell-derived cytokines, like IFN-γ, have been shown to stimulate production of molecules that are indicative of pro-inflammatory effector mechanisms of innate cells, like IL-1β and TNF-α (28).This indicates that RA is driven by T_h1 cells, which may be due to lack of counteracting T_h2 (or IL-4) or immunosuppressive T_{reg} cells (or T_{reg}-induced IL-10). Immune dominance of T_h1 cells in arthritis was demonstrated in *chapter 2* by the finding that at the time of onset of PGIA in 5/4E8 TCR-Tg mice the cytokine response to PG_{70-84} peptide by splenocytes of these mice had shifted to a higher IFN-γ/IL-4 ratio. In addition, the level of PG-specific antibodies shifted towards an IgG_{2a}/IgG_1 ratio that was about 10 fold higher during PGIA in TCR-Tg mice than in wild-type mice. Since antibody class switching to IgG_{2a} and IgG_1 has been described to be promoted by T_h1 and T_h2 cells and their cytokines respectively (29, 30), the IgG_{2a}/IgG_1 ratio may indicate dominance of T_h1- or T_h2 cells. Therefore the increase in PG-specific IgG_{2a}/IgG_1 in 5/4E8 TCR-Tg mice indicates interaction of T_h1 cells with B cells in disease progression of PGIA. Previous studies have demonstrated the need for T_h1-like responses (16, 17, 30-34) and the need for B cells as antigen-presenting cells for $CD4^+$ T cells and as producers of antigen-specific antibodies to induce full blown arthritis (18, 35-39).

The idea of IFN-γ being responsible for the induction of inflammation (28) was challenged by the finding that IFN-γ knockout mice are more susceptible to development of inflammation in some studies (40, 41). This indicates that IFN-γ may have immunoregulatory properties in some settings. Nevertheless, IFN-γ is important for induction of PGIA as IFN-γ deficient BALB/c mice show decreased susceptibility to PGIA, while IL-4 deficient knockout animals are highly susceptible to PGIA (30). Together with the finding of anti-arthritic activity of IL-4 and IL-10 in PGIA (30, 31) and other models of inflammatory disorders that are dominated by IFN-γ^+ T cells, this suggests potential of exaggerated T_h1-like immunity to induce inflammation due to insufficient activity or numbers of T_h2 or T_{reg} cells. This view on T cell biology in inflammation has recently been expanded by the discovery of the subset of T_h17 cells. These cells produce IL-17 and are involved in the induction of autoimmune-mediated inflammation (42, 43). Despite the finding that differentiation of T_h17 cells can be blocked by IFN-γ, both IL-17 and IFN-γ have been shown to be major players in induction of inflammation in different studies. With respect to autoimmune-mediated disease, immune homeostasis may therefore roughly be considered as a balance of pro-inflammatory T_h1 or T_h17 cells versus T_{reg} cells (43, 44).

Differential effects of IFN-γ show the complexity of cytokine networks involved in the autoimmune response. Such different effects found for a certain cytokine or cell may be explained by timing of action of such a cytokine or cell (45, 46). Timing may influence cytokine interactions with the present combination of cells and cytokines that varies at different times during the course of disease (47) and therefore the combined action of these immune mediators. For instance, TGF-β has been reported to induce regulatory T cells, but in combination with IL-6 this cytokine promotes differentiation of T_h17 cells (43, 48, 49). Furthermore, T_h17-differentiation through antigenic stimulation and cytokines was inhibited by IL-2 during the early T cell response, but during progressed stimulation IL-2 promoted growth of T_h17 cells (49). In addition, T_h1 cells have, besides their pro-inflammatory action, been reported to become producers of IL-10 with regulatory immune functions, probably as part of an eventual negative feedback mechanism (50, 51). Nonetheless, although T_h1-cells or related cytokines can have regulatory functions, tissue-antigen-specific T_h1-like immune responses are, together with T_h17 cells, functionally associated with and active in the induction of auto-immune mediated disease like arthritis.

Antigen-directed $CD4^+$ T cell mediated gene therapy targets arthritis

Autoimmune arthritis and many other autoimmune disorders have been shown to be provoked by T_h1 cells, whereas T_h2 cells and T_h2 associated cytokines can counteract on T_h1 cell responses (34, 52, 53). In addition, T_{reg} cells suppress T_h1- and T_h2 cell responses through, for example, IL-10 (54, 55). Such counteractive or suppressive action by these T cells and their cytokines is a mechanism that is held responsible for suppression or prevention of RA. Therefore, genes encoding cytokines like IL-4 and IL-10 seemed good candidates to be expressed in adoptive gene transfer with antigen-specific $CD4^+$ T cells. Also biologicals that neutralize the pro-inflammatory cytokines TNF-α or IL-1β, which are considered to be produced downstream of T cell-induced responses, are good candidates as targets for such therapy based on their successful therapeutic effect in the clinic (28).

As described in *chapter 3*, we generated retroviral constructs encoding IL-4, IL-10, TNF-α-RIg or IL-1RA to be used for retroviral transduction of $CD4^+$ T cells. PG-specific $CD4^+$ T cells were also forced to adopt a T_h1 cell- or T_h2 cell-phenotype by *ex vivo* antigenic stimulation in the presence of exogenous cytokines that have proven capacities to direct generation of T_h1 or T_h2 cells (32, 56, 57). Studies in several models for different autoimmune diseases have shown ameliorating effects of such transduced- and T_h2 $CD4^+$ T cell phenotypes on disease outcome (5, 6), indicating the beneficial potential of these agents. Genetic constructs that were generated for the retroviral transduction system were functional in our studies. However, $1x10^6$ transferred PG-specific TCR-Tg $CD4^+$ T cells were able to suppress arthritis only when expressing the transduced IL-10 gene. Moreover, exacerbation of arthritis was not found for any of the PG-specific $CD4^+$ T cell phenotypes tested. Studies in other labs have shown that the success of regulation by T cells depends on the number of immunomodulatory T cells used to interfere with T-cell mediated responses (58, 59). It is therefore likely that, except for $IL\text{-}10^+$ T cells, in our study the numbers of T cells used were not sufficient to regulate arthritis.

The cytokine IL-10 is a cytokine with immunoregulatory functions and is required for regulation by different subsets of T_{reg} cells. For example, expression of regulatory IL-10 is found in T_R1 cells (60) and has been shown to be responsible for regulation by natural $CD4^+$ T_{reg} cells that are induced by transcription factor Foxp3 and characterized by constitutive

expression of CD25 (54, 61). Depletion of $CD25^+$ cells by anti-CD25 antibodies have been shown to worsen PGIA, indicating regulatory activity of $CD25^+$ T_{reg} cells in PGIA (Roord *et al*., unpublished results). However, transfer of $1x10^6$ Foxp3-transduced PG-specific $CD4^+$ T_{reg} cells that showed stable expression of CD25 (data not shown) or $CD25^+$ enriched $CD4^+$ T cell populations (62) did not suppress PGIA. Because only IL-10 expressing PG-specific T cells suppressed PGIA, our study indicates that especially IL-10^+ T cells are a powerful means to regulate arthritis. Because of their regulatory function we name these IL-10-transduced $CD4^+$ T cells IL-10^+ T_{reg} cells.

In *chapter 4* we showed that IL-10^+ T_{reg} cells required recognition of the arthritogenic antigen to suppress arthritis. This was concluded from the finding that PG-specific IL-10^+ T_{reg} cells suppressed arthritis and IL-10^+ T_{reg} cells with an irrelevant TCR did not affect disease. This demonstrates the need for T cells to interact with cells that present cartilage derived antigens for successful regulation by these T cells. Cells in the joint synovia can present cartilage antigens like PG (63) that are released into the synovial fluid (64-66) and are therefore likely to retain antigen-specific regulatory T cell activity in the joint. Also other studies applying adoptive T cell transfer, showed requirement for recognition of antigen by T cells locally in the inflamed tissues to regulate inflammation (58) and to retain T cells in the joint (67). Therefore, it is suggested that antigen-specific IL-10^+ T_{reg} cells act locally at the site of inflammation and the lymph nodes that drain these sites because these are the most likely sites where cartilage antigens are processed, presented and thus recognized by cartilage antigen-specific T cells.

IL-10^+ T_{reg} cells suppress pro-inflammatory T- and B cell responses

IL-2 is expressed by and promotes growth of effector (T_h1) cells (53, 68, 69) and IL-2 also promotes long-term growth of T_h17 cells (49). Such T_h17 cells have been shown to be important for effective induction of arthritis and are characterized by the expression of IL-17 (42). In *chapter 4* we showed that PG-specific IL-10^+ T_{reg} cells reduce PG-specific expression of IL-2 and *in situ* expression of IL-17 in splenocytes during the chronic phase of arthritis. Together with the finding that PG-specific IL-10^+ T_{reg} cells tended to reduce PG-specific TNF-α expression, this suggests that PG-specific IL-10^+ T_{reg} cells suppress eventual function of T_h1 and/or T_h17 cells that mediate inflammation. In addition, the finding in *chapter 4* that PG-specific IL-10^+ T_{reg} cells suppress PG-specific IgG_{2a}, an antibody class that is known to be promoted by T_h1 cells, suggests reduced interaction of T_h1 cells with PG-specific B cells or direct effects of IL-10^+ T_{reg} cells on B cells that override effects of T_h1 cells.

Antibodies are crucial for induction of a full blown arthritis (18) and especially antibodies of the IgG_{2a} subclass have been associated with induction of arthritis (30). Antibodies provoke inflammatory responses at sites where antigen is available for recognition by the immune system through binding of immune complexes to FcγR (70, 71) or through complement activation (72, 73). In addition to producing antibodies, B cells are important for stimulation of T cells. B cells can provide a milieu that promotes formation of lymphoid structures in the joint and attracts T cells (73). Furthermore, especially antigen-specific B cells are efficient APCs for T cells that share the same antigen (18, 36, 74). Oligoclonal B cell populations are found in joints of RA patients (75, 76), indicating restriction of BCR usage directed to antigens that are present in the joint. The presence of abundant numbers of B cells in follicles (77) in the joint may therefore retain antigen-specific T cells by direct interactions of especially antigen(PG)-specific IL-10^+ T_{reg} cells with B cells in the joint,

where the cartilage antigen is available for presentation to T cells (18, 36, 37). However, the net antigen-specific immune response is not necessarily confined only to the actual site of B-T cell interaction at sites of inflammation because antibodies may also provoke inflammatory responses in non-inflamed tissues where the antigen is present. Thus, suppressing antibody responses of B cells may help to prevent spreading of disease between different joints.

Surprisingly, as described in *chapter 5*, no suppressive effect of PG-specific IL-10$^+$ T_{reg} cells was found on the early IFN-γ response of B cells and CD4$^+$ (PG-specific) T cells in PGIA. In addition, suppression of early proliferation of naive PG-specific T cells by IL-10$^+$ T_{reg} cells was not found *in vivo* in PGIA, although IL-10$^+$ T_{reg} cells can suppress proliferation of CD4$^+$ T cells *in vitro*. This finding implicates that translation of *in vitro* data to the more physiological and spatially relevant situation *in vivo* must be treated with caution. Altogether, the data suggest that PG-specific IL-10$^+$ T_{reg} cells do not ameliorate arthritis by suppressing the early (pre-arthritic) differentiation of pathogenic effector T_h1 cells. PG-specific IL-10$^+$ T_{reg} cells may rather ameliorate arthritis by suppressing exaggeration of pathogenic antigen-specific immune responses in a later stage of pathology and by suppression of production of cartilage-specific IgG_{2a} by B cells. Suppression of exaggerated pro-inflammatory immunity in a later stage of pathology is also suggested by the finding that, though they suppressed the (maximum) arthritis severity, PG-specific IL-10$^+$ T_{reg} cells did not affect the time of onset of disease (*chapter 3 and 4*).

IL-10$^+$ T_{reg} cells stimulate expression of anti-inflammatory IL-10 in T- and B cells

Besides the well known action of IL-10 to suppress production of cytokines, IL-10 has been shown to induce expression of IL-10 in several cell types, like monocyte-derived DCs and T cells *in vitro* (60, 78). These DCs suppressed activation and differentiation of T_h1 cells (78). T cells that are stimulated with antigen *in vitro* in the presence of IL-10 acquired regulatory functions that suppressed inflammation *in vivo* and T cell proliferation *in vitro* (60). Together with the finding, in *chapter 4*, of increased levels of IL-10 after transfer of PG-specific IL-10$^+$ T_{reg} cells, this led to the idea that induction of IL-10 expression in the endogenous cells of recipients may be a mechanism by which IL-10$^+$ T_{reg} cells suppress inflammatory responses *in vivo*. In *chapter 4* we showed that PG-specific IL-10$^+$ T_{reg} cells did not suppress arthritis in animals that are not able to produce endogenous IL-10. This confirmed our hypothesis that such induction of IL-10 expression in the endogenous system of recipients is needed to suppress arthritis.

As the results in *chapter 4* suggested that PG-specific IL-10$^+$ T_{reg} cells affected the antigen-specific response, we wondered whether IL-10 expression was propagated in CD4$^+$ T cells. *Chapter 5* showed that within the first week after transfer of PG-specific IL-10$^+$ T_{reg} cells IL-10 expression was found at increased levels in the endogenous CD4$^+$ T cells. This showed that IL-10 expression in CD4$^+$ T cells can be propagated by exogenous IL-10 expressed by CD4$^+$ T_{reg} cells *in vivo*. Although IL-10 has been shown to be involved in the generation of CD4$^+$CD25$^+$ T_{reg} cells that produce TGF-β *in vitro* (79), our data indicated that induction of IL-10 is the main regulatory mechanism by which PG-specific IL-10$^+$ T_{reg} cells regulate because expression of markers like Foxp3 and TGF-β that are also associated with regulation by T_{reg} cells was not elevated.

In addition to the exploding interest in IL-10-mediated T_{reg} cell biology, studies in several models of autoimmune diseases have demonstrated that critical regulatory activity is exerted through IL-10 by B_{reg} cells (80, 81). Antigen-specific B cells closely interact with T_h

cells sharing the same antigen. Moreover, T_h-B collaboration directs differentiation of effector (cytokine and antibody) responses of these lymphocytes and is required to induce arthritis (18, 35, 82). Therefore, we wondered how IL-10^+ T_{reg} cells would act on the cytokine responses of B cells in addition to the observed suppression of PG-specific IgG_{2a}. Although recently the induction of T_{reg} cells by B cells had been shown (83), the effect of T_{reg} cells producing IL-10 on the differentiation of cytokine responses of B cells was not known. *Chapter 5* showed that PG-specific IL-10^+ T_{reg} cells appear to promote stimulation of B cells as PG-specific IL-10^+ T_{reg} cells supported the number of B cells. This may be due to an enhanced expression of autocrine IL-2 we observed in the B cell population during the early response in arthritis. This expression was probably induced by IL-10 derived from PG-specific IL-10^+ T_{reg} cells as IL-10 has been shown to stimulate B cell growth *in vitro* by stimulating IL-2 production, which is a growth factor for B cells (84, 85). Moreover, PG-specific IL-10^+ T_{reg} cells promoted expression of IL-10 in the B cell population. Since primarily antigen-specific B cells interact with T cells specific for the same antigen (18, 36, 74), this indicates that PG-specific IL-10^+ T_{reg} cells may transfer regulatory activity to the antigen-specific B cell population by stimulation of IL-10^+ B cells. However, further studies should confirm regulatory capacities of such induced IL-10^+ B cells.

Thus, immunoregulatory effects of IL-10 produced by T cells may be envisioned not only to suppress inflammatory immune responses, but also to activate sufficient immunosuppressive responses in other lymphocytes to maintain immune homeostasis in healthy individuals. Regulatory B-T cell interactions may be reciprocally involved in active suppression of lymphocyte responses that lead to inflammation. Because lymphocytes are involved in all forms of antigen-mediated inflammation, this concept of regulatory interactive B-T mechanisms may not be restricted to autoimmunity in arthritis, but may be extended to immune responses in other autoimmune diseases, allergy and infectious diseases that are mediated by B- and T_h cells.

Concluding remarks

The proteoglycan-specific TCR-Tg mouse enables the use of a homogenous population of potentially arthritogenic $CD4^+$ T cells to study functions mediated by such cartilage-proteoglycan specific $CD4^+$ T cells in chronic B and T cell mediated arthritis. Expression of IL-10 by autoantigen-specific $CD4^+$ T cells is a promising device for restoring disrupted immune homeostasis to suppress the arthritic immune response. Such functional restoration of autoimmune homeostasis depends not solely on suppression of pro-inflammatory immunity but also requires stimulation of anti-inflammatory IL-10 expression (Fig. 1). Closely interacting B and T cells are potential candidates in which such regulatory IL-10 expression is promoted. Extending focus on IL-10-mediated suppression by T_{reg} cells to biology of regulatory IL-10^+ B_{reg} cells and regulatory B-T cell interactions may therefore provide more comprehensive insight in mechanisms that support antigen-specific regulatory capacity in the immune system. Thus, further exploration of induction and mechanisms of IL-10 dependent antigen-specific interactions of regulatory lymphocytes will support development of specific and more efficient treatment of antigen-driven inflammatory diseases like arthritis.

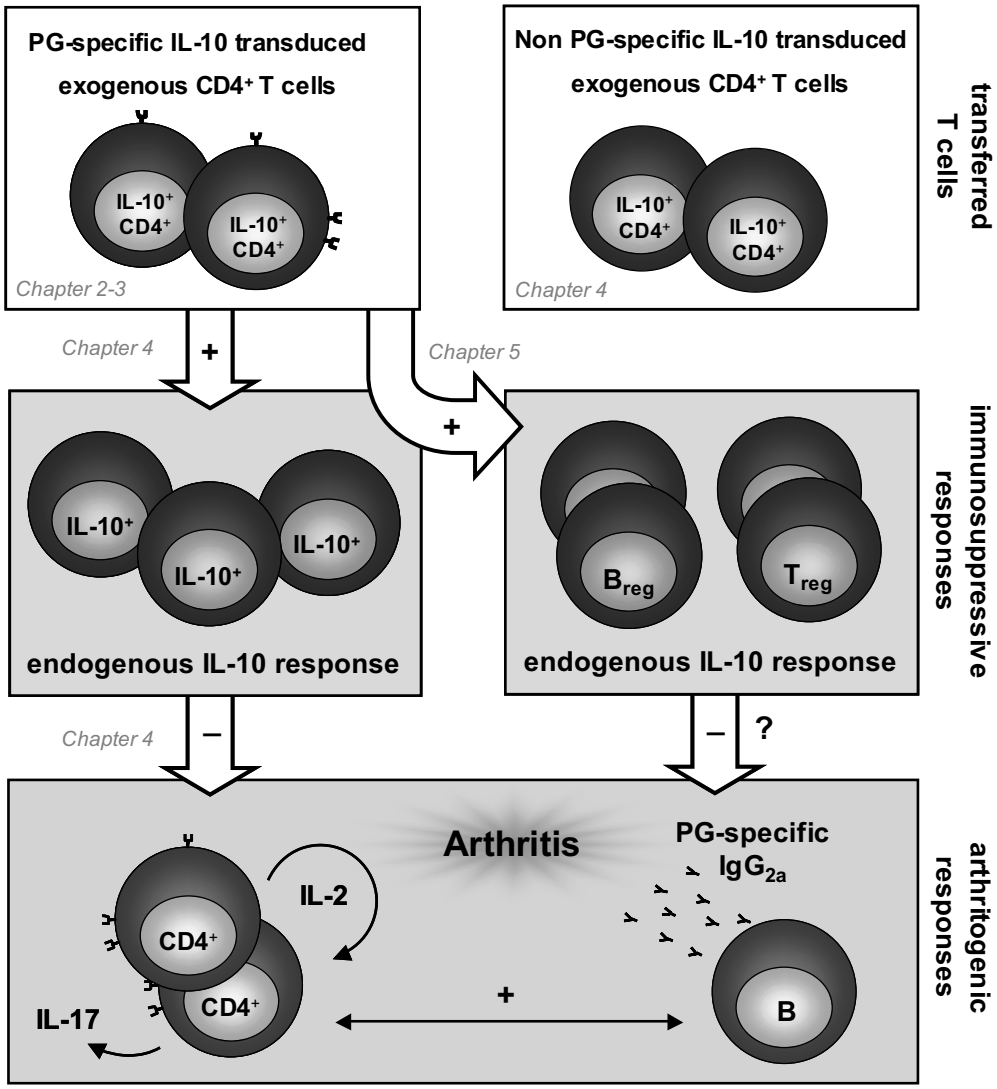

FIGURE 1. Summarizing interpretation of the major findings on immune regulation by T cells described in this thesis. A homogenous population of $CD4^+$ T cells that is potentially arthritogenic due to expression of a T cell receptor that is specific for cartilage proteoglycan (*chapter 2*), was transduced with an active gene coding for IL-10 to become functional T_{reg} cells (*chapter 3*). Transfer of such T_{reg} cells in PG-induced arthritis suppressed inflammation in the joints requiring their PG-specific T cell receptor (*chapter 4*). These T_{reg} cells also suppressed lymphocyte mediated responses which are associated with severity of arthritis, like production of IL-17, IL-2 and PG-specific IgG_{2a} antibodies, in the chronic phase of disease. Transferred PG-specific IL-10^+ T_{reg} cells needed to propagate endogenous production of IL-10 by the treated host to suppress arthritis (*chapter 4*). Furthermore, transferred PG-specific IL-10^+ T_{reg} cells promoted production of endogenous IL-10 by other $CD4^+$ T cells and also by B cells, which suggests that IL-10^+ T_{reg} cells promote IL-10^+ B_{reg} cells and other IL-10^+ T_{reg} cells that smother arthritogenic responses (*chapter 5*).

REFERENCES

1. Anderson, A. C., J. Reddy, R. Nazareno, R. A. Sobel, L. B. Nicholson, and V. K. Kuchroo. 2004. IL-10 plays an important role in the homeostatic regulation of the autoreactive repertoire in naive mice. *J Immunol* 173:828-834.
2. von Herrath, M. G., and L. C. Harrison. 2003. Antigen-induced regulatory T cells in autoimmunity. *Nat Rev Immunol* 3:223-232.
3. Weisman, M. H. 2002. What are the risks of biologic therapy in rheumatoid arthritis? An update on safety. *J Rheumatol Suppl* 65:33-38.
4. Skapenko, A., J. Leipe, P. E. Lipsky, and H. Schulze-Koops. 2005. The role of the T cell in autoimmune inflammation. *Arthritis Res Ther* 7 Suppl 2:S4-14.
5. Tarner, I. H., A. J. Slavin, J. McBride, A. Levicnik, R. Smith, G. P. Nolan, C. H. Contag, and C. G. Fathman. 2003. Treatment of autoimmune disease by adoptive cellular gene therapy. *Ann N Y Acad Sci* 998:512-519.
6. Tarner, I. H., E. Neumann, S. Gay, C. G. Fathman, and U. Muller-Ladner. 2006. Developing the concept of adoptive cellular gene therapy of rheumatoid arthritis. *Autoimmun Rev* 5:148-152.
7. Bluestone, J. A. 2005. Regulatory T-cell therapy: is it ready for the clinic? *Nat Rev Immunol* 5:343-349.
8. Chatenoud, L., and J. A. Bluestone. 2007. CD3-specific antibodies: a portal to the treatment of autoimmunity. *Nat Rev Immunol* 7:622-632.
9. Edwards, J. C., and G. Cambridge. 2006. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. *Nat Rev Immunol* 6:394-403.
10. Mikecz, K., T. T. Glant, M. Baron, and A. R. Poole. 1988. Isolation of proteoglycan-specific T lymphocytes from patients with ankylosing spondylitis. *Cell Immunol* 112:55-63.
11. Li, N. L., D. Q. Zhang, K. Y. Zhou, A. Cartman, J. Y. Leroux, A. R. Poole, and Y. P. Zhang. 2000. Isolation and characteristics of autoreactive T cells specific to aggrecan G1 domain from rheumatoid arthritis patients. *Cell Res* 10:39-49.
12. Guerassimov, A., Y. Zhang, S. Banerjee, A. Cartman, J. Y. Leroux, L. C. Rosenberg, J. Esdaile, M. A. Fitzcharles, and A. R. Poole. 1998. Cellular immunity to the G1 domain of cartilage proteoglycan aggrecan is enhanced in patients with rheumatoid arthritis but only after removal of keratan sulfate. *Arthritis Rheum* 41:1019-1025.
13. Karopoulos, C., M. J. Rowley, M. Z. Ilic, and C. J. Handley. 1996. Presence of antibodies to native G1 domain of aggrecan core protein in synovial fluids from patients with various joint diseases. *Arthritis Rheum* 39:1990-1997.
14. Glant, T. T., K. Mikecz, A. Arzoumanian, and A. R. Poole. 1987. Proteoglycan-induced arthritis in BALB/c mice. Clinical features and histopathology. *Arthritis Rheum* 30:201-212.
15. Glant, T. T., A. Finnegan, and K. Mikecz. 2003. Proteoglycan-induced arthritis: immune regulation, cellular mechanisms, and genetics. *Crit Rev Immunol* 23:199-250.
16. Buzas, E. I., F. R. Brennan, K. Mikecz, M. Garzo, G. Negroiu, K. Hollo, G. Cs-Szabo, E. Pintye, and T. T. Glant. 1995. A proteoglycan (aggrecan)-specific T cell hybridoma induces arthritis in BALB/c mice. *J Immunol* 155:2679-2687.
17. Bardos, T., K. Mikecz, A. Finnegan, J. Zhang, and T. T. Glant. 2002. T and B cell recovery in arthritis adoptively transferred to SCID mice: antigen-specific activation is required for restoration of autopathogenic CD4+ Th1 cells in a syngeneic system. *J Immunol* 168:6013-6021.
18. O'Neill, S. K., M. J. Shlomchik, T. T. Glant, Y. Cao, P. D. Doodes, and A. Finnegan. 2005. Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis. *J Immunol* 174:3781-3788.
19. Glant, T. T., E. I. Buzas, A. Finnegan, G. Negroiu, G. Cs-Szabo, and K. Mikecz. 1998. Critical roles of glycosaminoglycan side chains of cartilage proteoglycan (aggrecan) in antigen recognition and presentation. *J Immunol* 160:3812-3819.
20. Buzas, E. I., A. Hanyecz, Y. Murad, F. Hudecz, E. Rajnavolgyi, K. Mikecz, and T. T. Glant. 2003. Differential recognition of altered peptide ligands distinguishes two functionally discordant (arthritogenic and nonarthritogenic) autoreactive T cell hybridoma clones. *J Immunol* 171:3025-3033.
21. Buzas, E. I., A. Vegvari, Y. M. Murad, A. Finnegan, K. Mikecz, and T. T. Glant. 2005. T-cell recognition of differentially tolerated epitopes of cartilage proteoglycan aggrecan in arthritis. *Cell Immunol* 235:98-108.
22. Szanto, S., T. Bardos, Z. Szabo, C. S. David, E. I. Buzas, K. Mikecz, and T. T. Glant. 2004. Induction of arthritis in HLA-DR4-humanized and HLA-DQ8-humanized mice by human cartilage proteoglycan aggrecan but only in the presence of an appropriate (non-MHC) genetic background. *Arthritis Rheum* 50:1984-1995.
23. Nurieva, R. I., P. Treuting, J. Duong, R. A. Flavell, and C. Dong. 2003. Inducible costimulator is essential for collagen-induced arthritis. *J Clin Invest* 111:701-706.
24. Dolhain, R. J., A. N. van der Heiden, N. T. ter Haar, F. C. Breedveld, and A. M. Miltenburg. 1996. Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joints of patients with rheumatoid arthritis. *Arthritis Rheum* 39:1961-1969.
25. Yudoh, K., H. Matsuno, F. Nakazawa, T. Yonezawa, and T. Kimura. 2000. Reduced expression of the regulatory CD4+ T cell subset is related to Th1/Th2 balance and disease severity in rheumatoid arthritis. *Arthritis Rheum* 43:617-627.
26. Bucht, A., P. Larsson, L. Weisbrot, C. Thorne, P. Pisa, G. Smedegard, E. C. Keystone, and A. Gronberg. 1996. Expression of interferon-gamma (IFN-gamma), IL-10, IL-12 and transforming growth factor-beta (TGF-beta) mRNA in synovial fluid cells from patients in the early and late phases of rheumatoid arthritis (RA). *Clin Exp Immunol* 103:357-367.
27. Morita, Y., M. Yamamura, M. Kawashima, S. Harada, K. Tsuji, K. Shibuya, K. Maruyama, and H. Makino. 1998. Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. *Arthritis Rheum* 41:1669-1676.
28. McInnes, I. B., and G. Schett. 2007. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* 7:429-442.
29. Stevens, T. L., A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann, and E. S. Vitetta. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 334:255-258.
30. Kaplan, C., J. C. Valdez, R. Chandrasekaran, H. Eibel, K. Mikecz, T. T. Glant, and A. Finnegan. 2002. Th1 and Th2 cytokines regulate proteoglycan-specific autoantibody isotypes and arthritis. *Arthritis Res* 4:54-58.
31. Finnegan, A., K. Mikecz, P. Tao, and T. T. Glant. 1999. Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. *J Immunol* 163:5383-5390.

32. Maffia, P., J. M. Brewer, J. A. Gracie, A. Ianaro, B. P. Leung, P. J. Mitchell, K. M. Smith, I. B. McInnes, and P. Garside. 2004. Inducing experimental arthritis and breaking self-tolerance to joint-specific antigens with trackable, ovalbumin-specific T cells. *J Immunol* 173:151-156.
33. Wang, D., J. A. Hill, A. M. Jevnikar, E. Cairns, and D. A. Bell. 2002. Induction of transient arthritis by the adoptive transfer of a collagen II specific Th1 clone to HLA-DR4 (B1*0401) transgenic mice. *J Autoimmun* 19:37-43.
34. Finnegan, A., M. J. Grusby, C. D. Kaplan, S. K. O'Neill, H. Eibel, T. Koreny, M. Czipri, K. Mikecz, and J. Zhang. 2002. IL-4 and IL-12 regulate proteoglycan-induced arthritis through Stat-dependent mechanisms. *J Immunol* 169:3345-3352.
35. Shlomchik, M. J., J. E. Craft, and M. J. Mamula. 2001. From T to B and back again: positive feedback in systemic autoimmune disease. *Nat Rev Immunol* 1:147-153.
36. Yan, J., B. P. Harvey, R. J. Gee, M. J. Shlomchik, and M. J. Mamula. 2006. B cells drive early T cell autoimmunity in vivo prior to dendritic cell-mediated autoantigen presentation. *J Immunol* 177:4481-4487.
37. Takemura, S., P. A. Klimiuk, A. Braun, J. J. Goronzy, and C. M. Weyand. 2001. T cell activation in rheumatoid synovium is B cell dependent. *J Immunol* 167:4710-4718.
38. Crawford, A., M. Macleod, T. Schumacher, L. Corlett, and D. Gray. 2006. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. *J Immunol* 176:3498-3506.
39. Toellner, K. M., S. A. Luther, D. M. Sze, R. K. Choy, D. R. Taylor, I. C. MacLennan, and H. Acha-Orbea. 1998. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. *J Exp Med* 187:1193-1204.
40. Ferber, I. A., S. Brocke, C. Taylor-Edwards, W. Ridgway, C. Dinisco, L. Steinman, D. Dalton, and C. G. Fathman. 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol* 156:5-7.
41. Vermeire, K., H. Heremans, M. Vandeputte, S. Huang, A. Billiau, and P. Matthys. 1997. Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *J Immunol* 158:5507-5513.
42. Hirota, K., M. Hashimoto, H. Yoshitomi, S. Tanaka, T. Nomura, T. Yamaguchi, Y. Iwakura, N. Sakaguchi, and S. Sakaguchi. 2007. T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17+ Th cells that cause autoimmune arthritis. *J Exp Med* 204:41-47.
43. Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8:345-350.
44. Afzali, B., G. Lombardi, R. I. Lechler, and G. M. Lord. 2007. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin Exp Immunol* 148:32-46.
45. Boissier, M. C., G. Chiocchia, N. Bessis, J. Hajnal, G. Garotta, F. Nicoletti, and C. Fournier. 1995. Biphasic effect of interferon-gamma in murine collagen-induced arthritis. *Eur J Immunol* 25:1184-1190.
46. Sojka, D. K., A. Hughson, T. L. Sukiennicki, and D. J. Fowell. 2005. Early kinetic window of target T cell susceptibility to CD25+ regulatory T cell activity. *J Immunol* 175:7274-7280.
47. Adarichev, V. A., C. Vermes, A. Hanyecz, K. Ludanyi, M. Tunyogi-Csapo, A. Finnegan, K. Mikecz, and T. T. Glant. 2006. Antigen-induced differential gene expression in lymphocytes and gene expression profile in synovium prior to the onset of arthritis. *Autoimmunity* 39:663-673.
48. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235-238.
49. Laurence, A., and J. J. O'Shea. 2007. T(H)-17 differentiation: of mice and men. *Nat Immunol* 8:903-905.
50. O'Garra, A., and P. Vieira. 2007. T(H)1 cells control themselves by producing interleukin-10. *Nat Rev Immunol* 7:425-428.
51. Trinchieri, G. 2007. Interleukin-10 production by effector T cells: Th1 cells show self control. *J Exp Med* 204:239-243.
52. Liew, F. Y. 2002. T(H)1 and T(H)2 cells: a historical perspective. *Nat Rev Immunol* 2:55-60.
53. O'Garra, A., and N. Arai. 2000. The molecular basis of T helper 1 and T helper 2 cell differentiation. *Trends Cell Biol* 10:542-550.
54. O'Garra, A., and P. Vieira. 2004. Regulatory T cells and mechanisms of immune system control. *Nat Med* 10:801-805.
55. Mills, K. H. 2004. Regulatory T cells: friend or foe in immunity to infection? *Nat Rev Immunol* 4:841-855.
56. Nakamura, T., Y. Kamogawa, K. Bottomly, and R. A. Flavell. 1997. Polarization of IL-4- and IFN-gamma-producing CD4+ T cells following activation of naive CD4+ T cells. *J Immunol* 158:1085-1094.
57. Openshaw, P., E. E. Murphy, N. A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J Exp Med* 182:1357-1367.
58. Setoguchi, K., Y. Misaki, Y. Araki, K. Fujio, K. Kawahata, T. Kitamura, and K. Yamamoto. 2000. Antigen-specific T cells transduced with IL-10 ameliorate experimentally induced arthritis without impairing the systemic immune response to the antigen. *J Immunol* 165:5980-5986.
59. Mutis, T., R. S. van Rijn, E. R. Simonetti, T. Aarts-Riemens, M. E. Emmelot, L. van Bloois, A. Martens, L. F. Verdonck, and S. B. Ebeling. 2006. Human regulatory T cells control xenogeneic graft-versus-host disease induced by autologous T cells in RAG2-/-gammac-/- immunodeficient mice. *Clin Cancer Res* 12:5520-5525.
60. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737-742.
61. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.
62. Bardos, T., M. Czipri, C. Vermes, A. Finnegan, K. Mikecz, and J. Zhang. 2003. CD4+CD25+ immunoregulatory T cells may not be involved in controlling autoimmune arthritis. *Arthritis Res Ther* 5:R106-113.
63. Brennan, F. R., G. Negroiu, E. I. Buzas, C. Fulop, K. Hollo, K. Mikecz, and T. T. Glant. 1995. Presentation of cartilage proteoglycan to a T cell hybridoma derived from a mouse with proteoglycan-induced arthritis. *Clin Exp Immunol* 100:104-110.
64. Williams, J. M., C. Downey, and E. J. Thonar. 1988. Increase in levels of serum keratan sulfate following cartilage proteoglycan degradation in the rabbit knee joint. *Arthritis Rheum* 31:557-560.
65. Saxne, T., D. Heinegard, and F. A. Wollheim. 1987. Cartilage proteoglycans in synovial fluid and serum in patients with inflammatory joint disease. Relation to systemic treatment. *Arthritis Rheum* 30:972-979.
66. Lohmander, L. S., H. Wingstrand, and D. Heinegard. 1988. Transient synovitis of the hip in the child: increased levels of proteoglycan fragments in joint fluid. *J Orthop Res* 6:420-424.

67. Costa, G. L., M. R. Sandora, A. Nakajima, E. V. Nguyen, C. Taylor-Edwards, A. J. Slavin, C. H. Contag, C. G. Fathman, and J. M. Benson. 2001. Adoptive immunotherapy of experimental autoimmune encephalomyelitis via T cell delivery of the IL-12 p40 subunit. *J Immunol* 167:2379-2387.
68. Fehervari, Z., T. Yamaguchi, and S. Sakaguchi. 2006. The dichotomous role of IL-2: tolerance versus immunity. *Trends Immunol* 27:109-111.
69. Waldmann, T. A. 2006. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* 6:595-601.
70. Kaplan, C. D., Y. Cao, J. S. Verbeek, M. Tunyogi-Csapo, and A. Finnegan. 2005. Development of proteoglycan-induced arthritis is critically dependent on Fcgamma receptor type III expression. *Arthritis Rheum* 52:1612-1619.
71. Ioan-Facsinay, A., S. J. de Kimpe, S. M. Hellwig, P. L. van Lent, F. M. Hofhuis, H. H. van Ojik, C. Sedlik, S. A. da Silveira, J. Gerber, Y. F. de Jong, R. Roozendaal, L. A. Aarden, W. B. van den Berg, T. Saito, D. Mosser, S. Amigorena, S. Izui, G. J. van Ommen, M. van Vugt, J. G. van de Winkel, and J. S. Verbeek. 2002. FcgammaRI (CD64) contributes substantially to severity of arthritis, hypersensitivity responses, and protection from bacterial infection. *Immunity* 16:391-402.
72. Ji, H., K. Ohmura, U. Mahmood, D. M. Lee, F. M. Hofhuis, S. A. Boackle, K. Takahashi, V. M. Holers, M. Walport, C. Gerard, A. Ezekowitz, M. C. Carroll, M. Brenner, R. Weissleder, J. S. Verbeek, V. Duchatelle, C. Degott, C. Benoist, and D. Mathis. 2002. Arthritis critically dependent on innate immune system players. *Immunity* 16:157-168.
73. Martin, F., and A. C. Chan. 2004. Pathogenic roles of B cells in human autoimmunity; insights from the clinic. *Immunity* 20:517-527.
74. Brennan, F. R., K. Mikecz, E. I. Buzas, D. Ragasa, G. Cs-Szabo, G. Negroiu, and T. T. Glant. 1995. Antigen-specific B cells present cartilage proteoglycan (aggrecan) to an autoreactive T cell hybridoma derived from a mouse with proteoglycan-induced arthritis. *Clin Exp Immunol* 101:414-421.
75. Lee, S. K., S. L. Bridges, Jr., P. M. Kirkham, W. J. Koopman, and H. W. Schroeder, Jr. 1994. Evidence of antigen receptor-influenced oligoclonal B lymphocyte expansion in the synovium of a patient with longstanding rheumatoid arthritis. *J Clin Invest* 93:361-370.
76. Bridges, S. L., Jr., B. E. Clausen, J. C. Lavelle, P. G. Fowler, W. J. Koopman, and H. W. Schroeder, Jr. 1995. Analysis of immunoglobulin gamma heavy chains from rheumatoid arthritis synovium. Evidence of antigen-driven selection. *Ann N Y Acad Sci* 764:450-452.
77. Weyand, C. M., J. J. Goronzy, S. Takemura, and P. J. Kurtin. 2000. Cell-cell interactions in synovitis. Interactions between T cells and B cells in rheumatoid arthritis. *Arthritis Res* 2:457-463.
78. Corinti, S., C. Albanesi, A. la Sala, S. Pastore, and G. Girolomoni. 2001. Regulatory activity of autocrine IL-10 on dendritic cell functions. *J Immunol* 166:4312-4318.
79. Zheng, S. G., J. H. Wang, J. D. Gray, H. Soucier, and D. A. Horwitz. 2004. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol* 172:5213-5221.
80. Mizoguchi, A., and A. K. Bhan. 2006. A case for regulatory B cells. *J Immunol* 176:705-710.
81. Fillatreau, S., C. H. Sweenie, M. J. McGeachy, D. Gray, and S. M. Anderton. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 3:944-950.
82. Harris, D. P., L. Haynes, P. C. Sayles, D. K. Duso, S. M. Eaton, N. M. Lepak, L. L. Johnson, S. L. Swain, and F. E. Lund. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol* 1:475-482.
83. Reichardt, P., B. Dornbach, S. Rong, S. Beissert, F. Gueler, K. Loser, and M. Gunzer. 2007. Naive B cells generate regulatory T cells in the presence of a mature immunologic synapse. *Blood* 110:1519-1529.
84. Rousset, F., E. Garcia, T. Defrance, C. Peronne, N. Vezzio, D. H. Hsu, R. Kastelein, K. W. Moore, and J. Banchereau. 1992. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci U S A* 89:1890-1893.
85. Fluckiger, A. C., P. Garrone, I. Durand, J. P. Galizzi, and J. Banchereau. 1993. Interleukin 10 (IL-10) upregulates functional high affinity IL-2 receptors on normal and leukemic B lymphocytes. *J Exp Med* 178:1473-1481.

Nederlandse Samenvatting
(met inleiding voor niet-immunologen)

HET IMMUUNSYSTEEM

Voor degenen die niet bekend zijn met het immuunsysteem volgt hier een korte uiteenzetting van de immunologie die betrekking heeft op het onderzoek dat wordt beschreven in dit proefschrift. Daarna volgt een beknopte samenvatting van de hoofdstukken 1 tot en met 6 van dit proefschrift.

Het immuunsysteem is een georganiseerd leger van verschillende lichaamscellen die het lichaam verdedigen tegen ongewilde binnendringers

Dieren, waar mensen biologisch gezien ook toe behoren, moeten zich, om te overleven, beschermen tegen ziek makende micro-organismen (zoals bacteriën, virussen, etc.), mogelijke groei van kankercellen, en andere ongewenste materie. Deze bescherming komt tot stand door een ingenieus systeem van verschillende lichaamscellen die nauw samenwerken om de ziekmakers af te weren; het *immuunsysteem*. Het immuunsysteem, ook wel afweersysteem, vormt als het ware een leger dat het lichaam verdedigt tegen alles wat een gezond lichaam liever kwijt dan rijk is. De cellen van het immuunsysteem noemen we *witte bloedcellen*, of ook wel *leukocyten*. Deze cellen komen voor in veel verschillende vormen met verschillende functies. Ieder type leukocyt heeft zo zijn eigen gespecialiseerde rol in de mechanismen die bijdragen aan de afweer.

Leukocyten komen in grote aantallen voor in de bloedbaan. Als de leukocyten in actie moeten komen in bedreigde weefsels, verlaten ze de bloedbaan en verplaatsen ze zich naar het geïnfecteerde weefsel. Om de communicatie binnen het “leger” van leukocyten goed te laten verlopen zijn er communicatie centra waar leukocyten elkaar ontmoeten en informatie uitwisselen; de lymfeknopen. Door deze lymfeknopen loopt de lymfe (weefselvocht). De lymfe vervoert de informatie (zoals signalen van ziekteverwekkers) vanuit de naburige weefsels naar de lymfeknopen, waar de leukocyten de informatie krijgen over eventuele ontstekingen of andere problemen in het omliggende weefsel. De leukocyten kunnen hierdoor gealarmeerd raken, uit de lymfeknopen treden (de bloedbaan in) en naar de weefsels toe gaan waar hun actie nodig is. Naast een locatie waar leukocyten gealarmeerd kunnen worden is de lymfeknoop ook een efficiënte ontmoetingsplek voor leukocyten om een optimale samenwerking tussen de cellen tot stand te brengen.

Goed functioneren wordt bepaald door goede samenwerking, communicatie en een bijzonder vermogen tot het maken van onderscheid

Zoals een goed geolied leger, moet ook het “leger” van leukocyten aan een aantal basale eisen voldoen om goed te functioneren. Leukocyten moeten *onderscheid kunnen maken* tussen wat schadelijk is en wat onschuldig is. Om gepaste acties in gang te zetten moeten ze de aard van de “vijand” *herkennen*. Een geschikte verdedigingsactie schakelt hierbij zo efficiënt mogelijk de schadelijke bron uit, maar moet tegelijkertijd zo min mogelijk schade toebrengen aan het eigen lichaam. Een ander vereiste voor een optimaal functionerende verdediging is goede *communicatie* tussen de verschillende soorten leukocyten die deelnemen aan de immuunrespons.

Voor het herkennen van signalen uit hun omgeving dragen cellen *receptoren*. Receptoren functioneren als detectoren die kenmerkende structuren (*liganden*) op bijvoorbeeld micro-organismen of losse moleculen uit de omgeving kunnen binden, waardoor cellen deze waarnemen. De specifieke herkenning van een bepaald ligand door een receptor

zou je kunnen vergelijken met een sleutel (ligand) die specifiek past op een bepaald slot (receptor).

Een belangrijke groep receptoren zijn de zogenaamde “patroonherkennings receptoren” (*PRR’s*) die bepaalde patronen herkennen die kenmerkend zijn voor bepaalde groepen van ziekmakende organismen of onschuldige materie. De reactie van een cel op binding van een bepaalde receptor met haar ligand hangt af van het soort signaal dat door de binding afgegeven wordt, en kan variëren van “niets doen” tot het uitvoeren van “een dodelijk agressieve aanval”.

Twee soorten leukocyten worden gekenmerkt doordat ze receptoren dragen die nóg specifieker zijn dan de PRR’s. Dit zijn de B-cellen en de T-cellen (*lymfocyten*). De structuren die door deze uiterst specifieke *B-cel receptoren* of *T-cel receptoren* worden herkend heten respectievelijk B-cel- of T-cel *antigenen* omdat deze structuren een reactie van B- of T-cellen tegen (*anti-*) deze structuren genereren (*-gen*). Vanwege deze *antigeenspecifieke receptoren* spreken we bij lymfocyten van *antigeenspecifieke cellen*. Door deze hoogst verfijnde receptoren kunnen lymfocyten een immuunrespons zeer specifiek richten op de afzonderlijke soorten schadelijke materialen en micro-organismen. Kort gezegd zorgt herkenning door PRR’s voor het onderscheid tussen algemene eigenschappen van “gevaarlijke vijandige strijdkrachten en ongevaarlijke medestanders”, terwijl B- en T-cel receptoren zorgen voor herkenning van namen van “individuele soldaten”.

Voor een effectieve verdediging is nauwgezette samenwerking, en daarmee dus ook doelgerichte communicatie een vereiste. Een belangrijk middel waarmee cellen van het immuunsysteem elkaar instrueren is het uitzenden van *cytokinen*. Dit zijn moleculen die informatie tussen cellen overdragen over een relatief korte afstand, zoals wij woorden spreken om met elkaar te communiceren. Verschillende soorten cytokinen dragen elk hun eigen soort boodschap, van activerend tot onderdrukkend. Vaak komen tijdens immuunresponsen verschillende cytokinen tegelijkertijd voor. De combinatie ervan bepaalt de uiteindelijke boodschap die verzonden wordt. Wanneer je dit vergelijkt met woorden betekent een zin meer dan de losse woorden alleen. Zo krijgen bijvoorbeeld de woorden “aardig” en “gemeen” samen de betekenis “aardig gemeen”.

B- en T-cellen ondersteunen de antigeenspecifieke beschermende immuunreacties

De leukocyten die in dit proefschrift werden onderzocht waren de T- en B-cellen. Zoals hierboven beschreven zijn dit bijzondere leukocyten, omdat ze met hun extreem verfijnde receptoren uitermate precies de immuunrespons op hun doel (antigeen) richten. Het immuunsysteem omvat vele miljarden verschillende T- en B-cellen, die elk specifiek zijn voor één bepaald antigeen. Wanneer lymfocyten hun ligand tegenkomen en herkennen met hun specifieke receptor gaan ze delen. Daardoor ontstaat een groep klonen van dezelfde cel. Hiermee neemt het legertje van deze *antigeenspecifieke cellen* in omvang toe, waardoor de afweer tegen een bepaald antigeen snel versterkt wordt. Daarnaast zal een aantal van deze cellen langdurig overleven als *geheugencellen*. Door een latere ontmoeting van de geheugencellen met hetzelfde antigeen kan een snellere immuunrespons op gang worden gebracht tegen dat antigeen. Dit fenomeen is het zogenaamde *immunologische geheugen*, waarop bescherming tegen infectieziekten door vaccinatie berust.

T-cellen herkennen hun antigenen alleen met hun T-cel receptor als de antigenen in kleine herkenbare fragmentjes (*peptiden*)worden aangeboden door *antigeenpresenterende cellen* (*APC*) die in de buurt van de T-cellen aanwezig zijn. Deze APC kunnen hierdoor grote

invloed uitoefenen op de activering van de T-cellen. De T-cellen die in dit proefschrift werden bestudeerd zijn de zogenaamde helper T-cellen (*T_h-cellen*). Deze T-cellen heten “helper” cellen omdat ze vooral de acties van andere (immuun)cellen ondersteunen. T_h-cellen worden getypeerd door het molecuul CD4 op hun celmembraan, vandaar dat we de T_h-cellen ook wel *$CD4^+$ T-cellen* noemen. De immunologische ondersteuning door T_h-cellen wordt bepaald door de signalen, o.a. via cytokinen, die de geactiveerde T_h-cellen richten aan de cellen in hun nabije omgeving.

De *B-cel* is een ander belangrijk type antigeenspecifieke cel. In tegenstelling tot een T-cel kan een B-cel zijn antigeen direct herkennen met zijn B-cel receptor, zonder voorafgaande bewerking en presentatie door andere cellen. Daarnaast kunnen B-cellen via hun B-cel receptor hun specifieke antigenen in zich opnemen, verwerken en vervolgens presenteren aan T-cellen. B-cellen zijn dus actief als APC. Doordat B-cellen hun acties tot op zekere hoogte richten op hun antigenen door hun B-cel receptor, activeren B-cellen efficiënt T-cellen die specifiek zijn voor datzelfde antigeen. Zo kunnen antigeenpresenterende B-cellen interacties met T-cellen aangaan, waarbij ze de reacties van elkaar tegen dezelfde antigenen sturen en ondersteunen. Een andere kenmerkende eigenschap van B-cellen is dat ze antistoffen uitscheiden die specifiek gericht zijn tegen de antigenen die via B-cel receptoren de B-cel hebben geactiveerd. Deze antistoffen (ook wel *antilichamen* of *immuunglobulinen*) kunnen zich verspreiden over grote afstanden binnen het lichaam, en binden alleen aan ziekteverwekkers en andere materie waarop het antigeen aanwezig is. Dit gebeurt om deze antigene materie te neutraliseren of om een ontstekingsproces te stimuleren op de plaats waar de te elimineren ziekteverwekker/antigeen aanwezig is. B-cellen kunnen dus net als T_h-cellen van dichtbij betrokken zijn bij antigeenspecifieke immuunresponsen, maar kunnen antilichamen in de strijd brengen die dienen als een soort zeer doelgerichte langeafstandsraketten.

Ontspoorde B- en T-cel reacties kunnen leiden tot allergieën en auto-immuunziekten

De cellen van het immuunsysteem zijn er op gemaakt om schadelijk invloeden van buitenaf zo snel mogelijk te verwijderen uit het lichaam. Bij het verwijderingproces is het niet alleen essentieel dat een ontstekingsreactie de gevaarlijke materie voldoende opruimt, maar ook dat diezelfde ontstekingsreactie op tijd gestopt wordt om te veel onnodige lichaamsschade te voorkomen.

Sommige van de miljarden T_h- en B-cellen herkennen in plaats van ziekteverwekkers, onschuldige stoffen. Normaal gesproken zullen deze B- en T_h-cellen geen ontstekingreacties teweeg brengen. In sommige gevallen raakt het immuunsysteem echter ontspoord, waardoor de B- en T_h-cellen agressief gaan reageren wanneer zij onschuldige stoffen herkennen. Dit is het geval bij allergieën voor bijvoorbeeld graspollen; de ontstekingsreactie die wordt opgewekt door B- en T_h-cellen die reageren tegen pollen (hooikoorts) veroorzaakt meer schade dan de pollen zelf zouden doen. Wanneer B- en T_h-cellen overdreven reageren op lichaamseigen weefsel (*autoantigenen*), en daar een ontsteking veroorzaken, dan spreekt men van een auto-immuunziekte. Veel voorkomende auto-immuunziekten zijn multiple sclerose (reactie tegen centraal zenuwstelsel), diabetes (insuline producerende cellen van de alvleesklier als doelwit) en reumatoïde artritis (waarbij voornamelijk de gewrichten het doelwit vormen). Omdat T_h-cellen een belangrijke rol spelen in het aansturen van immuunresponsen, dus ook die leiden tot auto-immuunziekten, kunnen auto-

antigeenspecifieke T_h-cellen een nuttig middel zijn om het ziekteproces van bijvoorbeeld reumatoïde artritis te bestuderen of zelfs te beïnvloeden.

Verschillende gedaantes van T-cellen en de cytokinen die ze produceren brengen verschillende effecten teweeg

In de jaren ’80 werd ontdekt dat aan de hand van cytokinen die worden uitgescheiden door een bepaalde T_h-cel, verschillende vormen van T_h-cel functies onderscheiden kunnen worden. T_h-cellen die reageren tegen ziekteverwekkers die voornamelijk binnen cellen voorkomen, zoals virussen, maar ook tegen lichaamseigen weefsels (auto-immuniteit) worden gekenmerkt doordat ze veel van het cytokine interferon-γ (IFN-γ) produceren en weinig interleukine-4 (IL-4). Deze cellen staan bekend als *T_h1*-cellen. De T_h-cellen die voornamelijk betrokken zijn bij de ontwikkeling van afweer tegen bijvoorbeeld worminfecties en bij allergieën, worden klassiek gekenmerkt doordat ze juist veel IL-4 en weinig IFN-γ produceren. Zij worden *T_h2*-cellen genoemd. Recentelijk is een ander soort T_h-cel ondekt die ook een belangrijke rol speelt bij auto-immuunziekten; de *T_h17*-cel. De *T_h17*-cel heeft zijn naam gekregen door het ontstekingsbevorderende IL-17 dat hij uitscheidt.

Naast bovengenoemde zogenaamde “effector” T_h-cellen bestaat een type $CD4^+$ T-cel die de acties en ontwikkeling van effector T_h-cellen kan remmen. Deze remmende $CD4^+$ T-cellen, de regulator T-cellen (*T_{reg}*-cellen), zijn verantwoordelijk voor het voorkomen van de ontspoorde T_h-cel responsen die leiden tot onnodige weefsel schade. De regulerende functie wordt uitgevoerd door onder andere de afweeronderdrukkende cytokinen IL-10 en TGF-β. Momenteel wordt verondersteld dat overdreven ontstekingen bij auto-immuunziekten, (deels) het gevolg zijn van een onevenwichtige activiteit van T_h1-/T_h17-cellen en T_{reg}-cellen en hun cytokinen.

Reumatoïde artritis

Reumatoïde artritis (RA) is een aandoening waarbij de gewrichten chronische ontstoken zijn. Deze ziekte komt in ongeveer 1% van de bevolking van de westerse wereld voor. De gewrichtsontsteking veroorzaakt pijn en stijfheid en leidt uiteindelijk tot afbraak van kraakbeen en botvergroeiing in de aangedane gewrichten. De exacte oorzaak van reumatoïde artritis is niet bekend, maar een combinatie van genetische factoren en omgevingsfactoren, zoals bepaalde infecties en roken, draagt bij aan de kans op het ontwikkelen van reumatoïde artritis.

De gewrichtsholte (*synovium*) tussen de met kraakbeen bedekte botuiteinden bevat in gezonde personen een vloeistof die nauwelijks cellen bevat. Deze wordt omgeven door een dunne grenslaag van cellen (*synoviale membraan*). Tijdens artritis groeit de gewrichtsholte vol met een grote hoeveelheid aan verschillende cellen (*pannus*). Veel van deze cellen scheiden cytokinen uit die het ontstekingsproces bevorderen, zoals *TNF-α* (tumor necrose factor-alpha) en *IL-1β* (interleukine-1bèta), en ook enzymen die het kraakbeen en bot kunnen afbreken. Daarnaast worden in het gewricht soms lymfeknoop-achtige structuren gevormd die veel B- en T-cellen bevatten. Het immuunsysteem van artritis patiënten bevat relatief veel T-cellen (met name T_h1-cellen) en B-cellen die hun acties richten tegen antigenen uit gewrichtskraakbeen en daardoor verantwoordelijk zijn voor de ontsteking. Kraakbeen-antigeenspecifieke T_h-cellen kunnen de ontsteking in gang zetten en in stand houden door cytokinen te produceren. B-cellen hebben een belangrijke rol in de ontsteking als antigeenpresenterende cellen voor activering van T_h-cellen. Ook zijn B-cellen de

producenten van antilichamen die specifiek zijn voor kraakbeenantigenen. Proefdierstudies hebben bewezen dat immunisatie (inenting zoals bij vaccinatie) met antigenen uit kraakbeen reacties opwekt van kraakbeenspecifieke B- en T_h-cellen, en daardoor artritis veroorzaakt.

Samengevat dragen veel soorten cellen en hun cytokinen bij aan de ontsteking. Vooral B- en T_h-cellen die aanwezig zijn in de gewrichten, en door hun kraakbeen-antigeen specifieke receptoren geactiveerd worden, bepalen de locatie van de ontsteking; het gewricht. Een schematische voorstelling hiervan wordt weergegeven in figuur 1 van hoofdstuk 1.

SAMENVATTING VAN DIT PROEFSCHRIFT

Algemene doelstelling en belangrijke onderzoeksvragen

T-cellen die zich richten tegen gewrichtskraakbeen zijn verantwoordelijk voor het ontwikkelen van gewrichtsontsteking bij reumatoïde artritis. In dit proefschrift werd onderzocht hoe dergelijke T cellen kunnen worden ingezet in een bijzonder doelgerichte strijd tegen gewrichtsontsteking, door ze met gentechnologie ontstekingsremmende eigenschappen te geven.

De ontsteking bij reumatoïde artritis kan, evenals bij andere chronische ontstekingen, gedeeltelijk onderdrukt worden met ontstekingsremmers zoals TNF-α blokkers. De werking van huidige ontstekingsremmers beperkt zich echter niet tot de gewrichtsontsteking, maar onderdrukt ook de afweer tegen ziekteverwekkers. Een van de serieuze neveneffecten van het gebruik van ontstekingsremmers die hierdoor wordt veroorzaakt is een verhoogd risico op infecties door ziekmakers. Daarom is de ontwikkeling van therapieën die zich specifieker richten op de ontsteking in het gewricht, en het ontwikkelen van kennis over de antigeen-specifieke immuunresponsen die daaraan ten grondslag liggen, noodzakelijk.

Aangezien T_h-cellen die zich door hun antigeen-specificiteit richten tegen gewrichtskraakbeen betrokken zijn voor de ontwikkeling en in stand houding van gewrichtsontsteking, zijn deze T_h-cellen een potentieel middel voor doelgerichte interventies in de behandeling van reumatoïde artritis. De in dit proefschrift beschreven studies hadden als *doel* om kraakbeenspecifieke T_h-cellen die artritis kunnen induceren zodanig (genetisch) te veranderen dat ze ontstekingsremmende eigenschappen krijgen. De toegepaste kandidaat-genen waren genen die coderen voor middelen met ontstekingsremmende eigenschappen.

Belangrijke vragen die we met de gedane studies wilden beantwoorden waren:

1) *Kunnen (verschillende gemanipuleerde) kraakbeenspecifieke T_h-cellen de gewrichts-ontsteking beïnvloeden?*
2) *Wat zijn geschikte genen om kraakbeenspecifieke T_h-cellen mee te voorzien zodat ze gewrichtsontsteking dempen?*
3) *Moeten gemanipuleerde T_h-cellen kraakbeenantigeen herkennen om gewrichts-ontsteking te verminderen?*
4) *Beïnvloeden ontstekingsremmende kraakbeenspecifieke T_h-cellen de ontwikkeling en reacties van andere leukocyten?*

Het onderzoek

Om de gestelde vragen te beantwoorden gebruikten we een onderzoeksmodel dat belangrijke gelijkenissen vertoont met reumatoïde artritis. In dit model wordt artritis geïnduceerd in muizen door twee immunisaties met proteoglycaan (PG), een component van gewrichtskraakbeen. Als reactie op de immunisaties zullen T- en B-cellen die het PG herkennen geactiveerd worden, waardoor deze cellen een chronische ontsteking tegen het kraakbeen in gewrichten veroorzaken.

In *hoofdstuk 1* van dit proefschrift wordt een inleiding gegeven op de gedane studies. In dit hoofdstuk wordt uitgebreid aandacht besteed aan de rol van het immuunsysteem, en met name de rol van B- en T-cellen, in het ontstaan van RA.

Voor het uitvoeren van onze studies hadden we een uniforme populatie van fysiologisch relevante, *artritogene* (artritis-veroorzakende) PG-specifieke T_h-cellen nodig. Als bron hiervoor hebben we, door het inbrengen van een T cel receptor-gen, een transgene muis gemaakt die een PG-specifieke T-cel receptor (*TCR*) op de $CD4^+$ T-cellen droeg. Dit wordt beschreven in *hoofdstuk 2*. De meeste van de $CD4^+$ T-cellen uit deze TCR-transgene muizen zijn specifiek voor PG. Verder laten we zien dat de transgene muizen gevoeliger zijn voor voor PG-geïnduceerde artritis dan gewone muzien. Het feit dat de TCR transgene cellen een verhoogde capaciteit hebben om artritis over te brengen in immuundeficiënte ontvangers, toonde aan dat kraakbeenspecifieke T_h-cellen een aanzienlijke rol spelen in de ontwikkeling van artritis. De bevinding dat de T_h-cellen van TCR-transgene muizen zich tijdens artritis ontwikkelen tot T_h1-cellen ondersteunt het idee dat auto-immuunziekten veroorzaakt kunnen worden door ontspoorde auto-antigeenspecifieke T_h1-cellen. Hiermee werd dus een transgene muis ontwikkeld die artritogene T_h-cellen levert die als nuttig gereedschap gebruikt kunnen worden in studies naar het functioneren van kraakbeenspecifieke T_h-cellen in artritis.

In *hoofdstuk 3* werden PG-specifieke T-cellen voorzien van actieve genen die coderen voor bewezen immuunsuppressieve agentia. De geteste agentia waren TNF-α Receptor Ig (blokkade van TNF-α-gemedieerde ontsteking), IL-1Receptor antagonist (neutraliseren van IL-1β-gemedieerde ontsteking), IL-4 (remmen van T_h1-/ T_h17-responsen) en IL-10 (remmen van T_h1-responsen). Deze genen werden gekopieerd van muizen DNA en vervolgens gekloneerd in virale DNA-constructen. Deze constructen werden gebruikt om de betreffende genen te verpakken in virusdeeltjes (retrovirussen). Retrovirussen worden veel toegepast omdat ze genen kunnen overdragen op cellen (zie figuur 3 van hoofdstuk 1). De in dit proefschrift beschreven retrovirussen waren zodanig gemanipuleerd dat alleen hun vermogen om de gewenste genen over te brengen naar de T-cellen na infectie was behouden, maar dat eventuele ziekmakende eigenschapen uitgeschakeld waren. Met deze methode hebben we de genoemde genen *in vitro* in het genoom de PG-specifieke T_h-cellen gebracht. Naast deze genetisch veranderde T_h-cellen werden ook T_h-cellen *in vitro* gekweekt in aanwezigheid van cytokinen waardoor ze de gedaante van T_h1-cellen of T_h2-cellen gingen aannemen.

De gemanipuleerde PG-specifieke T_h-cellen werden ingebracht bij muizen waarin artritis opgewekt werd, om de effecten van deze T cellen op gewrichtsontsteking te bestuderen. Alleen T_h-cellen die het IL-10-gen tot expressie brachten ($IL\text{-}10^+$) waren in staat om de geïnduceerde artritis te remmen. De resultaten in deze studies laten zien dat kraakbeenspecifieke T_h-cellen die IL-10 produceren ($IL\text{-}10^+$ T-cel) een sterk vermogen hebben om ontstekingsreacties te dempen, en dat productie van IL-10 in immuunregulatie door T_h cellen nadere aandacht verdient in de zoektocht naar een succesvolle behandeling van RA.

De experimenten in *hoofdstuk 4* gaan in meer detail in op hoe de kraakbeen-specifieke $IL\text{-}10^+$ T-cellen artritis reguleren. Uit de resultaten blijkt dat deze cellen alleen effectief zijn doordat ze specifiek zijn voor kraakbeen, aangezien $IL\text{-}10^+$ T-cellen die niet specifiek zijn voor PG, maar voor een eiwit (ovalbumine) dat niet in muizen voorkomt, geen effect hebben op de gewrichtsontsteking. Daarnaast onderdrukten de PG-specifieke $IL\text{-}10^+$ T-cellen de productie van PG-specifieke IgG_{2a}, een klasse van antilichamen die voornamelijk met hulp

van T_h1-cellen door B-cellen geproduceerd wordt. Ook onderdrukten de PG-specifieke IL-10^+ T-cellen de productie van TNF-α, IL-17 (pro-inflammatoire cytokinen) en IL-2 (een T-celdelingsfactor) in de latere fase van artritis, en konden de IL-10^+ T_h-cellen *in vitro* de deling, en dus de toename, van andere T_h-cellen remmen.

Naast het onderdrukken van de productie van pro-inflammatoire cytokinen, stimuleerden de IL-10^+ T-cellen de aanmaak van IL-10 in de behandelde ontvangers. Wanneer PG-specifieke IL-10^+ T-cellen werden ingebracht in muizen die zelf geen IL-10 konden produceren, hadden ingebrachte IL-10^+ T-cellen geen effect meer op de ontsteking. Dit experiment liet daarom zien dat het stimuleren van de productie van IL-10 in de behandelde ontvangers een belangrijk mechanisme is waarmee PG-specifieke IL-10^+ T-cellen de gewrichtsontsteking remmen (zie ook figuur 1 van hoofdstuk 6).

Verschillende studies hebben laten zien dat er B_{reg}-cellen en T_{reg}-cellen bestaan, welke belangrijke anti-inflammatoire functies uitoefen door het produceren van IL-10. Daarnaast hebben verschillende studies laten zien dat T- en B-cellen de ontwikkeling van elkaar kunnen beïnvloeden. Er was echter weinig bekend over hoe IL-10-producerende B_{reg}- en T_{reg}-cellen ontstaan. PG-specifieke IL-10^+ T-cellen kunnen B-cellen beïnvloeden, zoals in hoofdstuk 4 bleek uit de veranderde antilichaamproductie die werd veroorzaakt door PG-specifieke IL-10^+ T-cellen. In *hoofdstuk 5* stelden we de vraag of de ontwikkeling van IL-10-producerende T_{reg}-cellen en B_{reg}-cellen gestimuleerd kan worden door PG-specifieke IL-10^+ T-cellen, en of deze PG-specifieke IL-10^+ T-cellen de vroege ontwikkeling van pro-inflammatoire T_h- en B-cellen kunnen remmen.

Tijdens de eerste (zes) dagen van de vroege immuunrespons tegen PG konden de ingebrachte PG-specifieke IL-10^+ T_{reg}-cellen de pro-inflammatoire kenmerken van T_h-cellen niet remmen. Echter, de aantallen IL-10-producerende T_h-cellen van de ontvangende muis zelf namen toe onder invloed van de ingebrachte PG-specifieke IL-10^+ T-cellen. Daarnaast nam het aantal B-cellen dat IL-10 produceerde significant toe, wat er op duidt dat IL-10 geproduceerd door T_{reg}-cellen een groei- of differentiatiefactor is voor B-cellen die zelf IL-10 produceren. Aanvullende studies moeten uitwijzen of deze geïnduceerde IL-10-producerende T- en B-cellen in de met PG-specifieke IL-10^+ T-cellen behandelde ontvangers ook daadwerkelijk functioneel regulerend werken als een T_{reg}- en B_{reg}-cel.

In *hoofdstuk 6* worden de resultaten van dit proefschrift samenvattend bediscussieerd.

Conclusies

De in dit proefschrift beschreven proteoglycaanspecifieke TCR-transgene muis kan worden gebruikt als unieke bron voor een uniforme populatie van $CD4^+$ T-cellen die specifiek zijn voor kraakbeen-proteoglycaan. Dit geeft de mogelijkheid om het functioneren (en de experimenteel-therapeutische toepassing) van kraakbeenspecifieke $CD4^+$ T-cellen in door B- en $CD4^+$ T-cellen veroorzaakte chronische artritis te onderzoeken. Expressie van het cytokine IL-10 door auto-antigeenspecifieke T-cellen is een veelbelovend middel om een verstoord immunologisch evenwicht tussen pro- en anti-inflammatoire immuunresponsen te herstellen en daarmee de auto-immuniteit die leidt tot ontsteking te onderdrukken. Dergelijk herstel van de immunologische balans is niet alleen afhankelijk van onderdrukking van de pro-inflammatoire immuniteit, maar vereist vooral ook stimulatie van de productie van het immuun-regulerende IL-10. Nauw samenwerkende kraakbeenspecifieke B- en T-cellen zijn

mogelijke kandidaten waarin expressie van IL-10 wordt gestimuleerd ten behoeve van immuunregulatie. Het verleggen van de huidige aandacht voor met name IL-10-gemedieerde suppressie door T_{reg}-cellen naar de biologie van regulerende IL-10^{+} B_{reg}-cellen en regulerende interacties tussen T- en B-cellen kunnen een waardevolle toevoeging zijn voor het inzicht in mechanismen die de antigeenspecifieke regulerende capaciteit van het immuunsysteem bevordert. Onderzoek aan IL-10-afhankelijke antigeenspecifieke interacties van regulerende lymfocyten kan daarom een nuttig handvat bieden voor verdere ontwikkeling van doeltreffender en krachtiger behandeling van ontsteking bij auto-immuunziekten.

Dankwoord

DANKWOORD

De omvang van dit boekje is voor een proefschrift niet overdreven groot. Een stukje tekst, een paar plaatjes erbij, en klaar is Kees. In plaats van dit boekje, had je ook een CD met een aantal mooie stukken muziek op de plank kunnen zetten. En dan te bedenken dat onderzoekers er gedurende hun loopbaan tientallen, of soms zelfs honderden, van verzamelen! Dat kost behoorlijk wat uurtjes muziek op de plank. Toch is het niet zo maar wat, zo'n boekje. Er zijn veel zware inspanningen geleverd om dit proefschrift te laten worden zoals je het nu in je handen hebt. Niet alleen door mij, maar vooral ook door vele anderen. Dus als je twijfelt tussen de nieuwe CD of dit boekje op de laatste beschikbare plek in je te krappe kast zetten; koop een grotere kast, zou ik nu zeggen.

Vele uren, waaronder lange uren, energie vretende uren, energieke uren, inspirerende uren, heel treurige uren, boze uren, wanhopige uren, frustrerende uren, slapeloze uren, te korte uren, soms ook gewoon uren van 60 minuten, leerzame uren, hoopvolle uren, opgewekte uren, gezellige uren, muzikale uren en ook gewoon leuke uren. Zoveel uren heeft het uiteindelijk ongeveer gekost om dit boekje tot stand te brengen. Maar dat is niet meer dan een ruwe schatting. En dat voor een stukje tekst met wat plaatjes. Inderdaad, dat zou je kunnen zeggen. Maar de manier waarop deze, voor mij belangrijke, uren zijn ingevuld, en de manier waarop waardevolle inhoud gegeven is aan dit boekje is bewonderenswaardig. Althans, dat vind ik. Hiervoor wil ik een aantal mensen bijzonder bedanken.

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Curriculum vitae and publications

CURRICULUM VITAE

Teun Guichelaar werd op 31 maart 1979 geboren in Hoogeveen. In 1997 behaalde hij het Atheneum diploma aan het Menso Alting College in Hoogeveen. In datzelfde jaar begon hij met een studie Biologie aan de Wageningen Universiteit. In 2003 behaalde hij hier het doctoraal examen Biologie met als specialisatie cel- en moleculaire biologie. Binnen deze specialisatie fase rondde hij met succes twee afstudeeronderzoeken en een onderzoeksstage af.

Het eerste afstudeeronderzoek, over stress, bevordering van antigeen-opname en distributie van leukocyten in vissen door immersievaccinatie, voerde hij uit bij de vakgroep Celbiologie en Immunologie (prof. dr. ir. Huub Savelkoul en prof. dr. Willem van Muiswinkel) van de Wageningen Universiteit. Het volgende afstudeervak, over de rol van fosforylering van virale transporteiwitten in het transport van het “Cowpea Mosaic Virus”, deed hij bij de vakgroep Moleculaire Biologie (prof. dr. Ton Bisseling) van de Wageningen Universiteit. De studie Biologie sloot hij af met een externe stage, over klasse switch van antilichamen tegen polysacchariden van pneumococcen, bij de afdeling Histologie en Immunologie (prof. dr. Frans Kroese) van de Rijksuniversiteit Groningen.

Van mei 2003 tot augustus 2007 was hij als assistent in opleiding (AIO) in dienst van de Universiteit Utrecht bij de divisie Immunologie (promotor: prof. Dr. Willem van Eden, co-promotor: dr. Femke Broere), departement Infectieziekten en Immunologie, faculteit Diergeneeskunde. Het promotieonderzoek aan regulatie van ontsteking bij reumatoïde artritis door T cellen dat hij daar deed, resulteerde in dit proefschrift.

Vanaf december 2007 werkt hij als als post-doctoraal onderzoeker bij de afdeling Klinische Chemie en Haematologie van het Universitair Medisch Centrum in Utrecht, waar hij, onder supervisie van dr. Tuna Mutis, onderzoek doet aan T cel regulatie van afstotingsreacties en anti-tumor immunologie bij transplantatie.

PUBLICATIONS

Guichelaar, T., C.B. Ten Brink, P.J. van Kooten, S.E. Berlo, C.P. Broeren, W. Van Eden, and F. Broere. 2008. Autoantigen-Specific Interleukin-10-Transduced T Cells Suppress Chronic Arthritis by Promoting the Endogenous Regulatory IL-10 Response. *J Immunol* 180: 1373-1381.

Berlo, S.E., T. Guichelaar, C.B. Ten Brink, P.J. van Kooten, F. Hauet-Broere, K. Ludanyi, W. van Eden, C.P. Broeren, and T.T. Glant. 2006. Increased arthritis susceptibility in cartilage proteoglycan-specific T cell receptor-transgenic mice. *Arthritis Rheum* 54:2423-2433.

Hauet-Broere, F., L. Wieten, T. Guichelaar, S. Berlo, R. van der Zee, and W. Van Eden. 2006. Heat shock proteins induce T cell regulation of chronic inflammation. *Ann Rheum Dis* 65 Suppl 3:iii65-68.

Huising, M.O., T. Guichelaar, C. Hoek, B.M. Verburg-van Kemenade, G. Flik, H.F. Savelkoul, and J.H. Rombout. 2003. Increased efficacy of immersion vaccination in fish with hyperosmotic pretreatment. *Vaccine* 21:4178-4193.

Pouwels, J., N. Kornet, N. van Bers, T. Guichelaar, J. van Lent, T. Bisseling, and J. Wellink. 2003. Identification of distinct steps during tubule formation by the movement protein of Cowpea mosaic virus. *J Gen Virol* 84:3485-3494.