Modulating T cell homeostasis via TNF and TNFR superfamily members : characterization and function of effector & regulatory T cells

van Olffen, R.W.

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"Characterization and Function of Effector & Regulatory T cells"

Ronald William van Olffen

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I do not know what I may appear to the world; but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding of a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

Sir Isaac Newton (1642-1727)

English physicist, mathematician.

Contents

Chapter 1

General Introduction

General introduction

The tumor necrosis factor receptor (TNFR) superfamily can be divided into two distinct groups: death domain and TNF Receptor-associated Factor (TRAF) binding receptors[1]. The death domain containing receptors can induce apoptosis via the initiation of caspase cascades by death domain signaling intermediates. In contrast, TRAF-binding receptors can regulate inflammation, lymphoid organization and activation of antigen presenting cells (APC's). Furthermore, TRAF binding receptors can directly influence B and T cell activation, differentiation and survival. The availability of TNFR-ligands is tightly controlled and this regulation is critical for normal immune function since modulation of the expression of either receptor or ligand results in abrogated responsiveness or rather hyper-responsiveness of the immune system. This thesis focuses on the role of the TRAF-binding receptors CD27 and GITR in immune activation. Transgenic mouse models have been used to determine the direct effects of increased ligand availability prior to and after deliberate immunological challenges.

The immune system

The immune system functions as a protection mechanism that defends the host against invading pathogens and malignant cells. The immune system is build on the basis of a layered system. Pathogens first have to penetrate physical barriers such as skin prior to activating cells of the immune system. Hereafter, these pathogens and distressed cells induce the activation of the immune system.

Cells and products of the immune system can be separated into two distinct categories, namely innate and adaptive. Although an interplay exists between cells of the innate and adaptive immune system, innate immunity is generally considered the first line of defense as it provides a direct but nonspecific response against pathogens. In contrast, the adaptive immune system improves a response for a (subsequent) better recognition of the pathogen.

The resulting increase in pathogen recognition and elimination is exploited to combat a secondary infection.

Innate immunity

Cells of the innate immune system have evolved to respond against non-self antigens (pathogens) and signals from distressed cells. Both plants and animals have elaborate innate immune systems, suggesting an origin prior to the division of these lifeforms[2]. Generally, during an inflammatory response cells of the innate immune system (e.g. macrophages, granulocytes, mast cells and dendritic cells) directly convert to highly activated short lived effector cells which attempt to clear the invading pathogen. Activation of these cells occurs through the identification of pathogens (pathogen-associated molecular patterns, PAMPs) via pattern recognition receptors (PRR). These PRR include membrane bound, intracellular and soluble receptors (e.g. mannan-binding lectin, C-reactive protein, and serum amyloid protein), which exert their function through opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and the induction of apoptosis of infected cells[3]. Furthermore, upon pathogen encounter antigen presenting cells such as DC's upregulate co-stimulatory molecules (CD80, CD86, TNFR superfamily members and/or their ligands) which may activate cells of the adaptive immune system, thereby complementing the innate immune response. In addition, inflammatory cytokines (e.g. IL4 and TNFα by mast cells[4, 5] and IL12 by DC's[6]) and chemokines (e.g. TARC, MDC, MIG, IP-10, ITAC by DC's[7-9]) produced upon activation of an inflammatory response may facilitate the activation of the adaptive immune system..

Adaptive immunity

The adaptive immune system consist of lymphocytes (NK, B and T cells) capable of generating a memory population for the recognition of specific pathogens, thereby providing a faster and stronger response each time the pathogen is encountered. T cell activation starts when naïve T cells passing through lymph nodes recognize antigens presented by activated dendritic cells on major histocompatibility complex (MHC) molecules. Pathogens present in the cytosol (endogenous pathogens; viruses/ bacteria) are generally digested by host cell enzymes and coupled to MHC class I. In addition, dendritic cells have the capacity to process exogenous proteins derived from other cells on MHC class I. This process, termed cross-presentation, promotes immunity against tissue-specific viruses which do not infect DC's[10].The resulting complex promotes $CD8^+$ T cell activation, clonal expansion and induces cytotoxic T lymphocyte (CTL) differentiation. CTL's function through the production and release of perforins and granzymes which are effector molecules involved in the actual killing of infected cells. In contrast to endogenous pathogens, exogenous pathogens are usually displayed on MHC class II molecules, and induce $CD4^+$ T cell activation.

Naïve T cell activation and subsequent polarization proceeds in response to different extracellular signals. The first signal, TCR triggering via the recognition of a specific antigen bound to the MHC complex results in T cell activation and is followed by a second, co-stimulatory signal which licenses T cell expansion. CD28 is a member of the Ig-superfamily and is constitutively expressed on naïve T cells. Ligation of CD28 by its ligands CD80 or CD86 can provide this secondary co-stimulatory signal. In contrast, the absence of a co-stimulatory signal can lead to T cell anergy. In addition to co-stimulatory signals, T cell polarization is determined via the cytokine milieu (the third signal of the three signal hypothesis) during T cell activation[11]. The resulting polarization of T helper progenitor cells results in the commitment of $CD4^+$ T cells to a specific lineage, which include the T_H1, T_H2,

T_H17 cells and the distinct regulatory lineages (T_{Reg}, T_H3, T_R1). The development, characterization and function of these different lineages is discussed in more detail below.

Subsets of differentiated helper T cells

T Helper 1 cells

A T_H1 response is characterized by the production of interferon-γ (IFNγ), tumor necrosis factor (TNF) and interleukin-2 (IL-2) and prototypically promotes cell mediated immunity. Cell mediated immunity is generally induced to combat viral infections and intracellular pathogens. This response maximizes expansion of cytotoxic $CD8^+$ T cells and promotes macrophage activation and differentiation. In a positive feedback loop IFNγ potentiates T_H1 lineage commitment through increasing IL12 production by macrophages and dendritic cells. IL12 induces the transcription factor T_{Bet} in T cells and thereby T_H1 commitment and IFNγ production. Conversely, IFNγ lowers the production of IL4 and thus limits T_H2 lineage commitment[12, 13].

T Helper 2 cells

In contrast to T_H1 reactions, T_H2 responses result in the secretion of IL4, IL5, IL6, IL10 and IL13, and promote humoral immunity. Humoral immune responses are characterized by the production of antibodies, which bind to invading pathogens (e.g. viruses and bacteria) and mark these pathogens for removal by the innate immune system. The cytokines associated with a T_H2 response are capable of promoting further T_H2 commitment. For instance, IL-10 is capable of downregulating IL12 production by macrophages and dendritic cells and lowers IFNγ production by helper T cells. In addition, IL4 promotes T_H2 cytokine secretion and T_H2 lineage commitment via upregulation of the transcription factor GATA-3, thereby preserving the humoral immune response[14]. The recognition of T_H2 promoting pathogens by antigen

presenting cells results in the upregulation of NOTCH ligands[15]. This promotes NOTCH activation and cleavage of its intracellular domain on T cells, thus initiating transcription of the IL4 gene and generating a T_H2 response[15].

T Helper 17 cells

In contrast to the T_H1 and T_H2 lineages, T_H17 cells have only recently emerged as a new T helper lineage. T_H17 cells are recognized as IL17 producing cells[16] and have been implicated as important players in autoimmunity. IL17 induces specific cytokines (IL6, IL1β, TNFα) and chemokines (CXCL1,2,8) associated with activation and migration of neutrophils. Following the identification of the T_H17 lineage, the factors resulting in the generation of T_H17 cells have been further characterized. Naïve T cells commit to the T_H17 lineage after TCR triggering in the presence of TGFβ and IL6 (or IL1β in humans) due to the induction of the transcription factor RORγt[17-21]. T_H17 cells are capable of producing IL17, IL21, IL23 and IFNγ. The upregulation of the IL23 receptor by IL21 and IL6 and the subsequent positive feedbackloop through IL23 production results in T_H17 cell proliferation and stabilization[17-19, 22, 23]. In this respect, formation of T_H17 and T_{Reg} are mutually exclusive, but do share similar growth factors for their differentiation. The production of IL6 (murine) or IL1β (human) during inflammation blocks T_{Reg} commitment and promotes T_H17 cell formation[24]. In addition, IL21 inhibits FoxP3 transcription further promoting T_H17 cell formation[25, 26].

$CD4^+$ Regulatory T cells

Regulatory T cells were first described in the early 70's as suppressor T cells[27]. These suppressor T cells remained a discredited subset of T cells until the 90's, when Sakaguchi and colleagues identified $CD4^+CD25^+$ regulatory T cells (T_{Reg}) which developed in the thymus and could be isolated from the periphery[28]. Over the last couple of years several T_{Reg}

populations have been identified. The natural occurring T_{Reg} (nT_{Reg}) inhibit proliferation and/or function of non-T_{Reg} through a contact dependent mechanism or via the production of IL10 in vivo[29]. In contrast to the nT_{Reg} which originate from the thymus, inducible T_{Reg} (iT_{Reg}) are generated after TCR triggering by tolerogenic DC's or in the presence of tolerogenic cytokines. Inducible T_{Reg} include the T_R1 cells and the T_H3 cells, which respectively suppress T cell responses via IL10 or TGFβ production[30-32]. Although characterization of T_{Reg} has established increased expression levels of CD25, CTLA-4 and GITR on regulatory T cells, it should be noted that these markers are not unique for T_{Reg} as they are also expressed by activated non-regulatory T cells. Despite variable expression in T_R1 and T_H3 cells, the transcription factor FoxP3 was found to be exclusively expressed in murine regulatory T cells resulting in the commitment to a regulatory phenotype[33, 34].

The Influence of the microenvironment on helper T cell polarization

The activation of antigen presenting cells by pathogen-associated molecular patterns via pattern recognition receptors results amongst others in the production of cytokines and chemokines. The cytokine microenvironment influences the commitment of naïve T cells to a T_H1, T_H2, T_H17 or a T_{Reg} lineage upon activation. Interleukin 12 produced by macrophages or activated B cells induce T_H1 commitment, whereas IL4 further promotes T_H2 lineage skewing. Although both T_H17 and T_{Reg} use TGF_β for lineage commitment, the presence or absence of IL6 determines which lineage will eventually develop.

Interestingly, negative feedback mechanisms via transcription factors result in a sustained lineage commitment (Fig 1)[35].

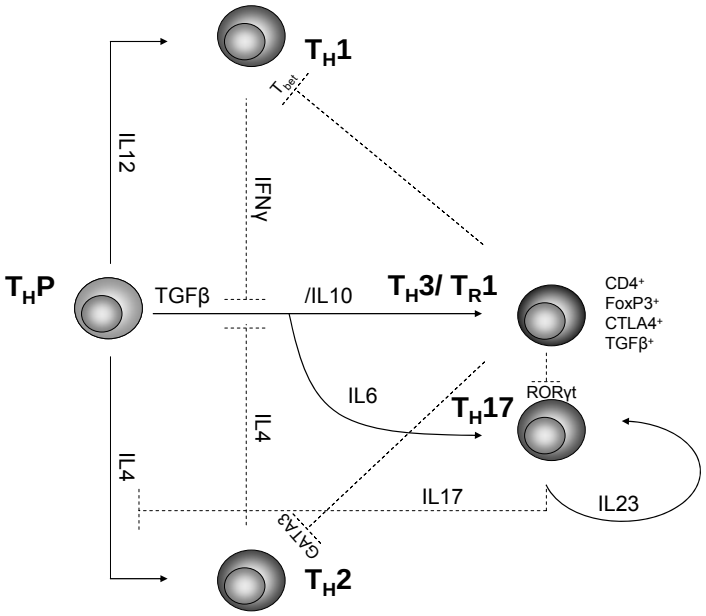

Figure 1. Representation of cytokine/growth factor influences on murine CD4$^+$ lineage commitment. T_H progenitor (T_HP) cells may commit to a specific lineage under influence of the specified cytokines. Induction of specific transcription factors result in negative and positive feedback mechanisms and sustained lineage commitment. Induction of the T_H1 lineage plays an important role in cell mediated immunity, whereas the T_H2 lineage promotes humoral immunity. The T_H17 lineage is associated with autoimmunity in contrast to the T_{Reg} lineage which regulates T cell activation. T_HP cells signify naïve CD4$^+$ T cells. Dashed lines denote a negative feedback mechanism. Straight lines show positive induction.

The role of TNFR superfamily members in adaptive immunity

For effective T cell activation, T cells require co-stimulatory signals next to TCR triggering. In addition to providing T cell activation via TCR triggering and influencing the cytokine microenvironment, antigen presenting cells upregulate several ligands of the TNFR superfamily. These signals result in effective T cell activation and polarization (Fig 2). Members of the TNFR superfamily have emerged as important mediators of survival, proliferation and differentiation of T cells during all phases of adaptive immune responses. The TRAF-binding co-stimulatory members of the TNFR superfamily, which include CD40, 4-1BB, CD27, OX40, CD30, HVEM and GITR, have distinct effects on T cell expansion.

Below, the effects of these TNFR superfamily members on B cell and $CD4^+$ and $CD8^+$ T cell function will be discussed.

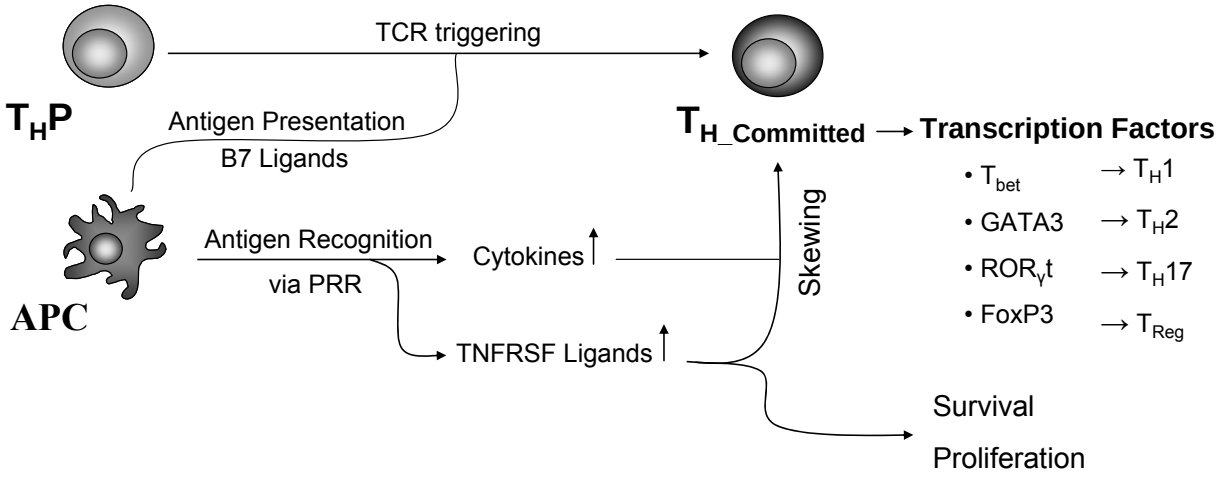

Figure 2. Schematic representation of APC effects on T helper cell activation and lineage commitment.

Antigen presenting cells present antigen to naïve $CD4^+$ T cells via MHC class II molecules thereby providing TCR triggering. The recognition of antigen via pattern recognition receptors induce cytokine secretion and can influence lineage commitment. The upregulation of TNFR superfamily member ligands further influence lineage commitment and affect T cell homeostasis.

CD40 - CD40L

Upon TCR triggering, CD40L is transiently upregulated on T cells[36]. The functions of CD40L upon interaction with its receptor CD40 include the upregulation of B7 molecules (i.e. CD80, CD86) on APC's and the induction of IL12, thereby promoting T_H1 responses. CD40L triggering plays an important role in germinal centre development, memory B cell formation and production of IgG, IgA and IgE antibody responses[37]. Indeed genetic defects in CD40-CD40L signaling result in hyper IgM syndrome[38], thus corroborating its pivotal role in B cell

function and humoral responses. Next to this, hyper IgM patients suffer from severe defects in T cell immunity that can be attributed to the function of CD40-CD40L interaction in the activation of APC.

Studies using CD40L$^{-/-}$ mice confirm its role in T_H1 skewing as these mice show a defect in T_H1 development[39]. In addition, blockade of CD40L during a typical T_H1 mediated autoimmune disease (experimental autoimmune encephalomyelitis, atherosclerosis, arthritis) results in significant protection in disease development[40-45]. CD40L blockade is associated with a strong modulation of cytokine profile in mice. The T_H1 cytokine IFNγ is suppressed whereas IL4 is enhanced[46]. Furthermore, CD40L expression on CD4$^+$ T cells may directly promote memory CD8$^+$ T cell formation[47].

Thus CD40L on T cells indirectly skews T cells to a T_H1 phenotype, promotes CD8$^+$ T memory cell formation and helps in memory B cell formation.

4-1BB - 4-1BBL

Similar to CD40L, 4-1BB is transiently upregulated on T cells upon TCR triggering. In addition to indirectly promoting T_H1 responses by enhancing IL12 production from myeloid DC's, 4-1BB is mainly associated with CD8 responses[48, 49]. Inhibiting signaling via 4-1BB on CD8$^+$ T cells does not directly affect the primary CD8 expansion, but does decrease the accumulation of CD8$^+$ effector type T cells at the peak of an immune response[50]. Furthermore, an agonistic 4-1BB antibody was capable of preventing apoptosis of CD8$^+$ T cells stimulated with superantigen[51]. Moreover, 4-1BBL$^{-/-}$ mice showed a defective CD8$^+$ T cell response following viral infection[52] and the absence of 4-1BB or its ligand had no effect on CD4$^+$ T cell responses during viral challenge with influenza, vesicular stomatitis virus or LCMV[52-54]. The transgenic overexpression of 4-1BBL under control of the MHC class II I-Eα promoter further revealed a prominent role of 4-1BB – 4-1BBL interactions in humoral

responses as these mice showed a progressive depletion of mature B cells[55]. Thus, the TNFR superfamily member 4-1BB mainly functions as a costimulatory receptor for $CD8^+$ T cells, has only slight effects on $CD4^+$ T cell responses and may influence B cell survival.

CD27 - CD70

The TNFR superfamily member CD27 is expressed on naïve, activated and memory T cells, hematopoietic progenitor cells, NK cells and a subset of B cells[56-59]. In collaboration with TCR engagement ligation of human CD27 by its ligand CD70, which is upregulated on B cells and DC's upon activation, promotes the expansion of IFNγ producing $CD4^+$ (i.e. T_H1 cells) and $CD8^+$ T cells[36, 60-62]. In addition, expression of the IL12 receptor is upregulated thereby making $CD4^+$ T cells receptive to T_H1 polarization and enhances the transcription of the transcription factor T_{Bet}[63]. Furthermore, studies using genetically modified mice have shown that CD27 ligation is important for memory T cell formation[61]. Loss of CD27 signaling decreased $CD4^+$ and $CD8^+$ T cell accumulation in the lung following influenza infection and decreased formation of influenza specific T cells in the periphery. In addition to defects in primary T cell responses, loss of CD27 signaling impairs the formation and optimal secondary expansion of memory T cells[61, 64, 65]. CD27 signaling promotes T cell accumulation via stimulating survival of activated T cell, and does not affect T cell proliferation[66].

Therefore, CD27 signaling promotes cellular immunity as this functions as a co-stimulatory receptor for both $CD4^+$ and $CD8^+$ T cell expansion.

OX40 – OX40L

Expression of the TNFRSF member OX40 is restricted to activated T cells, in which OX40 is preferentially expressed on $CD4^+$ T cells[67, 68]. Expression of the ligand (OX40L) is upregulated upon activation of B cells[69, 70], T cells[71] and DC's[72]. In addition, OX40L is expressed on epithelial cells[73]. The function of OX40 triggering on T cells in respect to

lineage commitment does not appear to be specific. OX40 has been implicated in increasing both T_H1 as well as T_H2 responses and promotes a response subsequent to lineage commitment depending on the cytokine environment[74-78].

CD30 – CD30L

The TNFRSF member CD30 is expressed on activated T cells, B cells, NK cells and on eosinophils[79-81], whereas its ligand is expressed on activated T cells and B cells. CD30 is preferentially expressed on T_H2 cells, although naïve T cells and T_H1 cells can express CD30[82]. The cytokine IL4, associated with T_H2 skewing, results in upregulation of CD30[79, 83]. In addition to its importance for the T_H2 lineage, CD30 can affect $CD8^+$ memory T cell survival[84].

HVEM - Light

The herpes virus entry mediator (HVEM) is expressed on resting T cells, monocytes and immature dendritic cells. Expression of its ligand (Light) is induced on T cells upon activation and is expressed on monocytes, NK cells and immature DC's[85-89]. In contrast to the TNFRSF members described above, HVEM is downregulated upon T cell activation; thereby limiting the time in which HVEM functions as a costimulatory receptor for T cells[89]. The costimulatory function of HVEM on T cells becomes apparent in Light transgenic mice, which show a clear autoimmune phenotype. $CD4^+$ and $CD8^+$ T cells are expanded and these mice showed severe inflammation in the intestine. In addition, splenomegaly and lymphadenopathy was observed[90-92]. Studies using Light KO mice suggest that HVEM-Light interactions have a significant impact on memory $CD8^+$ T cell formation, as CTL recall responses were significantly decreased following immunisations with a viral peptide[93]. Therefore, HVEM can be seen as an important player in T cell expansion and memory formation.

GITR - GITRL

The glucocorticoid-induced TNFR family-related gene (GITR) is highly expressed on activated T cells and regulatory T cells. Studies using recombinant GITRL or agonistic antibodies to stimulate GITR suggest that GITR functions as a costimulatory factor for T cells upon TCR triggering[94-97]. As of yet, the specific function for GITR on regulatory T cells remains unresolved. Ligation of GITR on T_{Reg} has first been associated with neutralization of the suppressive capacity of these cells and later dismissed as non-regulatory T cells escaped suppression upon GITR triggering[98]. The effects of GITR engagement in vivo on skewing of $CD4^+$ T cells to a T_H1, T_H2, T_H17 or T_{Reg} phenotype have not yet been determined.

The impact of genetic modification of TNFRSF member expression

The function of TNFRSF members on T cell homeostasis has extensively been investigated using knockout and/or transgenic mice. In table 1 an overview is given on the effects on T cell homeostasis using gene targeted and transgenic mice.

Table 1 Immunological consequences of gene targeting the TNFR superfamily members

Molecule	Expression	KO	TG	Phenotype	Refs
CD27	T & B cells NK cells Progenitors	Full KO	-	Reduced CD4 and CD8 T cell expansion and memory formation to influenza; normal primary T cell response, but reduced memory response to LCMV.	61, 99
		-	-	-	
CD70	T & B cells DCs Macrophages	-	-	-	
		-	B cell-TG	Enhanced CD4 and CD8 effector T cell formation; improved clearance of influenza and tumor; splenomegaly and IFNγ dependent B cell depletion; premature death due to opportunistic infection.	62, 100, 101
			DC-TG	Enhanced CD4 and CD8 effector T cell formation; lack of CD8 deletional tolerance; splenomegaly and B cell depletion.	102
			T cell-TG	Enhanced CD8 effector T cell formation, but impaired maintenance of memory CD8 T cells; only minor splenomegaly and B cell depletion.	103
4-1BB	T & B cells NK cells DCs	Full KO	-	Reduced CD8 T cell response to VSV; enhanced CD4 T cell response to protein immunization.	54, 104
		-	T cell-TG	Enhanced T cell proliferation in vitro and increased CHS.	105
4-1BBL	B cells DCs Macrophages	Full KO	-	Reduced CD8 T cell expansion and memory formation upon acute infections; normal CD8 T cell numbers but impaired function during chronic infection.	52, 53, 106, 107
		-	B cell-TG	Normal T cell response, but reduced APC function during allogeneic stimulation; splenomegaly, B cell depletion and reduced IgG responses.	55
CD30	T cells	Full KO	-	Reduced T cell expansion and IFN-□ production by CD4 T cells upon mycobacterium infection; contradictory reports on thymic negative selection.	108-110
		-	T cell-TG	Enhanced thymocyte apoptosis upon stimulation; splenomegaly.	111
CD30L	T & B cells Macrophage	Full KO		Reduced CD8 memory T cell formation upon Listeria infection; reduced CD4 effector T cell formation upon mycobacterium infection.	84, 112
		-	-	-	
HVEM	T & B cells DCs Macrophages	Full KO	-	Enhanced T cell activation and cytokine production.	113
		-	Soluble form	Resistant to infection with HSV-1, but not pseudorabies virus.	114
Light	T cells DCs	Full KO	-	Reduced CD8 T cell expansion and effector cell formation to SEB or peptide; reduced CD8 T cell proliferation and CD4 T cell IL-2 production in MLR.	93, 115
		-	T cell-TG	Enhanced CD4 and CD8 effector T cell formation; splenomegaly, autoantibodies and inflammation of several organs; reduced thymic output.	116 91, 92
OX40	T & B cells DCs	Full KO	-	Reduced CD4 T cell proliferation and effector cell formation to infection with influenza and LCMV, but not with L. major, N. brasiliensis or TMEV; reduced formation of effector and memory CD8 T cells to VACV infection.	117-119
		-	-	-	
OX40L	T & B cells DCs Macrophages	Full KO	-	Reduced CD4 T cell proliferation and effector cell formation to protein immunization; reduced CD8 T cell response to allogeneic stimulation.	69, 72
		-	DC-TG	Increased CD4 accumulation in B cell follicles.	120
			T cell-TG	Enhanced CD4 T cell responses, inflammation of lung and intestine; more severe EAE; enhanced Th2 response and impaired clearance of L. major.	121 122, 123
			All cell-TG	Increased CD4 T cell numbers, enhanced CHS and allogeneic response.	124
GITR	T cells	Full KO	-	Reduced regulatory CD4 T cell numbers; reduced effector T cell activity and disease intensity during experimental colitis or arthritis; enhanced CD4 effector T cell formation upon Candida infection.	98, 125 126, 127
		-	-	-	
GITRL	B cells	-	-	-	
		-	B cell-TG	Enhanced in vivo effector and regulatory CD4 T cell proliferation, delayed disease induction in EAE model.	This thesis

Expression profile of costimulatory members of the TNF-R superfamily and the phenotype of genetically mutated mice, in which these respective molecules have either been deleted (KO) or transgenically expressed (TG).

Scope of this thesis

The activation and interplay between both an innate and adaptive immune response is highly regulated though cellular ligands and receptors on cells of the immune system. Here, we assessed the function of two members of the TNFR superfamily in T cell homeostasis and immune activation, namely CD27 and GITR, via modulation of the expression of their respective ligands CD70 and GITRL.

GITR ligation has been implicated in T cell costimulation and abrogation of the suppressive capacity of regulatory T cell in vivo[94, 95, 128-130]. To assess the effects of constitutive GITR ligation, we have generated mice in which GITRL was constitutively expressed on B cells (chapter 2). GITRL TG mice showed a specific increase in $CD4^+$ effector and regulatory type T cells due to enhanced proliferation of these T cells, whereas the naïve T cell compartment was not affected. In addition, GITR triggering did not affect the suppressive capacity of regulatory T cells. Thus, GITR ligation in vivo influences the maintenance $CD4^+$ effector and regulatory T cells via enhanced division. In chapter 3, we investigated the effects of GITR-GITRL signaling in humoral immunity. Terminal differentiation of splenic B cells remained unaffected in GITRL TG mice, whereas numbers of peritoneal B1 and B2 B cells were modulated. GITRL TG mice showed increased IgA serum immunoglobulin levels, correlating with an increase in numbers of peritoneal B2 B cells. Chapter 4 describes the indirect effects of CD27 ligation on T cells on innate immune responses. Although CD70 TG mice had a highly activated monocyte compartment, it was found that these monocytes showed a reduced response to sterile induced peritonitis and were protected against atherosclerosis in an atherosclerotic model. In chapter 5 we investigated the effects of CD70 co-stimulation by non-APC's. To this end, we generated CD70 TG mice which expressed CD70 on T cells. These CD70 TG mice showed increased numbers of $CD8^+$ effector T cells and enhanced CD8

T cell responses to influenza A infection. Due to increased activation induced cell death, $CD4^+$ T cell help was hampered resulting in decreased T and B cell memory responses. Chapter 6 describes the function of CD27 ligation on T cell polarization. We found that CD27 ligation promoted formation of IFNγ producing T_H1 polarized cells in T_H1 prone C57Bl/6J mice, but did not enhance helper T cell formation in T_H2 prone Balb/c mice. However, induction of allergic airway inflammation in CD70nTG Balb/c mice showed that CD27 signaling plays a supportive role in T_H1 differentiation, without modulating the classical T_H2 response. In conclusion, chapter 7 discusses how immunodeficiency's may occur through deregulated expression of TNFR and ligand superfamily members.

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Chapter 2

GITR triggering induces expansion of both effector and regulatory CD4$^+$ T cells in vivo.

Ronald W. van Olffen*, Nathalie Koning*,†, Klaas P.J.M. van Gisbergen*, Felix M. Wensveen*, Robert M. Hoek*, Louis Boon‡, Jörg Hamann*, René A.W. van Lier* and Martijn A. Nolte*.

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*Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands.

†Netherlands Institute for Neuroscience, an institute of the Royal Netherlands Academy for Arts and Sciences, Meibergdreef 47, 1105 BA Amsterdam, The Netherlands.

‡Bioceros BV, Yalelaan 46, 3584 CM Utrecht, The Netherlands.

Abstract

Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR)[4] is expressed on activated and regulatory T cells, but its role on these functionally opposing cell types is not fully understood. Here we describe that transgenic expression of GITR's unique ligand (GITRL) induces a prominent increase of both effector and regulatory $CD4^+$ T cells, but not $CD8^+$ T cells. Regulatory T cells from GITRL-transgenic mice are phenotypically activated and retain their suppressive capacity. The accumulation of effector and regulatory T cells is not due to enhanced differentiation of naïve T cells, but a direct result of increased proliferation. Functional consequences of increased numbers of both regulatory and effector T cells were tested in an autoimmune model and show that GITR stimulation is protective, as it significantly delays disease induction. These data indicate that GITR regulates the balance between regulatory and effector $CD4^+$ T cells by enhancing proliferation of both populations in parallel.

Introduction

Members of the TNF receptor (TNFR) superfamily are able to directly and indirectly affect the course of an immune response [1]. The glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is a member of this family and has been implicated in regulating both innate and adaptive immune responses [2, 3]. Regulatory T cells are well-known for their expression of GITR, though this receptor is also expressed on activated non-regulatory T cells, as well as B cells, monocytes and macrophages, dendritic cells and mast cells [4-8]. Its ligand (GITRL) can be found on a variety of cells, including dendritic cells, macrophages and B cells [9-11]. GITRL is transiently upregulated on these antigen presenting cells upon stimulation via the transcription factor NF-1 [9] and it most likely exerts its main function during inflammatory responses. Indeed, agonistic antibodies and (cells expressing) recombinant GITRL enhance T cell proliferation in vitro upon TCR triggering [5, 6, 9, 12, 13], which suggests that GITR acts as a costimulatory factor for T cells. GITR stimulation on regulatory T cells in coculture with effector T cells has been suggested to neutralize the suppressive capacity of regulatory T cells [5, 6, 9]. However, it was later shown that GITR ligation on regulatory T cells does not directly affect their suppressive capacity, but that GITR stimulation on non-regulatory T cells allows them to escape suppression by regulatory T cells [14]. GITR is not essential for regulatory T cell function, as regulatory T cells from $GITR^{-/-}$ mice display a normal capacity to suppress T cell proliferation in vitro [15, 16]. This leaves unanswered what the function of GITR is on regulatory T cells.

Studies using $GITR^{-/-}$ mice showed that the absence of GITR was protective in several disease models, which was attributed to an impaired effector function of T cells [17-19]. Correspondingly, studies that have directly addressed the function of GITR on T cells in vivo by deliberate stimulation of the receptor with agonistic antibodies conclude that GITR has a pro-inflammatory role within the immune system through its costimulatory effects on T cells

[6, 20-22]. However, these antibodies have their limitations when studying the impact of GITR stimulation in complex disease models, in particular since anti-GITR antibodies have been reported to cause depletion of regulatory T cells [23]. Moreover, recent studies on the crystal structure of GITRL have revealed that this ligand can exist in multiple oligomerization states that depend on binding to the receptor [24, 25], and it therefore remains to be addressed whether crosslinking GITR with agonistic antibodies exerts the same downstream effects as signaling induced by membrane-bound GITRL. Thus, in order to properly address the consequence of direct GITR stimulation on T cell function in vivo, we generated transgenic (TG) mice in which GITRL is constitutively expressed on B cells. Our findings demonstrate that GITR stimulation in vivo very effectively increases the absolute number of both effector and regulatory CD4$^+$ T cells through enhanced proliferation of both cell types. In agreement with increased regulatory T cell numbers, GITRL TG mice showed a marked delay in disease onset upon induction of experimental autoimmune encephalomyelitis (EAE), an experimental model for multiple sclerosis. We propose that GITR plays an important role in the regulation of both regulatory and effector CD4$^+$ T cell numbers in vivo by enhancing their turnover.

Results

Generation of B cell specific GITRL TG mice.

To study the function of GITR on T cells in vivo, we generated B cell specific GITRL TG mice by expressing GITRL cDNA under control of the human CD19 promoter (Fig. 1A).

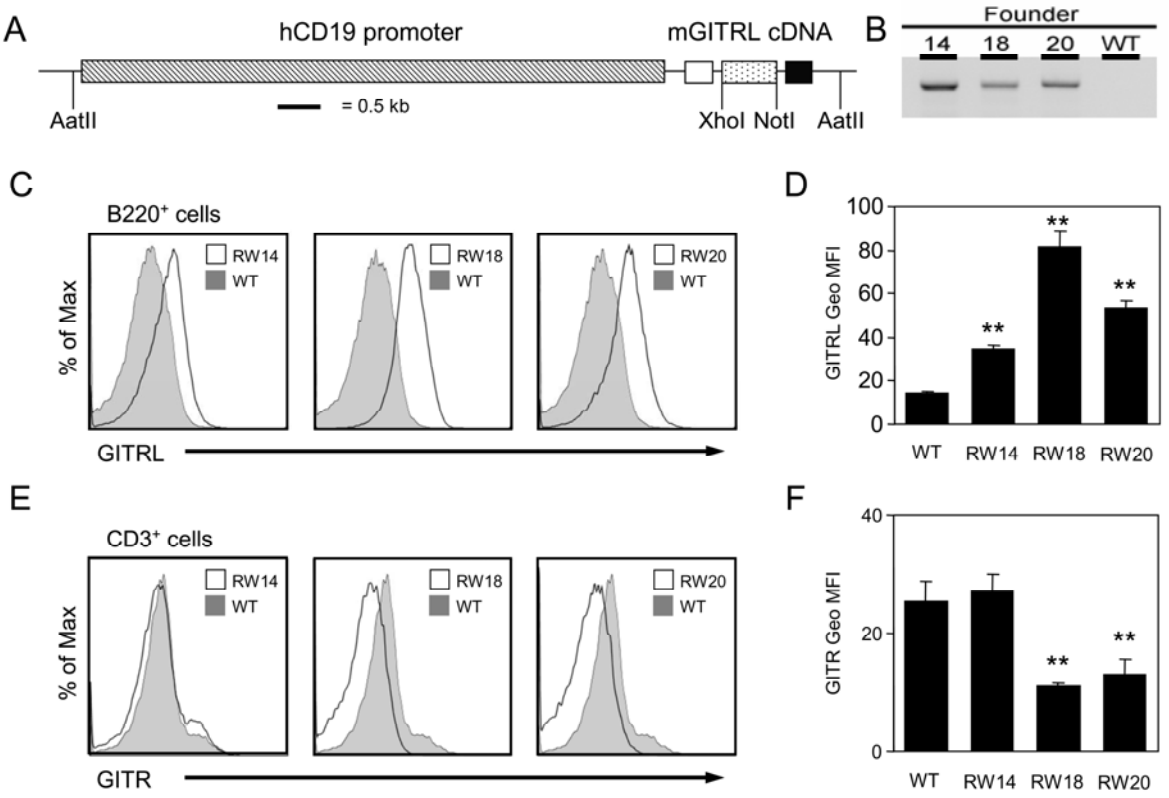

Figure 1. Generation of B cell specific GITRL TG mice. (A) Schematic representation of the hCD19-mGITRL DNA construct. The human CD19 promoter (hatched box) is followed by a chimeric intron (white box), mGITRL cDNA (dotted box) and a poly A tail (black box). (B) PCR analysis of genomic tail DNA from WT or GITRL TG mice (founder lines RW14, 18 and 20). (C) Representative staining for GITRL on splenic $B220^+$ cells from WT, RW14, RW18 and RW20 mice and (D) the expression of GITRL on splenic $B220^+$ B cells as the average geometric mean fluorescence intensity (geoMFI) ± SD for 3 mice per group. (E) Representative staining for GITR expression on splenic $CD3^+$ cells from WT, RW14, RW18 and RW20 mice and (F) the expression of GITR on $CD3^+$ T cells as the average geoMFI ± SD. Asterisks denote significant differences (** $p<0.005$).

Through microinjection of fertilized oocytes, we acquired three founder lines (RW14, RW18 and RW20), which were identified by genomic PCR analysis (Fig. 1B). GITRL TG mice were fertile, born at expected Mendelian frequencies and appeared as healthy as their littermate controls. Flow cytometry showed that GITRL was indeed significantly expressed on B cells in all founder lines, with highest expression on the RW18 line (Fig. 1C-D). As receptor shedding upon ligand stimulation is a hallmark of various TNFR-superfamily members [26-30], we determined the expression of GITR on T cells. T cells from GITRL TG mice showed decreased GITR expression compared to WT mice, which correlated with the level of GITRL expression (Fig. 1E-F). This indicates that GITR is indeed functionally engaged by its ligand in these mice. The data shown below are obtained from experiments with the GITRL TG RW18 line, though a similar, but less pronounced phenotype was also found in the other founder lines (data not shown).

GITRL TG mice have more CD4$^+$ effector memory-like and regulatory type T cells.

To establish the effects of GITR triggering in vivo, we analyzed the primary and secondary lymphoid organs of GITRL TG mice. T cell differentiation in the thymus of these mice was comparable to WT littermates (data not shown), as was cellularity of bone marrow, thymus, peripheral and mesenteric lymph nodes (Fig. 2A). However, spleens of GITRL TG mice contained significantly more leukocytes than WT mice (Fig. 2A). This increase was due to elevated numbers of CD4$^+$ T cells (Fig. 2B-C), whereas numbers of B cells and CD8$^+$ T cells were not significantly altered (Fig. 2B-C). Analysis of FoxP3 expression indicated that a substantial part of this increase in CD4$^+$ T cells could be attributed to an enlarged regulatory T cell compartment, as up to three times more CD4$^+$FoxP3$^+$ regulatory T cells were present in the spleens of GITRL TG mice (Fig. 2D-E). Phenotypic analysis of FoxP3$^-$CD4$^+$ T cells indicated that the non-regulatory fraction was also affected in GITRL TG mice, as

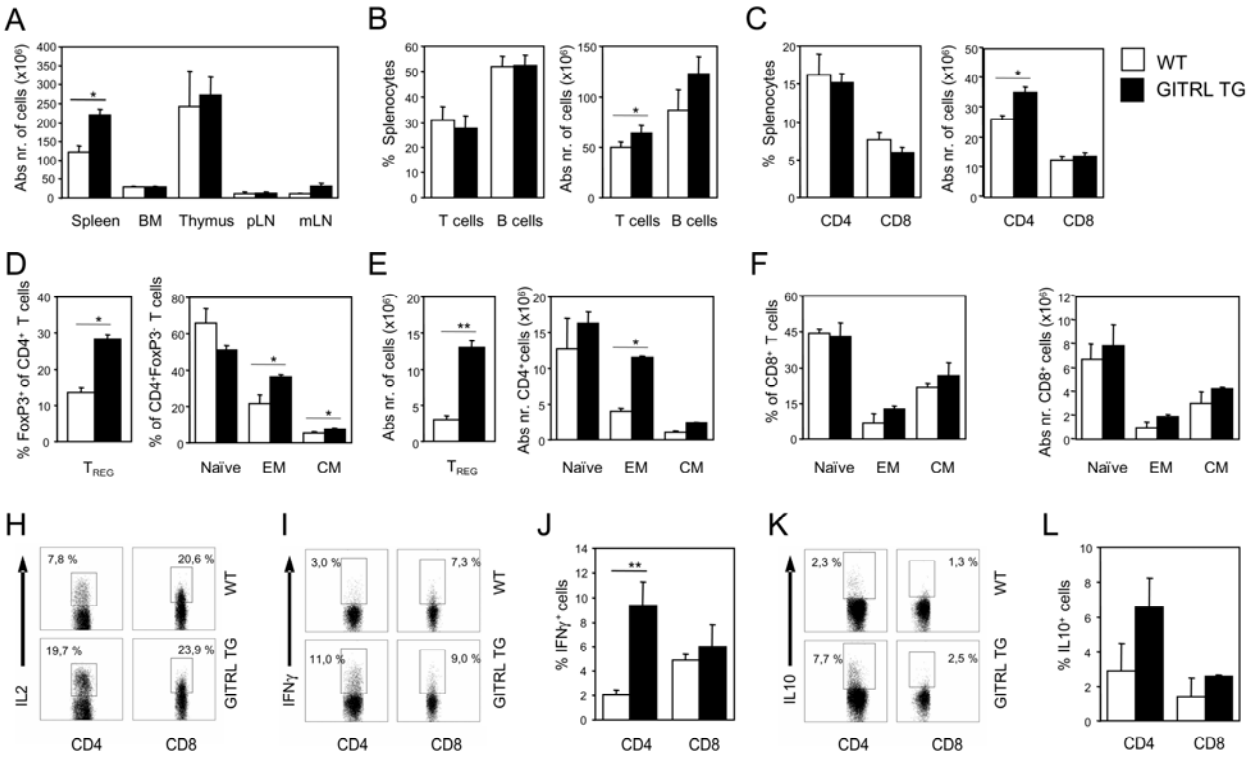

Figure 2. GITRL TG mice have more effector and regulatory type CD4$^+$ T cells.

(A) Absolute number of cells in spleen, bone marrow (BM), thymus, peripheral (pLN) and mesenteric (mLN) lymph nodes in 4-8 weeks old WT (white bar) and GITRL TG (black bar) mice. Percentage and absolute number of (B) T and B cells or (C) CD4$^+$ and CD8$^+$ T cells in the spleen of WT and GITRL TG mice. (D, E) Percentage and absolute number of splenic regulatory (FoxP3$^+$) and non-regulatory (FoxP3$^-$) CD4$^+$ T cells in WT and GITRL TG mice. Non-regulatory CD4$^+$ T cells were subdivided in naïve (CD44$^-$CD62L$^+$), effector memory (EM) (CD44$^+$CD62L$^-$) and central memory (CM) (CD44$^+$CD62L$^+$) cells. (F, G) Percentage and absolute number of naïve, EM and CM cells of splenic CD8$^+$ T cells. Production of (H) IL2, (I-J) IFNγ and (K-L) IL10 by CD4$^+$ or CD8$^+$ T cells in WT and GITRL TG mice after stimulation with PMA/ionomycin. Asterisks denote significant differences (* $p<0.05$; ** $p<0,005$). Data represent the average value ± SD of 3-5 mice and are representative for 2-4 independent experiments.

significantly more $CD4^+$ T cells with an effector memory-like ($CD44^+CD62L^-$) and central memory-like ($CD44^+CD62L^+$) phenotype were identified (Fig. 2D-E). This increase of $CD4^+$ T cells with either a regulatory or a memory-like phenotype in GITRL TG mice apparently did not develop at the cost of the naïve $CD4^+$ population, since absolute numbers of naïve $CD4^+$ T cells were comparable with WT littermates (Fig. 2E). No differences were found for $CD8^+$ T cells with respect to their naïve, effector memory-like and central memory phenotype (Fig. 2F-G). Corroborating the specific increase in $CD4^+$ effector T cells in GITRL TG mice, splenocyte stimulation with PMA-ionomycin showed increased production of the effector cytokines IL2 (Fig. 2H) and IFNγ (Fig. 2I-J) by $CD4^+$, but not $CD8^+$ T cells. Consistent with the increase in regulatory T cell numbers, we observed a trend towards more IL10-producing CD4 T cells, but this difference was not significant (Fig. 2K-L). These data thus indicate that GITR triggering in vivo enhanced the number of both regulatory and effector $CD4^+$ T cells.

Distribution and activation status of regulatory T cells in GITRL TG mice.

To determine whether the strong increase of regulatory T cells in GITRL TG mice was restricted to the spleen, we analyzed the presence of these cells in bone marrow, thymus, peripheral and mesenteric lymph nodes and liver in these mice. We found that GITRL TG mice have a systemic increase in regulatory T cell numbers, as all analyzed compartments, except for the bone marrow, showed a significantly higher fraction of $FoxP3^+$ $CD4^+$ cells compared to WT mice (Fig 3A).

Next, we analyzed the activation status of splenic regulatory and non-regulatory T cells. Apart from the described changes in CD44 and CD62L expression (see Fig. 2D-E), non-regulatory $CD4^+$ T cells in GITRL TG mice were comparable with their WT counterparts on the basis of several other costimulatory and activation molecules (Fig 3B). On the other hand, we found that $CD4^+FoxP3^+$ regulatory T cells from GITRL TG mice consistently expressed lower

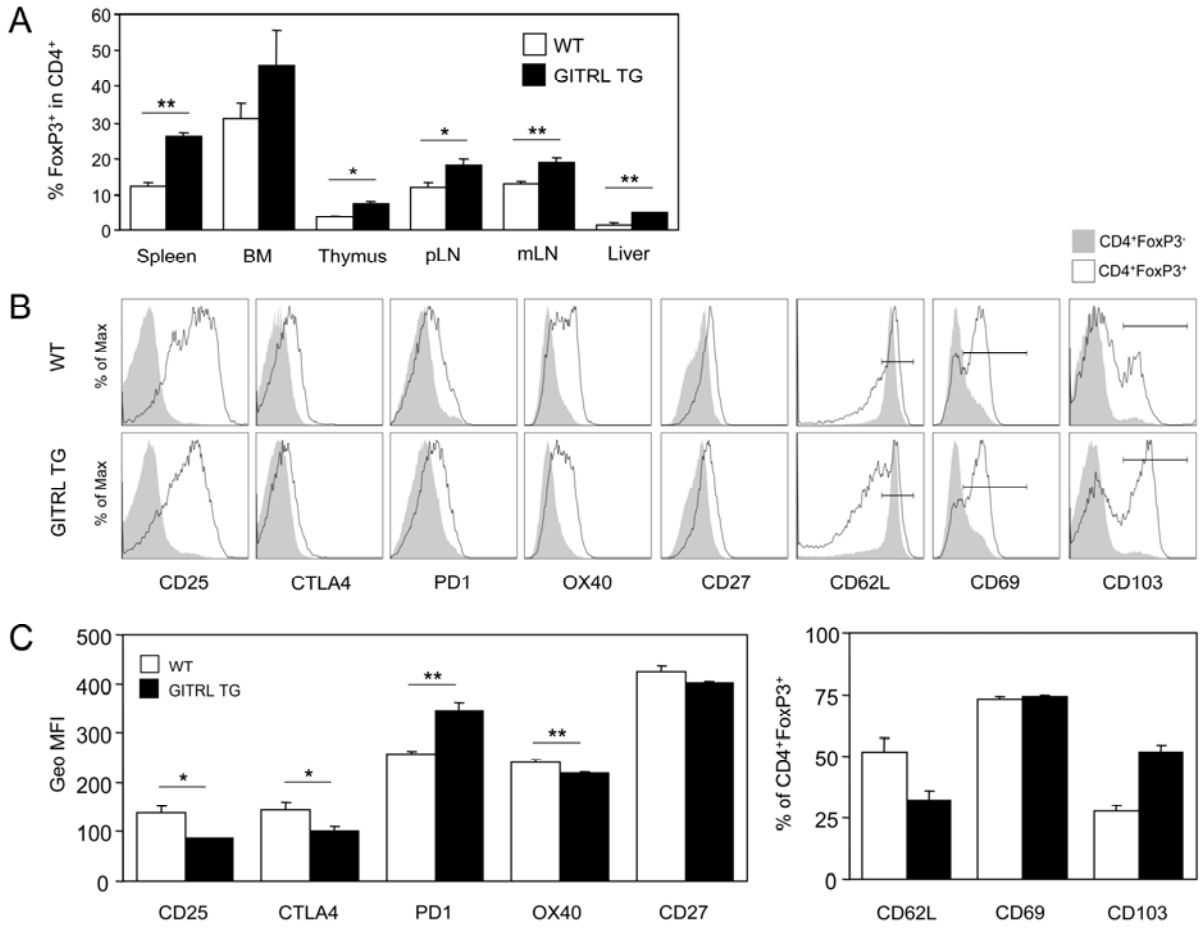

Figure 3. Systemic increase of regulatory T cells via GITR signaling.

(A) The percentage of FoxP3$^+$ cells within the CD4$^+$ T cell compartment in spleen, bone marrow (BM), thymus, peripheral (pLN) and mesenteric (mLN) lymph nodes and liver in WT and GITRL TG mice. (B) Phenotypic analysis of splenic FoxP3$^-$ (filled graph) and FoxP3$^+$ (open graph) CD4$^+$ T cells for surface expression of CD25, CTLA4, PD1, OX40, CD27, CD62L, CD69 and CD103 in WT and GITRL TG mice. (C) Average intensity (geoMFI ± SD) or percentage positive cells (% ± SD) of these molecules on FoxP3$^+$ CD4$^+$ T cells. Asterisks denote significant differences (* $p<0.05$, ** $p<0.005$). Data are representative for 2 independent experiments with each at least 3 mice per group.

levels of CD25, CD62L and CTLA4 compared to their counterparts in WT mice (Fig 3B-C). In addition, the expression of PD1 was increased in GITRL TG mice, while a large fraction of regulatory T cells from GITRL TG mice expressed the adhesion molecule CD103 (α_E integrin) on their surface (Fig 3B-C). The expression of OX40, CD27 and CD69 was

comparable to WT mice (Fig 3B-C). As FoxP3$^+$ regulatory T cells can be divided in two functionally distinct subsets, namely naïve (CD62L$^+$ CD103$^-$) and effector regulatory T cells (CD62L$^-$ CD103$^+$) [31], we conclude that constitutive GITR stimulation not only leads to more regulatory T cells, but specifically stimulates the formation of regulatory T cells with an effector phenotype.

GITR engagement in vivo does not affect the suppressive capacity of regulatory T cells.

To determine if the altered phenotype of regulatory T cells in GITRL TG mice mirrored a change in their function, we performed in vitro proliferation assays, in which WT responder T cells (CD4$^+$CD25-) were stimulated with anti-CD3/CD28 in the presence of increasing numbers of regulatory T cells (CD4$^+$CD25$^+$) from WT or GITRL TG mice. From these experiments it can be concluded that regulatory T cells from GITRL TG are fully capable to suppress responder T cell proliferation and were equally anergic as regulatory T cells from WT mice (Fig. 4A). This conclusion challenges previous reports, which have suggested that GITR stimulation on regulatory T cells is sufficient to abrogate their suppressive capacity [5, 6].

We also analyzed the susceptibility of GITRL TG vs WT derived responder T cells to the suppressive capacity of WT regulatory T cells, as it has been reported that GITR stimulation allows T cells to escape suppression by regulatory T cells [14]. These experiments revealed that responder T cells from GITRL TG mice could still be adequately suppressed by regulatory T cells (Fig. 4B), thereby indicating that chronic GITR stimulation in vivo is not sufficient to induce an enduring state of insensitivity to regulatory T cell activity. Instead, it rather suggests that this previously described capacity of GITR is only effective when given together with TCR stimulation [14].

To establish whether regulatory T cells in GITRL TG mice are indeed functionally active in vivo, we examined several organs of 12 months old GITRL TG mice for cellular infiltrates as

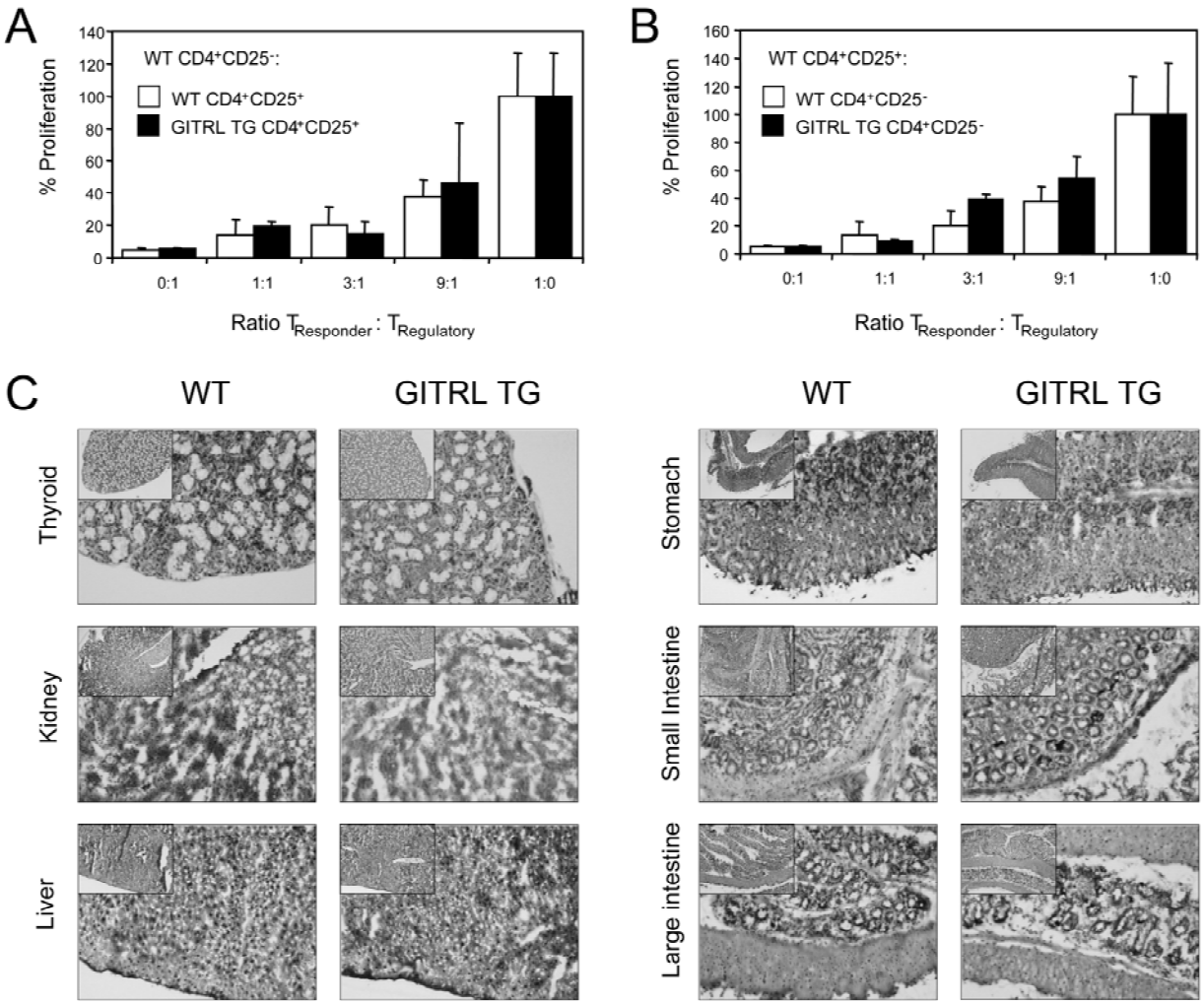

Figure 4. Regulatory T cell function of WT and GITRL TG mice.

(A) Ability of purified WT and GITRL TG regulatory ($CD4^+CD25^+$) T cells to suppress WT responder ($CD4^+CD25^-$) T cells. (B) Ability of WT regulatory T cells to suppress proliferation of WT and GITRL TG responder T cells. Cells were cultured at different ratios for 4 days with 10 μg/ml soluble anti-CD3 mAb in the presence of irradiated WT splenocytes as APCs; for the final 16 hours [3H]thymidine was added and incorporation was measured. Data are depicted as the percentage proliferation compared to responder T cells alone (average of triplicate wells ± SD) and are representative of 2 independent experiments. (C) Wright Giemsa staining of cryo-sections from thyroid gland, kidney, liver, stomach, and small and large intestine of 12 month old WT and GITRL TG mice. Data are representative for 2 mice per group.

a sign of organ inflammation and autoimmunity. Loss of function of regulatory T cells has been associated with increased numbers of autoreactive T cells, which can induce several forms of autoimmunity, including thyroiditis, glomerulonephritis, gastritis and inflammatory bowel disease [32-34]. But despite the strong increase of effector CD4 T cells observed in lymphoid organs of GITRL TG mice (Fig. 2), we did not find any sign of inflammation or cellular infiltration in either the thyroid gland, kidney, liver, stomach or intestines of these mice (Fig. 4C). This indicates that GITR stimulation does not impair the function of regulatory T cells in vivo and considering the increase of effector T cells, this suggests that the regulatory T cells in GITRL TG mice are rather competent in preserving the homeostasis within this more active immune system.

GITR costimulation enhances CD4$^+$ T cell proliferation and IL2 production

To investigate whether the increase in effector and regulatory type T cells could be explained by an increased survival potential mediated through GITR signaling, we examined the expression profile of approximately 40 pro- and anti-apoptotic proteins using an advanced PCR approach called multiplex ligation-dependent amplification (MLPA [35]). However, this comprehensive analysis did not reveal any significant differences in the apoptotic gene expression profile of naïve, effector and regulatory CD4 T cells isolated from GITRL TG mice compared to WT mice (data not shown).

Next, we set out to determine the effects of GITR triggering on T cell proliferation. CFSE labeled T cells from WT mice were stimulated with suboptimal anti-CD3 in a 1:1 ratio with WT or GITRL TG irradiated B cells for a period of 3 days. We found that increased GITRL availability enhanced CD4$^+$ T cell proliferation, but did not affect CD8$^+$ T cell proliferation (Fig. 5A). The enhanced proliferation of CD4$^+$ T cells via GITR engagement was no longer apparent when extra IL2 was added to these cultures, indicating that GITR engagement

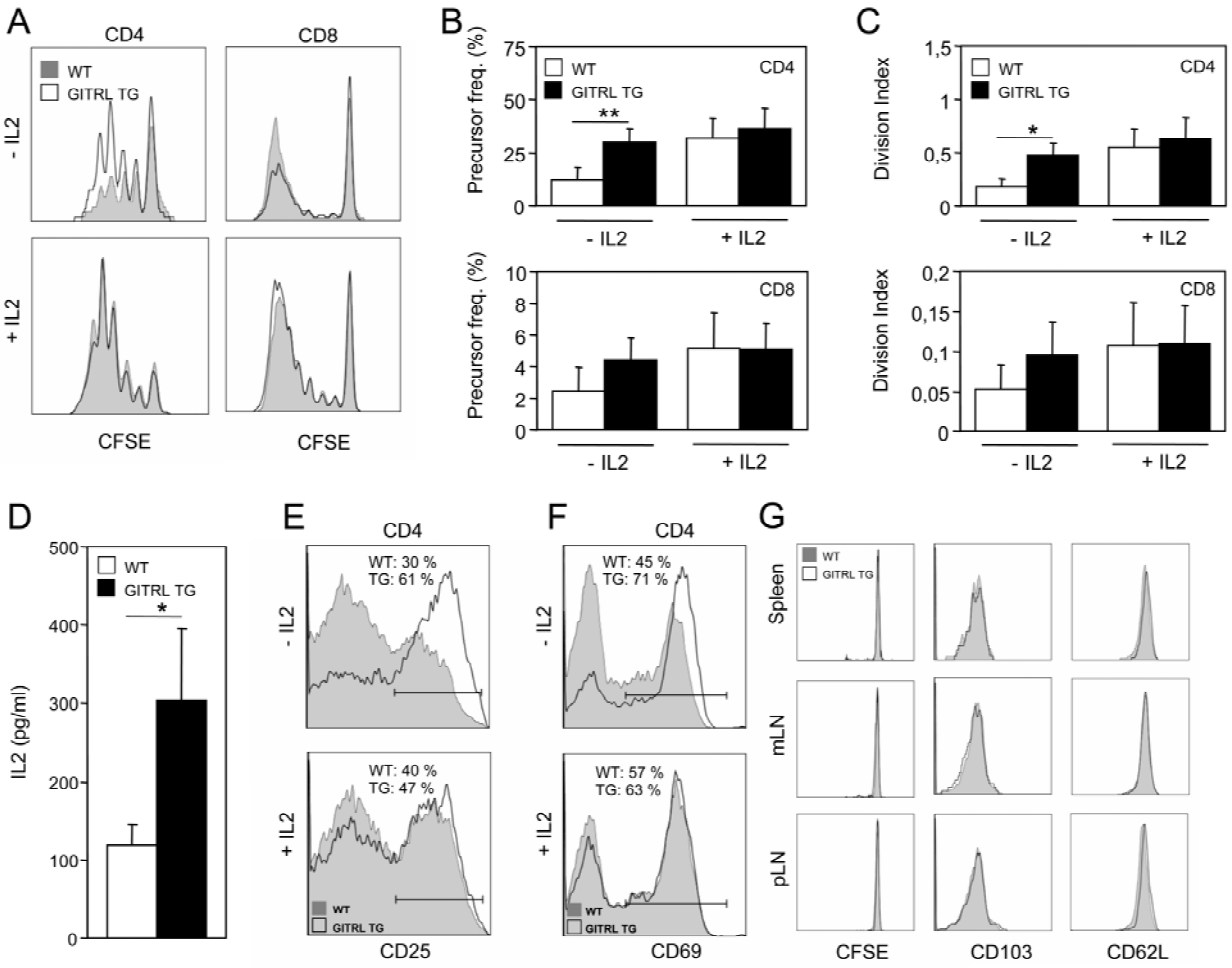

Figure 5. GITR costimulation in vitro enhances CD4$^+$ T cell proliferation and IL2 production.

(A) Anti-CD3-induced proliferation of CFSE-labelled WT T cells cultured for 3 days in the presence of WT (filled graph) or GITRL TG (open graph) B cells with or without IL2. Triplicate wells analyzed for (B) the average precursor frequency and (C) the average division index (i.e. the number of divisions that the dividing population underwent). (D) IL2 concentration in supernatants (average of triplicate wells ± SD) after stimulating WT T cells as in (A) for 24 hours in the presence of a blocking antibody against CD25 to prevent IL2 consumption. Expression of CD25 (E) and CD69 (F) on CD4$^+$ T cells stimulated as in (A) after 24 hours. (G) Naïve WT CD4$^+$CD25$^-$ T cells from Ly5.1 mice were CFSE-labeled and injected intravenously in WT (filled graph) or GITRL TG (open graph) mice. Donor cells, gated on CD45.1$^+$CD4$^+$ T cells, were analyzed 3 days after transfer for expression of CFSE, CD103 and CD62L expression. A representative staining from 3 mice per group is shown. Asterisks denote significant differences (* $p<0.05$, ** $p<0.005$).

affects the early proliferative capacity of $CD4^+$ T cells. We found that increased GITR ligation raised the percentage of $CD4^+$ T cells entering cell division (Fig. 5B), as well as the number of divisions that these cells underwent (Fig. 5C). Since the addition of IL2 enhanced T cell proliferation to a similar extent as the addition of GITRL TG B cells, we questioned whether GITR stimulation induced IL2 production. Indeed, when WT T cells were stimulated with anti-CD3, the addition of GITRL TG B cells induced almost 3-fold more IL2 than WT B cells (Fig. 5D). Moreover, GITR ligation increased the expression of CD25 and CD69 on $CD4^+$ T cells, confirming the enhanced IL2 production and increased activation induced by GITR stimulation (Fig 5E-F).

To determine if constitutive GITR triggering alone was sufficient to induce activation and/or proliferation of naïve T cells in vivo, we isolated naïve non-regulatory $CD4^+CD25^-$ T cells from $Ly5.1^+$ WT donor mice, labeled them with CFSE and transferred them into WT or GITRL TG ($Ly5.2^+$) recipients. Three days after transfer, naïve T cells transferred to both WT and GITRL TG mice showed no CFSE dilution and did not alter their expression levels of CD62L or CD103 (Fig. 5G). Thus, despite the fact that GITRL TG mice contained more T cells with an effector memory-like phenotype, these data imply that stimulation through GITR alone is not sufficient to induce activation or proliferation of naïve T cells. Yet, when TCR-triggering is provided, GITR stimulation enhanced the production of IL2 and increased proliferation of $CD4^+$ T cells in vitro.

GITR engagement in vivo increases the proliferation of effector and regulatory $CD4^+$ T cells.

As GITR engagement could directly and specifically enhance $CD4^+$ T cell proliferation, we investigated the proliferative capacity of $CD4^+$ T cells in WT and GITRL TG mice in vivo. As measured by Ki-67 expression, GITRL TG mice had more non-regulatory T cells

($CD4^+FoxP3^-$) in cell cycle in the spleen than WT mice (24% ± 2.2 in GITRL TG mice vs 14% ± 1.2 in WT mice) (Fig 6A-B). The fraction of regulatory T cells ($CD4^+FoxP3^+$) that stained positive for Ki-67 was not significantly different between WT and GITRL TG mice, but the fraction of Ki-67^+ cells within the regulatory T cell compartment is already high (~30%) in WT mice (Fig. 6A-B).

To directly assess T cell proliferation in vivo, WT and GITRL TG mice were injected i.p. with BrdU and incorporation of this compound was analyzed 16 hours later (Fig 6C-D). Within the non-regulatory $CD4^+$ T cell compartment, we found that GITRL TG mice contained more $BrdU^+$ cells than WT mice and this increase was most pronounced in the $CD62L^-$ effector fraction (Fig. 6C-E). A GITR-mediated increase in proliferation was even more profound for regulatory T cells, as the percentage of $CD4^+FoxP3^+$ T cells that had incorporated BrdU had more than doubled compared with WT littermates (Fig 6C-D). In this case, it was the $CD62L^+$ $CD103^-$ fraction of regulatory T cells that showed the most profound increase in BrdU incorporation (Fig. 6E-F). No effect of GITRL overexpression on BrdU incorporation was found in $CD8^+$ T cells (data not shown). Overall, these data indicate that GITR affects the numbers of regulatory T cells as well as the memory/effector pool of non-regulatory $CD4^+$ T cells in vivo by regulating their proliferation.

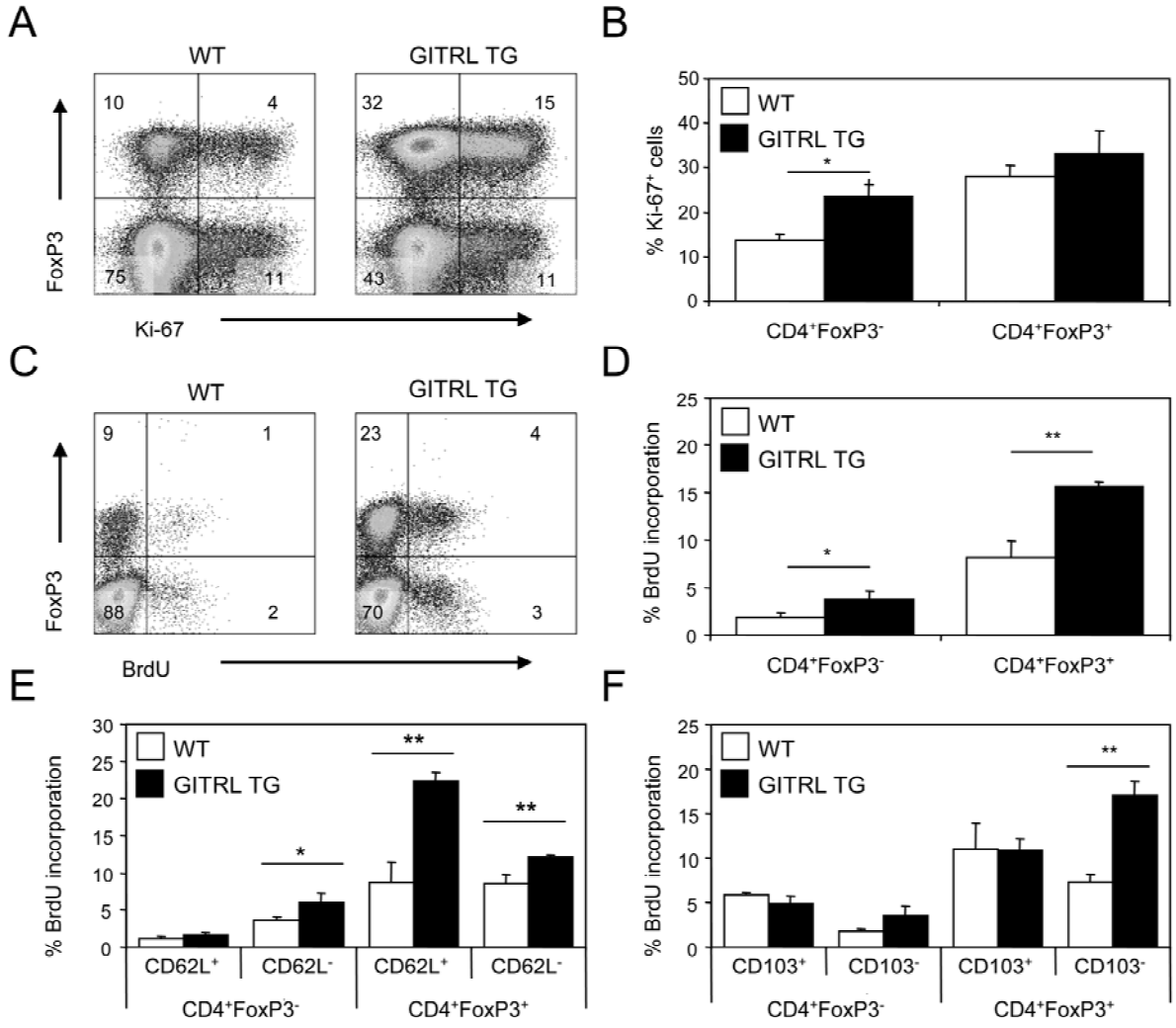

Figure 6. GITR triggering enhances proliferation of effector and regulatory T cells in vivo.

(A) Representative intracellular staining for FoxP3 and Ki-67 on splenic CD4+ T cells from WT and GITRL TG mice and (B) the percentage Ki-67$^+$ cells of FoxP3$^-$ and FoxP3$^+$ CD4$^+$ T cells (average ± SD). (C) Representative intracellular staining for BrdU and FoxP3 on splenic CD4+ T cells from WT and GITRL TG mice, 16 hours after i.p. injection of 1 mg BrdU. (D) The percentage BrdU$^+$ cells of FoxP3$^-$ and FoxP3$^+$ CD4$^+$ T cells (average ± SD). Characterization of proliferating FoxP3$^-$ and FoxP3$^+$ CD4$^+$ T cells based on CD62L (E) or CD103 (F) expression. The percentage of BrdU$^+$ cells in each fraction is depicted for WT (white bar) and GITRL TG (black bar) mice (average ± SD). Data are representative for 2 independent experiments with each at least 3 mice per group. Asterisks denote significant differences (* $p<0.05$, ** $p<0.005$).

Enhanced GITR ligation delays experimental autoimmune encephalomyelitis.

To examine the significance of the expansion of both regulatory and effector $CD4^+$ T cells on a complex immune response in vivo, GITRL TG mice were subjected to EAE, an experimental model for multiple sclerosis. We selected this model as it induces autoimmunity by selective depletion and loss of function of regulatory T cells through treatment of immunized mice with pertussis toxin, which probably facilitates autoreactive T cells to develop and cause nerve damage in the central nervous system [36, 37]. It is therefore conceivable that GITRL TG mice are protected from disease (i.e. limb paralysis), because they have more regulatory T cells, but it could also be that they display enhanced susceptibility to EAE compared to WT mice, because of their increased effector T cell formation. We found that regulatory mechanisms dominated the EAE response in GITRL TG mice, as they had a significant delay in disease onset compared to WT mice, which correlated with a lower clinical score (Fig. 7A-B). Although disease was delayed in GITRL TG mice, it was not inhibited, as the cumulative incidence and clinical score were similar between both groups at the experimental endpoint (Fig. 7A-B), suggesting that autoreactive cells did develop in these mice.

To obtain more insight in the mechanism of this difference in disease progression, we examined the CD4 T cell response in draining lymph nodes from WT and GITRL TG mice early after immunization. After 5 days, the impact of the immunization was readily visible in both mouse strains, as the draining (inguinal and lumbar), but not non-draining (axillary and brachial) lymph nodes, had considerably increased in size (data not shown). In contrast to what might be expected from the delay in disease progression, we found that the development of effector CD4 T cells was not perturbed in GITRL TG mice, as they contained even more effector CD4 T cells than WT mice (Figure 7C). Absolute numbers of regulatory CD4 T cells were also increased in GITRL TG mice (Figure 7C). Restimulation of lymph node cells with

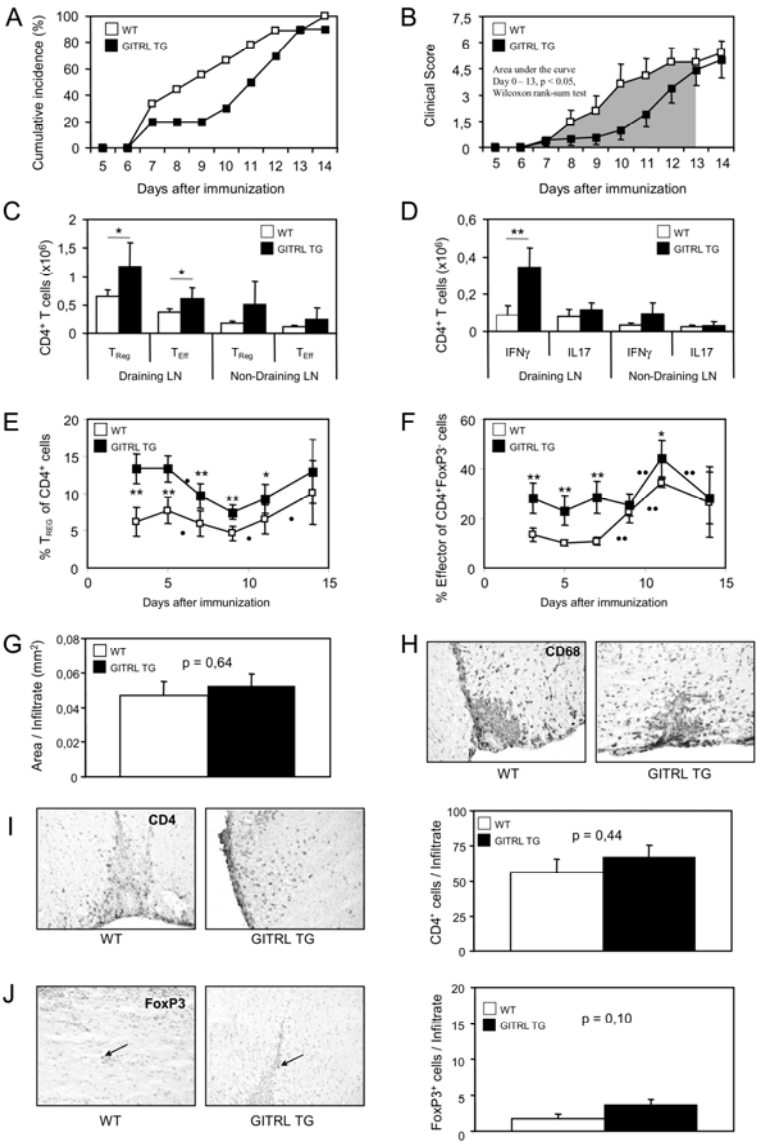

Figure 7. Delayed EAE induction though GITR ligation.

(A) The cumulative incidence and (B) the average clinical score following EAE induction in WT (□) and GITRL TG mice (■). Experiment depicted contains 10 animals per group and is representative of 2 independent experiments. Significant differences ($p<0.05$) of the area under the curve were determined by Wilcoxon rank sum test (highlighted area). The absolute number of (C) CD4$^+$ T cells and (D) IFNγ and IL17 producing CD4$^+$ T cells on day 5 following EAE induction in the draining (inguinal and lumbar) and non-draining (axillary and brachial) lymph nodes from WT and GITRL TG mice. (E) Percentage regulatory (FoxP3$^+$) and (F) non-regulatory effector (FoxP3$^-$CD62L$^-$) T cells within the CD4$^+$ compartment was determined in peripheral blood following immunization (day 0) and pertussis toxin injection (day 0 and 2) of WT (□) and GITRL TG (■) mice. Data represent the average ± SD of 4 mice. Asterisks denote significant differences between WT and GITRL TG mice on a particular day (* $p<0.05$, ** $p<0.005$). Black dots denote significant differences between consecutive days for either group (• $p<0.05$, •• <0.005). Cellular infiltrates in brain and spinal cord were analyzed for mice with a clinical score of five and in which disease onset occurred four to five days prior to experimental endpoint. (G) Size of cellular infiltrates in the spinal cord of WT and GITRL TG mice. Representative immunohistochemical staining and quantification of the spinal cord of WT and GITRL TG mice shows the presence of (H) CD68$^+$ macrophages, (I) CD4$^+$ T cells and (J) FoxP3$^+$ regulatory T cells on a heamatoxylin background staining (20x magnification). Arrow indicates a single FoxP3$^+$ cell. For quantification purposes, at least 6 infiltrates were analyzed per staining per mouse.

PMA-ionomycin corroborated an increase in effector T cells, as GITRL TG mice contained more IFNγ producing cells, whereas IL17 production was not affected (Figure 7D). Thus, GITR stimulation does not negatively affect the formation of autoreactive T cells following EAE induction, but, if anything, rather enhances it.

To further investigate the relative contribution of regulatory and effector $CD4^+$ T cells in disease development in these mice, we examined the levels of both cell types in circulation following EAE induction. During the first 9 days, the fraction of regulatory $CD4^+$ T cells decreased in both groups of mice, but GITRL TG mice continuously displayed more regulatory T cells in circulation than WT mice (Fig. 7E). Both GITRL TG and WT mice showed a disease-related increase of effector type $CD4^+$ T cells in peripheral blood, but this occurred later in GITRL TG than in WT mice, which is interesting as it correlates with the observed delay in disease development (Fig. 7F). At 14 days after immunization, when both mouse strains had a comparable clinical score, there were no significant differences in effector or regulatory CD4 T cells in the blood of GITRL mice compared to WT mice.

Finally, to investigate whether the observed differences in EAE signified merely a difference in time or also in quality of the immune response, we analyzed the cellular infiltrates in the central nervous system of mice with a similar clinical score. The infiltrated areas were comparable in size between GITRL TG and WT mice (Fig. 7G). Moreover, these mice showed a comparable influx of macrophages (Fig. 7H) and $CD4^+$ T cells (Fig. 7I) in the central nervous system and the influx of $FoxP3^+$ cells was very low in both groups (Fig. 7J). Therefore, these data suggest that GITR engagement does not change the quality of this autoimmune response, but rather delays disease development. This is not due to a decrease in the formation of disease-related effector type $CD4^+$ T cells, but might be related to an altered recirculation of these cells.

Discussion

Since its discovery in 1997, GITR has been the focus of many studies that address its biological function in cellular immunology [5, 6, 9, 38, 39]. These studies have indicated that GITR has costimulatory effects during T cell activation, but it is still not fully understood at what level GITR triggering affects both T cell activation and regulatory T cell function and how this influences immune responses in vivo. Here we describe that in vivo GITR stimulation through its natural ligand increased absolute numbers of both effector and regulatory type CD4$^+$ T cells. Detailed analysis revealed that this accumulation was a direct consequence of enhanced proliferation of both cell types in GITRL TG mice. The increase of effector and regulatory CD4$^+$ T cells was not at the expense of the naïve CD4$^+$ T cell pool. Together with the finding that transferred naïve WT T cells do not get activated in GITRL TG mice (Fig. 5G), this indicates that enhanced GITR triggering is not sufficient to activate naïve T cells, but that this process is still fully dependent on TCR activation. When TCR triggering is provided, GITR stimulation does enhance the expansion of newly activated CD4$^+$ T cells, as can be concluded from the in vitro stimulation (Fig. 5A-C) and EAE immunization experiments (Fig. 7C). However, the BrdU incorporation experiments indicate that constitutive GITR triggering also enhances proliferation of effector and regulatory CD4$^+$ T cells during the steady state situation (Figure 6E). To what extent the TCR is also required for this increased level of homeostatic proliferation in GITRL TG mice is not yet clear, as this expansion might also be driven by increased availability of cytokines like IL2. Transfer experiments with TCR-transgenic T cells could shed further light on this issue.

GITR ligation in vivo does not affect the anergic state of regulatory T cells in vitro, nor does it influence the suppressive function of regulatory T cells (Fig. 4A). The fact that GITRL TG mice do not develop any sign of organ inflammation or autoimmunity (Fig. 4C), despite the expansion of effector T cells, supports this notion and indicates that regulatory T cells are

fully functional in vivo in these mice and maintain homeostasis. The hypothesis that GITR regulates the size, but not the function of the regulatory T cell pool is supported by the observation that GITR$^{-/-}$ mice have normally functioning regulatory T cells, but fewer absolute numbers [14, 40]. In vitro studies have shown that agonistic anti-GITR antibodies can induce proliferation of regulatory T cells in vitro in an IL2-dependent manner, also without affecting their suppressive activity [5, 14]. We found that GITRL expression on B cells increased IL2 production by CD4$^+$ T cells in vivo (Fig. 2H) and in vitro (Fig. 5A.), which is most likely a direct effect, as GITR crosslinking with antibodies can induce IL2 production through TRAF-5 mediated NF-κB activation [41]. These results have two important implications for our understanding of the biological function of GITR on T cells. First, since regulatory T cells depend on exogenous IL2 for their proliferation [42], these findings indicate that GITR drives proliferation of both regulatory and effector T cells through the induction of IL2 from the latter. Second, it explains why GITR stimulation on non-regulatory T cells allows them to escape suppression by regulatory T cells [14], since it was recently shown that regulatory CD4$^+$ T cells exert their suppressive function through consumption of IL2 produced by activated T cells, leading to apoptosis of the latter [43]. Since GITR triggering increases the production of IL2, non-regulatory T cells can thereby escape from or delay cytokine deprivation-induced apoptosis. These implications fit in a previously postulated model for GITR function [44], in which it was also suggested that when GITRL expression decreases at the end of an immune response, this would render effector T cells susceptible to suppression by an expanded, activated regulatory T cell pool. Transgenic GITRL expression does not allow us to test this hypothesis in our system, but it is worth following up this idea, as it implies that GITR is indirectly involved in termination of a T cell response.

Detailed analysis revealed that GITR ligation in vivo modified the expression of several key proteins expressed by regulatory T cells (Fig. 3). We found that the IL2 receptor is

downregulated on regulatory T cells of GITRL TG mice, which is most likely a direct consequence of increased IL2 consumption driving enhanced proliferation [45]. This is in agreement with recent findings that homeostatically proliferating regulatory $CD4^+$ T cells in vivo express lower levels of the IL2 receptor than non-proliferating cells [46]. Furthermore, GITRL TG mice contained more regulatory T cells with an activated phenotype, expressing low levels of CD62L and high levels of CD103 (Fig. 3) [47]. This is interesting, because we found that BrdU predominantly incorporated in the $CD62L^+$ and $CD103^-$ population of regulatory T cells in GITRL TG mice (Fig. 6). This would thus indicate that GITR ligation induces proliferation of $CD62L^+CD103^-$ regulatory T cells and that during this proliferation they become activated and accumulate as $CD62L^-CD103^+$ regulatory T cells. This would be in agreement with an earlier study, which described that regulatory T cells with a high turnover downmodulate CD62L after several cell divisions [48]. Since CD62L is required for HEV-dependent lymphocyte entry into lymph nodes and CD103 is an integrin necessary for the homing and retention of cells at inflammatory sites, these data suggest that regulatory T cells in GITRL TG mice are more prone to enter (inflamed) peripheral tissues than secondary lymphoid organs compared to their WT counterparts. Indeed, we found that liver and bone marrow of GITRL TG mice accumulate more $CD62L^-CD103^+$ regulatory T cells than WT mice (data not shown), but since the supply of regulatory T cells is also increased in these mice, it requires more specific migration experiments to adequately address this issue.

An intriguing finding from our analysis of GITRL TG mice is that the functional consequences of GITR engagement were restricted to $CD4^+$ T cells, as no effects on the proliferation or effector cell formation of $CD8^+$ T cells could be detected, neither in vitro nor in vivo (Fig. 2, 5A-C and data not shown). This is in contrast with other studies in which a role for GITR on $CD8^+$ T cell responses was demonstrated, using agonistic GITR antibodies or $GITR^{-/-}$ mice [49-51]. We found that both $CD4^+$ and $CD8^+$ T cells in GITRL TG mice had

downmodulated surface expression of GITR compared to WT mice (Fig. 1 and data not shown), which indicates that GITR was functionally engaged by its ligand on both cell types. In WT mice, GITR expression is higher on $CD4^+$ non-regulatory T cells than on $CD8^+$ T cells [52] (and data not shown), which could be the reason why GITRL expression has a stronger effect on $CD4^+$ T cells than $CD8^+$ T cells. This might also relate to the finding that the costimulatory effect of GITR crosslinking with an anti-GITR antibody is apparent at a lower anti-CD3 concentration in $CD4^+$ T cells than in $CD8^+$ T cells [14]. Moreover, GITR upregulation following T cell activation is dependent on CD28 engagement in $CD4^+$, but not $CD8^+$ T cells [14, 53, 54]. Thus, although GITR functions on both $CD4^+$ and $CD8^+$ T cells, it is differently regulated in these subsets. In our hands, deliberate triggering of GITR on $CD8^+$ T cells in vivo by its natural ligand clearly does not translate into functional consequences, or at least not as strong as the effects found on $CD4^+$ T cells.

The synchronized expansion of regulatory and effector $CD4^+$ T cells that is induced upon GITR stimulation might seem contradictory for protective immunity, as these cell types obviously have opposite functions. However, recent in vivo studies have shown that regulatory T cells expand with similar kinetics as effector $CD4^+$ T cells upon HSV-2 infection [55] or immunization with Freund's complete adjuvants [56], so that their ratio remains relatively constant. Coincident expansion of regulatory and effector T cells could be a direct consequence of responsiveness of regulatory T cells to IL2 produced by effector T cells [57] and our data suggest that GITR could play a role in this process. It is most likely that the simultaneous increase of regulatory and effector T cells is the reason why GITRL overexpression induces a mild phenotype compared to transgenic overexpression of other members of the TNF-superfamily, such as CD70, OX40L, 4-1BBL and LIGHT, which leads to severe immunopathology induced by effector T cells [26, 58-62]. The clinical consequence of an immune response might even depend on this ratio of effector vs. regulatory T cells, as the

experimental induction of both adjuvant arthritis and type 1 diabetes correlates with an increase of this ratio [63-65]. The same might apply for the EAE model, as depletion of regulatory T cells resulted in enhanced disease progression and severity [66]. We found that the delay in disease induction observed in GITRL TG mice was not due to a inhibition in the formation of effector CD4$^+$ T cells in the draining lymph nodes (Fig. 7C), but rather correlated with a delay in the increase of effector CD4$^+$ T cells in circulation (Fig. 7E-F). As no differences were observed in final disease severity nor cellular infiltrates in the brain parenchyma, these observations suggest that GITR stimulation enhances both formation of effector and regulatory CD4$^+$ T cells in lymph nodes and might delay autoimmunity by regulating emigration of effector CD4$^+$ T cells from the lymph nodes. Whether GITR triggering has a direct effect on the egress of activated T cells from lymph nodes, or that this is an indirect effect mediated by regulatory T cells awaits further investigation.

In conclusion, we have shown that GITR serves as a costimulatory molecule in that it induces proliferation of regulatory as well as effector CD4$^+$ T cells in vivo. We suggest that upregulation of GITRL on antigen presenting cells during the initiation of an immune response, through the increase of pro-inflammatory stimuli, enhances IL2 production and thereby the proliferation of cognate CD4$^+$ T cells, which also makes them less susceptible to suppression by regulatory T cells. At the same time, GITRL expression during this early phase induces the expansion of regulatory T cells, aided by the presence of exogenous IL2 from proliferating non-regulatory T cells. These regulatory T cells might be important to re-establish the status quo of the immune system at later stages of the response.

Materials and Methods

Generation of GITRL TG mice.

cDNA encoding murine GITRL was obtained via PCR on total splenic cDNA and cloned into the pGEM-T plasmid (Promega). This construct was digested with NotI and XhoI to obtain a 600 bp fragment containing the mGITRL cDNA, which was cloned into the NotI – XhoI site of a CD19-pC3 plasmid (kindly provided by Patrick Derksen, Academic Medical Center, The Netherlands), resulting in the GITRL expression construct under control of the human CD19 promoter. This construct was linearized via AatII digestion (see Fig. 1A) and microinjected into pronuclei of C57BL/6 fertilized oocytes and implanted into pseudopregnant female C57BL/6 mice. Transgenic founders were identified by PCR analysis of tail or ear DNA, using the following PCR primers: pC3s1 (5'-GCAGTGACTCTC TTAAGGTAGCC-3') and mGITRL4a (5'CTTGAGTGAAGTATAGATCAGTGTA-3'). Three GITRL TG founder lines (RW14, RW18, RW20) were propagated by mating with wild type (WT) C57BL/6 mice and offspring were tested for the presence of the transgene by PCR analysis of tail or ear DNA with the same primers.

Mice.

GITRL TG mice were maintained on a C57BL/6 background and bred in the animal department of the Academic Medical Center (Amsterdam, The Netherlands) under specific-pathogen-free conditions. Mice were used at 6-24 weeks of age, age- and sex-matched within experiments and were handled in accordance with institutional and national guidelines. All experiments have been reviewed and approved by the AMC Animal Ethics Committee. For measurement of in vivo T cell proliferation, mice were injected i.p. with 1 mg (+)-5-Bromo-2'deoxyuridine, (BrdU, Aldrich) and sacrificed for analysis 16 hours later.

Cell staining and flow cytometry.

Single-cell suspensions were obtained by mincing the specified organs through 40 μm cell strainers (Becton Dickinson). Erythrocytes were lysed with an ammonium chloride solution and cells were subsequently counted using an automated cell counter (Casy, Schärfe System). Cells (5 x 10^5- 5 x 10^6) were collected in staining buffer (PBS with 0.5% bovine serum albumin (Sigma)) and stained for 30 min at 4˚C with antibodies in the presence of anti-CD16-CD32 (clone 2.4G2). The following fluorescently or biotin-labelled monoclonal antibodies (and clone names) were obtained from Pharmingen: anti-B220 (RA3-6B2), anti-CD3ε (145-2C11), anti-CD4 (L3T4), anti-CD8 (Ly-2), anti-CD62L (clone MEL-14), anti-CD69 (H1.2F3) or from eBioscience: anti-GITRL (ebioYGL386), anti-FoxP3 (NRRF-30), anti-GITR (DTA-1), anti-CD44 (IM7), anti-CTLA4 (4C10-4B9), anti-PD1 (RMP1-30), anti-CD134 (OX86), anti-CD27 (LG.7F9), anti-CD103 (M290), anti-CD45.1 (104 or A20). PE-conjugated anti-CD25 was obtained from Miltenyi Biotec. For the detection of biotinylated antibodies, streptavidin-PE (Caltag Laboratories, CA), streptavidin-APC (Pharmingen) or streptavidin-conjugated PerCP-Cy5.5 (Pharmingen) was used. Intracellular stainings for FoxP3 and/or BrdU were performed using Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience), according to the manufacturer's protocol. For BrdU staining, FITC-conjugated anti-BrdU/Dnase (Becton Dickinson) was added during the FoxP3 staining step and stained for 25 min at room temperature. Data were collected on a FACSCalibur or FACSCanto (Becton Dickinson) and were analyzed with using FlowJo software (Treestar).

Intracellular cytokine staining

To determine direct ex vivo cytokine production, splenocytes were plated at 1 x 10^6 cells/well in a 96-well round-bottom plate and stimulated with 1 ng/ml PMA and 1 uM ionomycin. After 2 hours incubation at 37°C, 1 ug/ml of the protein-secretion inhibitor Brefeldin A was

added (Sigma) and cells were cultured for another 4 hours. Hereafter, cells were washed and stained for CD3 and CD4 or CD8, followed by fixation and permeabilization (Becton Dickinson). Cells were then incubated for 30 min with fluorescently labelled antibodies against IFNγ, IL2, IL10 or IL17 (eBioscience), thoroughly washed and analyzed by flow cytometry.

T cell proliferation assay

To analyze the effect of GITR engagement on the proliferative capacity of WT responder cells, T cells were enriched from spleens of WT mice by negative selection using $CD19^+$ beads (Miltenyi Biotec). T cell enriched splenocytes were labeled with 0,25 μM carboxyfluorescein succinimidyl ester (CFSE) in PBS at 37˚C for 10 min and stimulated with 100 ng/ml anti-CD3 (clone 145-2C11) for 3 days in the presence of irradiated (10 Gray) WT or GITRL TG B cells with or without 200 U/ml IL-2. B cells were isolated by positive selection using $CD19^+$ beads (Miltenyi Biotec). For the analysis of the expression of CD25 and CD69, non-CFSE labeled enriched WT T cells were used, stimulated similarly and analyzed after 1 day. To determine the effects of GITR ligation on IL2 production in vitro, T cell enriched splenocytes were stimulated as described above, in the presence of 10 μg/ml blocking anti-CD25 antibody (clone PC61) to prevent IL-2 consumption. Culture supernatant was harvested after 1 day of stimulation and frozen at -20°C. The IL2 ELISA (Becton Dickinson) was performed according to instructions from the manufacturer.

Regulatory T cell assay.

Splenic $CD4^+CD25^-$ (responder cells) and $CD4^+CD25^{hi}$ (regulatory cells) T cells were isolated by cell sorting using a FACSAria (Becton Dickinson) and purity of sorted populations was consistently >96%. Responder cells were mixed with regulatory T cells at different ratios in

96-well tissue culture plates. The cells were stimulated with 10 µg/ml soluble anti-CD3 (clone 145-2C11) plus irradiated (10 Gray) WT splenocytes (APCs) at 37˚C for 72 hours. Hereafter, cells were pulsed for 16 hrs with 1 µCi 3H-TdR ([Methyl-3H]Thymidine, Amersham Pharmacia)/well, and incorporation of 3H-TdR was determined using a Beta Plate scintillation counter (Wallac, 1450 microbeta Plus Liquid Scintillation counter). Data are presented as percentage proliferation compared to maximum responder cell proliferation of triplicate assays.

Adoptive transfer of naïve T cells into WT and GITRL TG mice.

For adoptive transfers, naïve ($CD25^-$) $CD4^+$ T cells were purified from spleens and peripheral lymph nodes of Ly5.1 mice by negative selection using the $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec). Purified $CD4^+CD25^-$ cells (purity >90%) were labeled with 0,25 µM carboxyfluorescein succinimidyl ester (CFSE) in PBS at 37˚C for 10 min and injected after washing (± $1x10^6$ in 200 µl PBS) i.v. into WT and GITRL TG recipient mice. Distribution and phenotype of transferred cells was analyzed 3 days later by flow cytometry.

EAE induction

EAE was induced by s.c. immunization of mice in the hind flanks using 50 µg of MOG35-55 peptide

in CFA containing 1 mg/ml heat-inactivated Mycobacterium tuberculosis (Difco) on day 0. Mice also received 200 ng of pertussis toxin (Sigma) i.v. on days 0 and 2. Disease severity was assessed according to the following scale: 0, no disease; 1, flaccid tail; 2, loss of hind leg spreading reflex; 3, hind limb weakness; 4, unilateral hind limb paralysis; 5, bilateral hind limb paralysis; 6, abdominal paralysis; 7, moribund; 8, dead. All mice were sacrificed 14 days

following EAE induction after which brain and spinal cord was frozen in Tissue TEC (Sakura Finetek, The Netherlands) at -80 °C for immunohistochemical analysis.

Immunohistochemistry

Cryostat sections (8 μm) of spinal cord and brain of 4 WT and 4 TG mice were fixed in acetone, containing 1% H_2O_2 for 10 minutes. Then, sections were incubated with monoclonal rat anti-mouse antibodies to CD68 (a kind gift from Siamon Gordon, Oxford, UK, clone FE-11), CD4 and FoxP3 (eBioscience), diluted in PBS with 8% bovine serum albumine (BSA), 10% normal mouse serum (NMS) and 0.05% NaN_3 for 1 h at 4°C. After washing in PBS, the sections were incubated with anti-rat HRP diluted in PBS/ 8% BSA/ 10% NMS/ 0.05% NaN_3/ 350 mM NaCl. Staining was visualized with DAB (Sigma Laboratories, St. Louis, MO, USA) applied for 10 minutes. Sections were counterstained with heamatoxylin for 30 seconds, dehydrated and mounted in entallan (Merck, Darmstadt, Germany). As negative controls, primary antibodies were either left out or substituted with an isotype control antibody. No immunoreactivity was seen for all negative controls.

For the visualization of cellular infiltrates in organs obtained from WT and GITRL TG mice, cryostat sections (7 μm) of the thyroid, kidney, liver, stomach, and small and large intestine were stained by Diff-Quick (Dade Behring) according to the manufactures instructions and analyzed by light microscopy.

Statistical analysis.

Statistical analysis of the data was performed using the unpaired Student's *t*-test or Wilcoxon rank-sum test where mentioned. For the EAE experiments, effect on mean clinical score was assessed by calculating the area under the curve using the trapezoidal rule, followed by the

Wilcoxon rank-sum test. Differences in cumulative incidence were analyzed on a per day basis, using a χ^2 test of a contingency table.

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Chapter 3

Enhanced GITR/GITRL interactions reduce IgG3 and enhance IgA secretion.

Manuscript in Preperation

Ronald W. van Olffen[1], Klaas P.J.M. van Gisbergen[1], Jörg Hamann[1], Rene A.W. van Lier[1] and Martijn A. Nolte[1].

[1]Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands.

Summary

The interaction between the glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) and its ligand regulate both adaptive and innate immune responses. In this study, we investigated the role of GITR-GITRL signaling on humoral immune responses by overexpression of GITRL on B cells. GITRL-TG mice had a normal B cell development, but had elevated levels of serum IgA and strongly diminished IgG3 titers. These changes in isotypes correlated with increased numbers of B2 cells and decreased numbers of B1 cells in the peritoneal cavity. Interestingly, stimulation of splenic B cells *in vitro* revealed no intrinsic B cell defect by transgenic overexpression of GITRL on immunoglobulin production, suggesting that extrinsic factors through T cells modulate the humoral responses. Influenza A infection resulted in elevated virus-specific IgA levels in GITRL TG mice, without further modulating adaptive immune responses. Thus, transgenic overexpression of GITRL specifically affects mucosal related B cell subsets and promotes IgA responses at mucosa related sites.

Introduction

The basis of a humoral immune response is the production of protective, high-affinity antibodies. Antibodies play an essential role in the defense against pathogens: their primary function entails binding to the invading pathogen (thereby inhibiting dissemination), whereas their secondary function involves initiation of effector functions (opsonisation and complement activation).

Terminal differentiation of B cells takes place in the spleen following emigration of immature B cell from the bone marrow. Immature B cells go through several developmental stages; first forming transitional type 1 (T1) and transitional type 2 (T2) B cells, after which they differentiate to either marginal zone or mature follicular B cell (1). The different stages of B cell development can be distinguished based on IgD, IgM and CD21/35 expression (1). Apart from these mature B cells present in secondary lymphoid organs, quite distinct subsets of B1 and B2 cells can be found in peritoneal and pleural cavities, which develop either from bone marrow-derived immature B cells (2;3) or from fetal liver progenitor cells (4-6). B2 B cells have an intermediate expression of IgM, no CD11b expression and are $B220^{+}$ (7), whereas B1 B cells express CD11b, are $B220^{-}$ and IgM^{+}. In addition, the B1 lineage can be subdivided into $CD5^{+}CD11b^{+}B220^{-}IgM^{+}$ B1a B cells and $CD5^{-}CD11b^{+}B220^{-}IgM^{+}$ B1b B cell (8). With respect to class switch recombination and immunoglobulin production, the B1 lineage mainly responds to T cell-independent antigens, is responsible for a minor contribution of serum IgA and is the main secretor for IgG3 (9;10). In contrast, the B2 lineage requires T cell help to undergo class switch recombination and affinity maturation. Mucosa-associated B2 B cells are the main producers of IgA and are responsible for up to 99% of secretory IgA levels (11).

Signaling via tumor necrosis factor receptor (TNFR) superfamily members and their ligands regulates both adaptive and innate immune responses, and modulation of either receptor or ligand expression can dramatically affect the course of T and B cell responses (reviewed in

chapter 7 of this thesis and (12)). Defects in the interaction between CD40 and its ligand result in decreased germinal center development, and consequently loss of memory B cell formation and decreased class switch recombination (13;14). In addition, loss of CD40 signaling on APC's results in decreased cytokine secretion (e.g. IL12), thereby negatively affecting helper T cell formation and hindering an adequate immune response (15). We have previously shown that constitutive stimulation of CD27 on T cells by its ligand CD70 induces IFNγ dependent depletion of B cells (16). In addition, "reverse signaling" through CD70 on B cells enhances IgM production and diminishes IgG secretion (16;17). Another member of the TNFR superfamily that directly affects adaptive immune responses is the glucocorticoid-induced TNFR family-related protein (GITR), which is expressed on activated T cells and regulatory T cells and enhances proliferation of both effector and regulatory $CD4^+$ T cells (Chapter 2 of this thesis and (18)). Studies using $GITR^{-/-}$ mice have established that GITR-GITRL interaction regulates T cell activation and survival (19;20). Moreover, signaling via GITRL on pDC's promotes the induction of IDO and may thus be an important mediator in inducing tolerance (21). However, it is not yet known whether GITR-GITRL interactions also influence B cell responses.

We set out to obtain insight in the role of GITR-GITRL signaling in humoral immunity, using recently generated GITRL TG mice, which constitutively express GITRL on all B cells (Chapter 2 of this thesis). We found that enhanced GITR triggering induced a striking increase in serum IgA titers and inhibited the production of IgG3, both in the steady-state situation and upon T cell-dependent immunization. In agreement with increased serum IgA titers, we identified a significant increase in the mucosa-related peritoneal B2 B cell population. Moreover, overexpression of GITRL on B cells enhanced virus-specific serum IgA titers following Influenza A infection, but these were insufficient to enhance protective immunity based on the viral load. Thus, our data suggest that GITR-GITRL interactions

modulate humoral immune responses via altering mucosal B cell subsets and promoting IgA responses.

Results

Normal development of splenic B cell populations in GITRL TG mice.

GITRL TG mice have enhanced numbers of $CD4^+$ effector and regulatory T cells due to increased proliferation of these cells (chapter 2 of this thesis). Interestingly, high numbers of IFNγ-producing T cells induced by constitutive CD27 ligation alters terminal differentiation of B cells in the spleen and destroys normal splenic architectural structure (16). To determine if the enhanced GITRL expression on B cells and the resulting accumulation of effector and regulatory $CD4^+$ T cells would also affect terminal B cell development in GITRL TG mice, we examined their splenic B cell compartment, based on IgD, IgM, B220 and CD21/35 expression (Fig. 1A) (1). GITRL TG mice were found to have normal numbers of transitional type 1, transitional type 2, marginal zone B cells and mature follicular B cells (Fig. 1B). This indicates that the enhanced effector cell formation in GITRL TG mice does not appear to affect B cell development in the bone marrow and final B cell maturation in the spleen. Moreover, immunohistochemical analysis showed a normal architectural structure of the spleen and no differences were observed for T cell areas and B cell follicles between WT and GITRL TG mice (Fig. 1C). In addition, a distinct ring of marginal zone B cells could be identified upon staining for sinus-lining cells (MAdCAM-1^+) and B cell ($B220^+$) staining (Fig. 1C), which indicates that marginal zone B cells are located at the correct anatomical location in these mice.

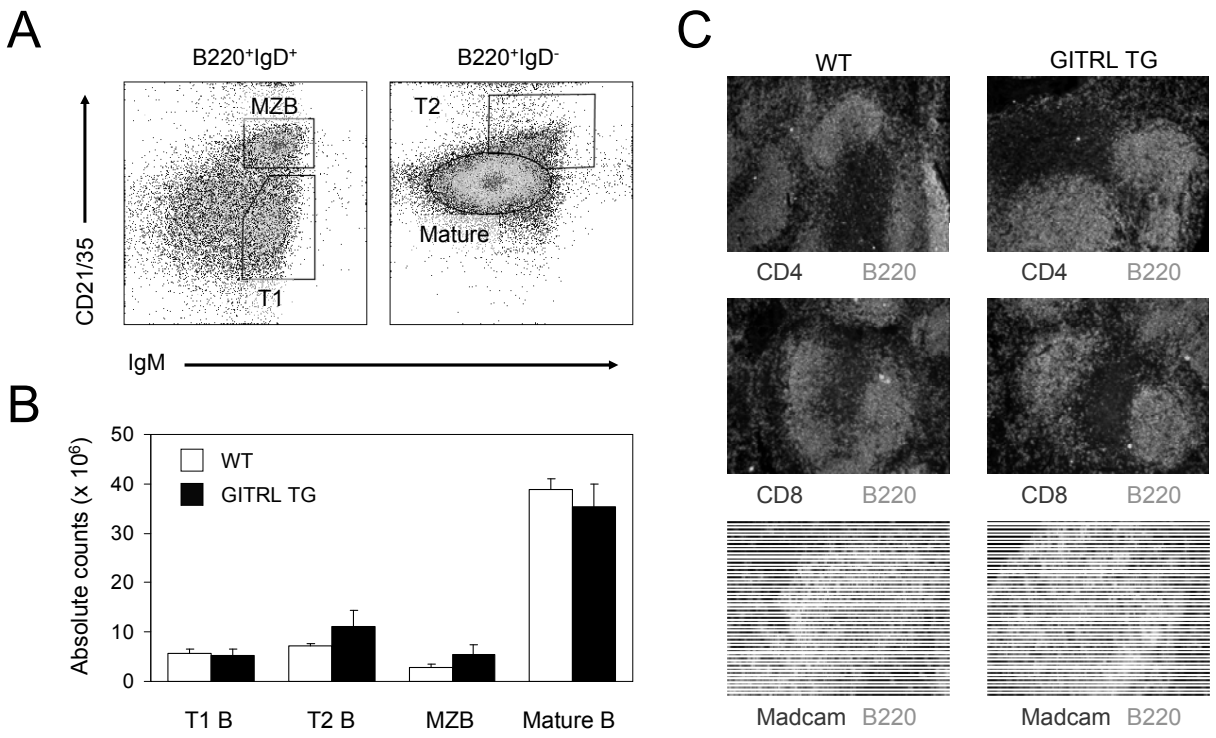

Figure 1. Normal distribution of splenic B cell populations.
(A) Representative staining of Transitional type 1 B cells (T1), Transitional type 2 B cells (T2), marginal zone B cells (MZB) and mature B cells (mature) in spleen from a WT mouse. (B) Absolute numbers of T1, T2, MZB and mature splenic B cells in WT (white bars) and GITRL TG (black bars) mice. Data represent the average value ± SD of 3-5 mice (C) The architectural structure of spleens from WT (left) and GITRL TG (right) mice was analyzed by staining for T cells ($CD4^+$ or $CD8^+$), B cells ($B220^+$) and sinus-lining cells ($MAdCAM\text{-}1^+$).

Modulated immunoglobulin secretion via constitutive GITRL overexpression.

Transgenic overexpression of GITRL on B cells results in increased numbers of IFNγ producing effector T cells (chapter 2 of this thesis). As helper T cells orchestrate class switching of activated B cells, we investigated whether serum immunoglobulin levels were altered in GITRL TG mice. GITRL TG mice showed an age-dependent modulation of serum immunoglobulin levels. In 4 week old mice only marginal changes were identified, but older mice (11 weeks) showed reduced IgG3 titers and enhanced serum levels of IgA, while the

other isotypes were normal (Fig 2A). To ascertain if the observed modulation of immunoglobulin levels in GITRL TG mice was a consequence of intrinsic B cell defects, due to constitutive GITRL reverse signaling, we investigated their immunoglobulin production capacity *in vitro*. When stimulated for 15 days *in vitro*, we found that splenic GITRL TG B cells could still produce IgG3 and showed normal IgA production (Fig. 2B). These results suggest that the observed changes in serum immunoglobulin levels in GITRL TG mice were not caused by changes evoked by the high GITRL expression on the transgenic B cells. In support of these observations, no effects of reverse GITRL signaling on immunoglobulin production were found by the addition of a mAb specific to GITRL (YGL386.2.2) to the B cell cultures (data not shown). However, at this time it is not known whether this antibody has any agonistic (or antagonistic) effects upon binding to GITRL.

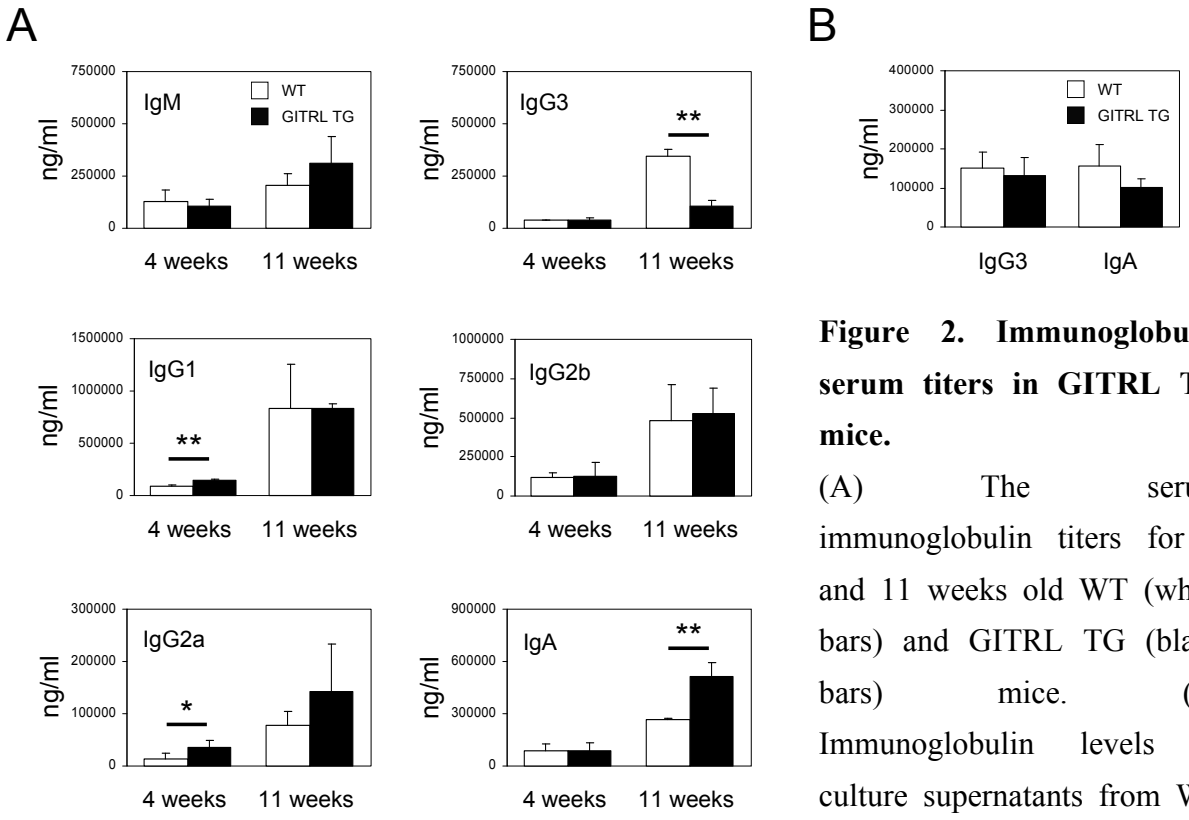

Figure 2. Immunoglobulin serum titers in GITRL TG mice.

(A) The serum immunoglobulin titers for 4 and 11 weeks old WT (white bars) and GITRL TG (black bars) mice. (B) Immunoglobulin levels in culture supernatants from WT (white bars) and GITRL TG (black bars) derived splenic B cells stimulated for 15 days with αIgM, IL2 and LPS. Data are expressed as average value ± SD of 3-4 mice per group.

Consequently, the modulation of serum immunoglobulin levels in GITRL TG mice compared to WT mice, might arise from B cell-extrinsic factors, such as alterations in helper T cell subsets. Therefore, we set out to determine if T cell-dependent B cell responses were affected in GITRL TG mice. WT and GITRL TG mice were immunized with TNP-KLH and TNP-specific serum immunoglobulin titers were analyzed 14 days later. GITRL TG mice showed a normal induction of most isotypes, but they failed to generate an IgG3 response and showed a slight reduction in IgG1 compared to WT mice (Fig. 3). As expected, antigen-specific IgA levels were equally low in both mouse strains, because this type of immunization does not induce isotype switching to IgA.

Thus, we conclude that constitutive GITR-GITRL interactions result in a specific modulation in immunoglobulin isotype switch, i.e. increased IgA and decreased IgG3 production.

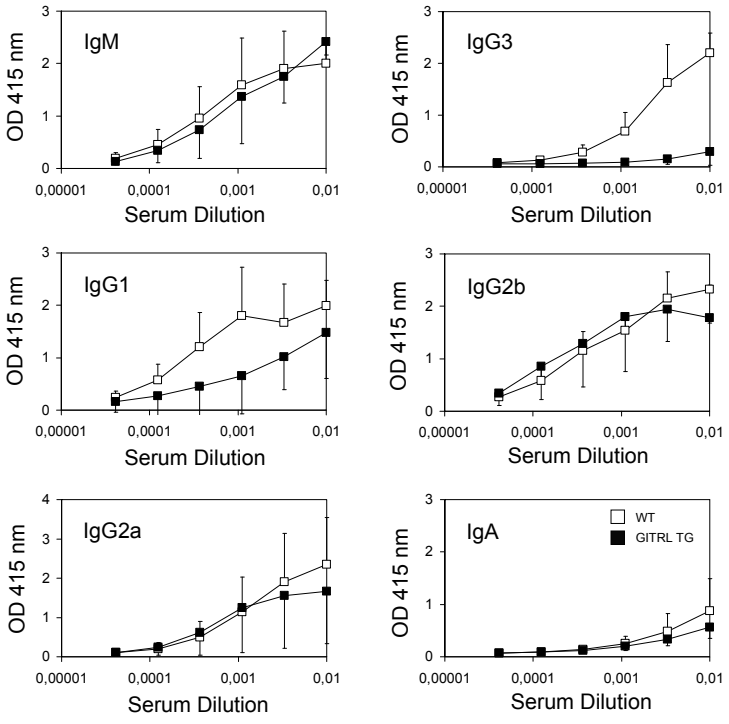

Figure 3. T cell dependent Ig responses to TNP-KLH.
WT (white bars) and GITRL TG (black bars) mice were immunized i.p. with 100 µg of TNP-KLH emulsified in alum. Isotype specific anti-TNP titers were determined in sera by TNP-specific ELISA on day 14 after immunization. Data are expressed as average value ± SD of five mice per group.

Altered mucosal B cell subsets in GITRL TG mice.

Based on these observations, we decided to investigate the mucosa-associated B cells in these mice, as these cells have also been implicated in the production of IgA and IgG3 (9-11). Therefore, we analyzed cells from the peritoneal cavity of WT and GITRL TG mice to determine if constitutive GITRL expression altered the numbers of mucosal B1 and B2 cell lineages (Fig. 4A). We found that GITRL TG mice had increased numbers of peritoneal B2 B cells and a decrease in B1a B cell numbers compared to WT mice (Fig. 4B). No differences were found in peritoneal B1b B cell numbers (Fig. 4B).

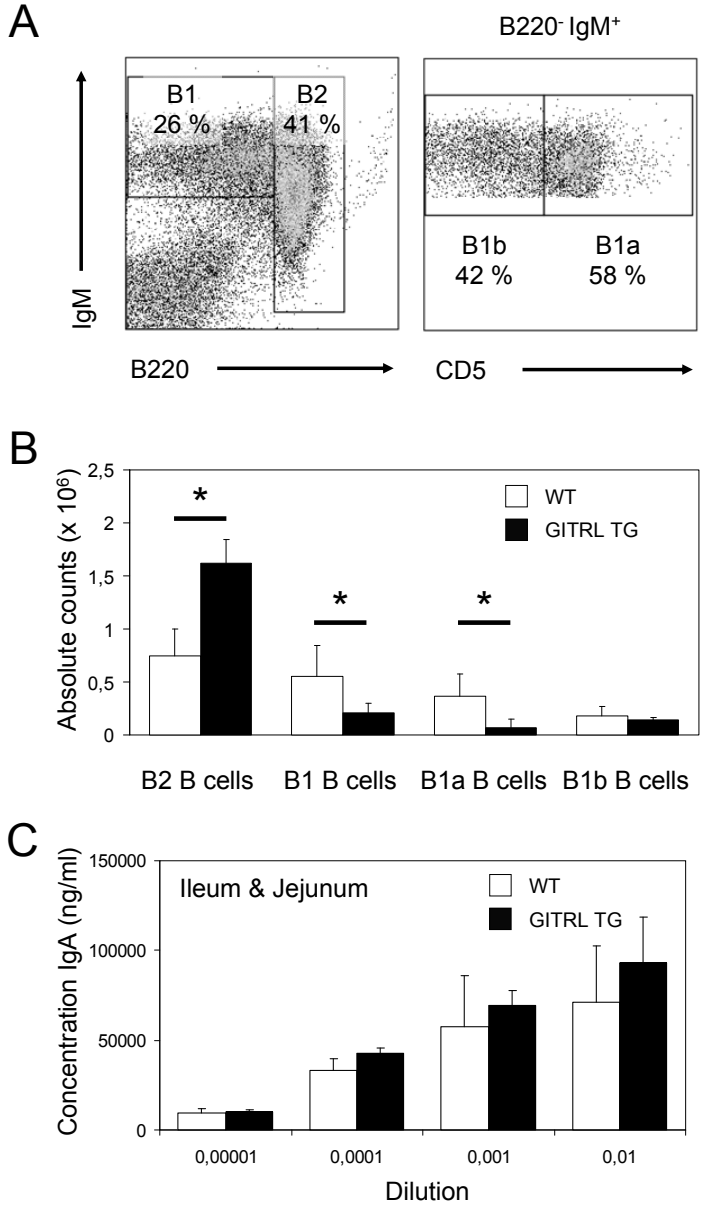

Figure 4. Modulation of peritoneal B cell repertoire in GITRL TG mice.

(A) Representative staining in a WT mouse of peritoneal B1a, B1b and B2 B cells. (B) Absolute numbers of peritoneal B2, total B1, B1a and B1b B cells in WT (white bars) and GITRL TG (black bars) mice. (C) Concentration of IgA found in serial 10-fold stool sample dilutions in the small intestine of WT (white bars) and GITRL TG (black bars) mice. Data represent the average value ± SD of 3-5 mice.

As peritoneal B2 B cells are main producers of secretory IgA (sIgA), we investigated the concentration of fecal IgA found in the small intestine in WT and GITRL TG mice. We found similar concentrations of fecal IgA, based on serial 10-fold stool sample dilutions in WT and GITRL TG mice (Fig. 4C). These data suggest that constitutive GITR-GITRL interactions specifically enhance serum IgA titers, correlating with the increase in peritoneal B2 B cell numbers, but not sIgA. In addition, the loss of an IgG3 response could be related to the reduced numbers of B1a B cells.

Enhanced IgA responses are not protective against influenza A infection.

IgA deficient mice show similar levels of viral replication and mortality following a lethal influenza challenge (22), but it has also been shown that the passive transfer of influenza specific IgA antibodies were protective for subsequent influenza infection by inhibiting viral replication or the infection itself (23;24). As GITRL TG mice have elevated levels of serum IgA, we questioned whether these mice were protected against an influenza A infection. GITRL TG mice developed a normal influenza-specific CD8 T cell response, based on tetramer staining in blood, spleen and lungs (data not shown). However, GITRL TG mice showed reduced IgM and IgG2a titers, a low IgG3 response and an increased IgA response compared to WT mice at the peak of influenza A infection (day 10) (Fig. 5A). However, GITRL TG mice were not protected from influenza A infection, as no difference was observed in viral load compared to WT mice (Fig. 5B).

In conclusion, GITR-GITRL interactions affect numbers of mucosa-associated B cell subsets, reduces IgG3 production and enhances IgA secretion, but this increase in IgA levels is not sufficient to protect GITRL TG mice during a primary influenza infection.

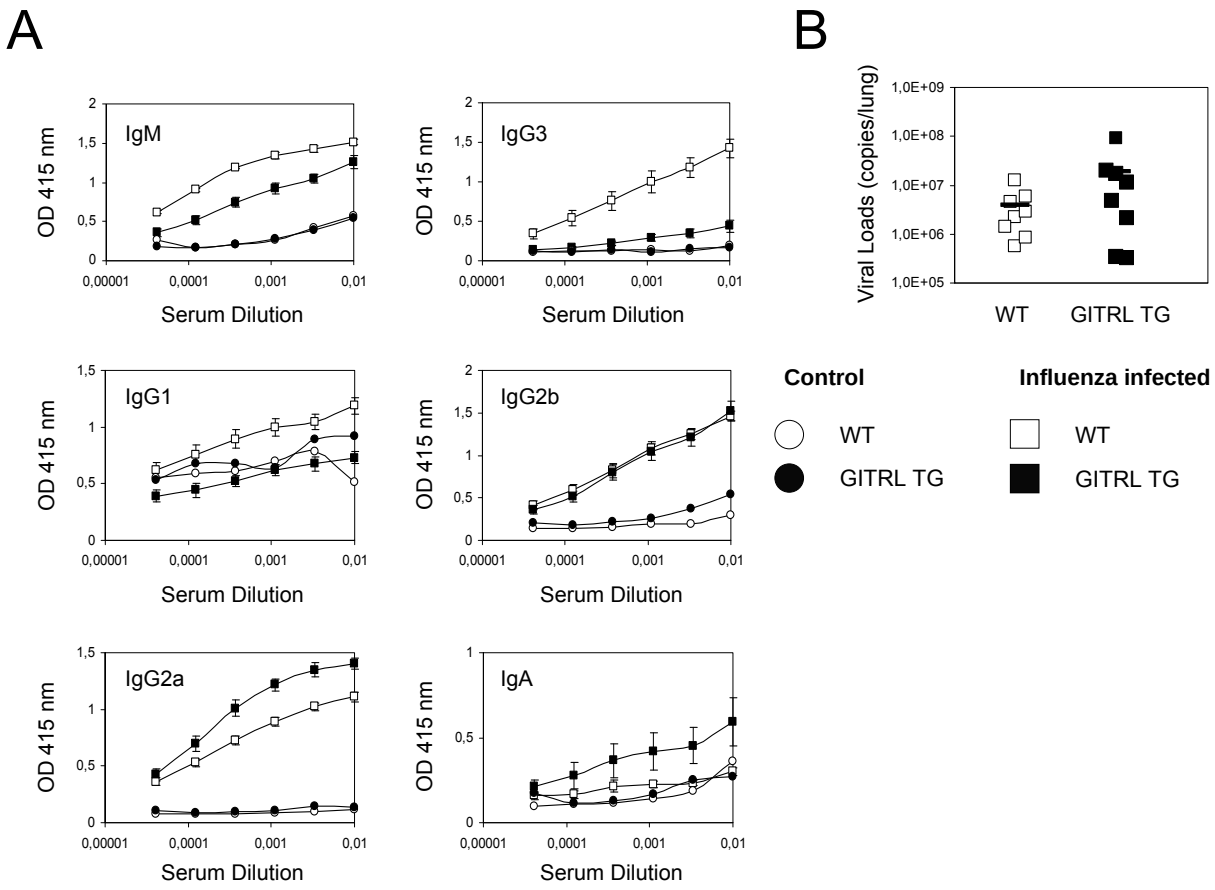

Figure 5. GITRL TG mice do not modulate immune response to influenza A infection.

WT and GITRL TG mice were intranasally infected with the influenza virus A/PR8/34. (A) Influenza A specific immunoglobulin titers were determined in sera of WT (white box) and GITRL TG (black box) mice by Influenza A-specific ELISA on day 10 after infection. WT (white circle) and GITRL TG (black circle) mice were intranasally challenged with PBS as control. Error bar indicates standard error of the mean of 8 individual mice. (B) Viral loads within the lungs of WT and GITRL TG mice were examined upon primary influenza infection at day 10.

Discussion

Humoral immune responses play an essential role in the defense against pathogens. The production of antibodies can inhibit pathogen entry by receptor shielding, and antibody binding can promote phagocytosis and pathogen neutralization via opsonisation and complement activation, respectively. In this respect and compared to other isotypes, IgA is most highly prevalent in mucosal areas and plays an important role in immunity. IgA can be produced in either monomeric form (as found in serum) or as polymeric IgA, which is transported through epithelial cells expressing a polymeric Ig receptor (pIgR). Proteolysis of dimeric IgA bound to pIgR results in release of secretory IgA (sIgA) in external secretions (25). Although IgA has inferior complement activation and opsonization capabilities compared to IgM and IgG, it functions as the first line of defense by its ability to prevent pathogens breaching mucosal barriers.

In the present study, we have investigated the effects of GITR-GITRL interaction on the humoral immune system. We found that GITRL TG mice have normal B cell differentiation in the spleen, but increased numbers of peritoneal B2 B cells and decreased numbers of peritoneal B1a B cells. In addition, we found that GITRL TG mice have elevated serum levels of IgA and decreased IgG3. As GITR is expressed on activated T cells and regulatory T cells, we assessed the effects of GITR-GITRL interaction on T cell dependent B cell responses by immunizing WT and GITRL TG mice with TNP-KLH. GITRL TG mice also failed to develop an IgG3 response to T cell dependent immunizations, correlating with decreased B1 B cell numbers.

As no intrinsic B cell defect was observed following stimulation of splenic B cells, we believe that the difference in immunoglobulin levels between WT and GITRL TG mice are directly correlated to the variance in numbers of mucosal B cell subsets, which might be due to extrinsic factors derived from the altered T cell compartment. Indeed, GITRL TG mice show

increased numbers of activated and regulatory T cells in all compartments (chapter 2 of this thesis), and it is thus possible that mucosal B cell subsets are influenced through an altered cytokine environment. TGF-β is a known isotype switch factor for IgA and downregulates IgG3 production (26), and is amongst others produced by a specific regulatory T cell subset (27). Interestingly, regulatory T cells have also been shown to infiltrate Peyer's patches and differentiate into $CD4^+$ follicular helper T (T_{FH}) cells, where they promote IgA production (28). Another explanation for the observed differences could be a direct effect on the mucosa-associated B cells, as it cannot be excluded that GITRL reverse signaling does occur *in vivo* and preferentially affects the differentiation or the survival of B2 versus B1 peritoneal B cells, resulting in increased IgA and reduced IgG3 production. More indepth experiments that address these possibilities will be required to expose the molecular mechanism by which GITRL can affect mucosal immunity.

We postulated that increased IgA levels might protect mice against Influenza A infection. Infected GITRL TG mice showed enhanced production of influenza-specific IgA titers in the serum compared to WT mice. Yet, based on viral load in the lung of GITRL TG mice, these increased IgA titers did not improve the anti-viral response in GITRL TG mice compared to WT mice. Nevertheless, these results do suggest that GITRL TG mice will be better protected against a secondary infection with influenza virus, which will be addressed in future experiments. In addition, it will also be interesting to determine the effects of increased monomeric IgA titers in others mucosa-related models, such as oral tolerance induction, as this also induces production of IgA (reviewed in (29)).

In conclusion, we show that the GITR-GITRL costimulatory axis can be an important player in mucosal immunity, as GITRL overexpression affects mucosa-associated B cell populations in the perioneum, but not B cells in the spleen. The resulting increase in IgA production and decrease in IgG3 production in GITRL TG mice are either the consequence of intrinsic effects

in the mucosa-associated B cells, such as GITRL reverse signaling, or of B cell extrinsic factors, as can be derived from the expanded regulatory and/or effector $CD4^+$ T cell subsets present in these mice.

Experimental Procedures

Mice.

GITRL TG (see chapter 2 of this thesis) mice and WT littermates were maintained on a C57BL/6J background and bred in the animal department of the Academic Medical Center (Amsterdam, The Netherlands) under specific-pathogen-free conditions. Mice were used at 6-24 weeks of age, age- and sex-matched within experiments and were handled in accordance with institutional and national guidelines.

Cell staining and flow cytometry.

Single-cell suspensions were obtained by mincing the specified organs through 40 μm cell strainers (Becton Dickinson). Erythrocytes were lysed with an ammonium chloride solution and cells were subsequently counted using an automated cell counter (Casy, Schärfe System). Cells (5×10^5- 1×10^6) were collected in staining buffer (PBS with 0.5% bovine serum albumin (Sigma)) and stained for 30 min at 4˚C with antibodies in the presence of anti-CD16-CD32 (clone 2.4G2; kind gift from Dr. Louis Boon, Bioceros, The Netherlands). The following fluorescently or biotin-labeled monoclonal antibodies were obtained from Pharmingen: anti-B220 (clone RA3-6B2), anti-CD5 (clone 53-7.3), anti-CD21/35 (clone 7G6) or from Southern Biotechnology Association: anti-IgM (clone 1B4B1) and anti-IgD (clone 11-26). For the detection of biotinylated antibodies, streptavidin-PE (Caltag Laboratories, CA), streptavidin-APC (Pharmingen) or streptavidin-conjugated PerCP-Cy5.5 (Pharmingen)

was used. Data were collected on a FACSCalibur (Becton Dickinson) and were analyzed with using FlowJo software (Treestar).

Immunohistochemistry

Spleen from 8 week old WT and GITRL TG mice were isolated and snap-frozen in liquid nitrogen. Cryostat sections (6μm) were fixed in 100% acetone for 10 min, air-dried and rehydrated in PBS. For immunofluorescent stainings, splenic sections were successively incubated for 45 min with unconjugated rat anti-mouse mAbs and Alexa 594-conjugated anti-rat IgG (molecular Probes). Sections were blocked for 5 min with 20% normal rat serum and subsequently incubated with biotinylated rat anti-mouse mAbs and Aalexa 488-conjugated streptavidin (Molecular Probes). The following rat anti-mouse mAbs were used: anti-B220 (clone 6B2), anti-MAdCAM-1 (clone MECA-367), anti-CD4 (clone RM4-5) and anti-CD8 (clone 53-6.7). Sections were extensively washed with PBS between each step and finally coverslipped with Vectashield (vectorlabs). Fluorescent stainings were analyzed using a Nikon Eclipse E800 microscope, connected to a digital camera.

TNP-KLH immunization.

Mice were immunized by intraperitoneal injection with 25 μg TNP-KLH (Biosearch Technologies, Novato, CA) emulsified in incomplete freund's adjuvant (Sigma). Sera were collected on day 14. Anti-TNP-specific immunoglobulin (Ig) levels were determined by ELISA as described below

Immunoglobulin Elisa.

The antibody titers were determined by an enzyme-linked immunosorbent assay. 96 wells plates were coated overnight at 4˚C with unlabeled rat-anti-mouse Ig (1 μg/ml) of the

indicated isotype (SBA) or with TNP-BSA (1 μg/ml) in coating buffer (0.1 M NaH_4CO_3). Subsequently, plates were blocked with 5% milk in PBS (blocking buffer) for 1 hour at room temperature. Sera or stool samples (total small intestine content in 2 ml PBS) from WT and GITRL TG mice were diluted in blocking buffer and incubated in duplo for 2 hours at room temperature. The quantification of bound immunoglobulin was performed by incubating the plates with a biotin-conjugated goat-anti-mouse IgG (SBA) followed by a 1 hour incubation at room temperature with streptavidin-conjugated alkalic phosphatase (Sigma). Plates were then developed using pNPP substrate (Sigma). The optical density was measured at 415 nm using a microplate reader. Between the various steps the plate underwent extensive washing using 0,05% Tween 20 in PBS..

In vitro B cell cultures.

Spleen from WT and GITRL TG mice were harvested and single cell suspensions prepared. B cells were isolated by positive selection using anti-$CD19^+$ microbeads (Miltenyi Biotec) and MACS separation system (Miltenyi Biotec), according to the manufactures protocol. B cells were cultured for 15 days with 5 μg/ml α-IgM (Jackson ImmunoResearch), 10 ng/ml IL-2 (Invitrogen),and 5 μg/ml LPS (Sigma). To induces reverse signalling of GITRL on B cells, a mAb specific to GITRL was added to the specified B cell cultures (clone YGL386.2.2). Immunoglobulin levels were determined as described above.

Influenza infection.

Mice were intranasally infected with 10x $TCID_{50}$ of the H1N1 influenza A virus A/PR8/34 for analysis of primary immune responses. After 10 days mice were sacrificed, and blood and lungs were collected for analysis. Viral loads within the lungs were quantified using qPCR as previously described (30). Influenza A specific immunoglobulins were determined in serum

and lung homogenates (½ lung in 500 µl staining buffer) via ELISA as described above using 96 wells plates coated with influenza A virus (10^6 x $TCID_{50}$).

Statistical analysis.

Figures represent means and error bars denote standard deviation. Student's T-test was used to analyze for statistical significance. $P < 0.05$ was considered statistically significant.

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Chapter 4

CD70 driven chronic T cell stimulation decreases inflammation-induced macrophage accumulation by enhancing apoptosis.

Ronald W. van Olffen[1], Mariska Vos[2], Anna D. Staniszewska[1], Robert M. Hoek[1,3], Jörg Hamann[1], Carlie J. M. de Vries[2], Martijn A. Nolte[1] and René A. W. van Lier[1]

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[1] Department of Experimental Immunology and [2] Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands.

[3]Current address: Netherlands Institute for Neuroscience, Department of Neuromedical Genetics, Meibergdreef 47, 1105 BA Amsterdam, The Netherlands.

Abstract

Persistent co-stimulation of T cells via constitutive expression of CD70 on B cells *in vivo* results in a strong increase in the number of IFNγ producing effector-type T cells. CD70 transgenic (TG) mice show characteristics of patients with chronic active infectious disease such as HIV, as they eventually exhaust their naïve T cell pool and show early mortality due to opportunistic pulmonary infections caused by *Pneumocystis carinii*. Since macrophages play a central role in the defense against pulmonary pathogens and IFNγ is the central cytokine in classical macrophage activation, we investigated whether CD70-induced immune stimulation affects the myeloid compartment. We found that CD70 TG mice have increased numbers of activated circulating monocytes expressing high levels of MHC class II. These monocytes have normal phagocytosis and migration characteristics *in vitro*. Interestingly however, monocytes from CD70 TG mice display enhanced IFNγ-dependent susceptibility to apoptosis *in vitro* and fail to accumulate at inflammatory sites *in vivo*, as is illustrated by a remarkable protection of these mice against atherosclerosis. This indicates that CD70-mediated immune activation affects innate cell function, which may contribute to a diminished ability to deal with opportunistic pathogens.

Introduction

The binding of TNF-like receptors (TNFR) to their specific TNF ligand family members regulates T cell activation, differentiation and survival. The TNFR family includes death domain containing receptors and TNF receptor-associated factor (TRAF) binding receptors. Ligation of death domain containing receptors results in the activation of caspase cascades leading to apoptosis. TRAF binding receptors, such as OX40, CD30, 4-1BB, HVEM, GITR and CD27 (reviewed in [1]), on the other hand are associated with signaling that leads to activation, differentiation and survival.

CD70, the unique ligand of CD27, is expressed both in humans and mice on activated T cells, B cells and dendritic cells [2-4]. Its receptor, CD27, is found on the majority of T cells, on subsets of antigen-experienced B cells, NK cells and hematopoietic progenitor cells [5-8]. Signaling via CD27 involves the association of TRAF2 and TRAF5 to the receptor and subsequent activation of c-JUN N-terminal kinase (JNK) and the transcription factor NF-κB [9, 10]. *In vitro*, ligation of CD27 increases the expression of the anti-apoptotic factor Bcl-X_L [11]. We have recently generated CD70 transgenic (TG) mice that constitutively express CD70 on all B cells [12]. CD70 TG mice accumulate effector-type T cells that produce ample amounts of IFNγ, which is dependent on both CD27-triggering and antigen-recognition [12, 13]. This increased secretion of IFNγ caused by constitutive signaling through CD27-CD70 interaction results in the inhibition of B cell development in the bone marrow, a phenotype that can be rescued if CD70 TG mice are crossed on an IFNγ$^{-/-}$ background [12].

CD70 TG mice show similarities to individuals with chronic active viral infections such as HIV-1. Compared to wild type (WT) mice, young CD70 transgenic mice have superior responses to viruses and tumors [13]. However, as CD70 TG mice age, the thymus involutes, naïve T cell numbers decline and proliferative responses to mitogenic stimuli wane. Interestingly, these mice show early mortality to opportunistic *Pneumocystis carinii*

pneumonia (PCP) [14] analogous to untreated HIV-infected patients. We observed that the occurrence of PCP was more frequent in CD70 TG mice than in nude mice housed in the same specific pathogen free (SPF) facility, suggesting that next to the naïve T cell demise, other alterations in the immune system might contribute to the increased susceptibility for opportunistic pathogens. As selective depletion of alveolar macrophages leads to an impaired clearance of *Pneumocystis carinii* resulting in pneumonia [15], we here analyzed phenotypic and functional traits of the monocyte/macrophage lineage in CD70 TG mice. We observed a significant increase of activated monocytes in the circulation and these monocytes showed a strong upregulation of MHC class II. Furthermore, the monocytes in CD70 TG mice have a normal capacity to phagocytose and are capable of responding to the chemoattractant MCP-1 *in vitro*. Interestingly, under conditions that simulate acute and chronic immune activation *in vivo*, in sterile peritonitis and a model for atherosclerosis [16], monocytes are unable to accumulate at inflammatory sites. Finally, we found that monocytes from CD70 TG mice are more susceptible to apoptosis, which is dependent on IFNγ. We conclude that chronic immune activation impairs monocyte function *in vivo* via specific enhancement of apoptosis.

Results

CD27-induced effector T cell formation affects monocyte maturation.

$B220^+$ cells in both spleen and bone marrow of CD70 TG mice display a strong upregulation of MHC class II, reflecting the high IFNγ levels *in vivo* [12]. To analyze the effect of constitutive CD27-CD70 interactions and the resulting pro-inflammatory cytokine profile on monocyte function, we first analyzed the cell surface phenotype of blood myeloid cells from CD70 TG mice in comparison to WT, $IFN\gamma^{-/-}$ and CD70 TG x $IFN\gamma^{-/-}$ mice. Within the circulating myeloid ($CD11b^+$) compartment, CD70 TG mice had normal percentages of monocytes ($GR1^-F4/80^+$ cells, Figure 1A-B). Like $B220^+$ cells, also $CD11b^+F4/80^+$

monocytes from CD70 TG mice showed a strong upregulation of MHC class II (Figure 1C-D). Furthermore, this upregulation was indeed IFNγ dependent as monocytes from CD70 TG x IFNγ$^{-/-}$ mice had normal MHC class II expression.

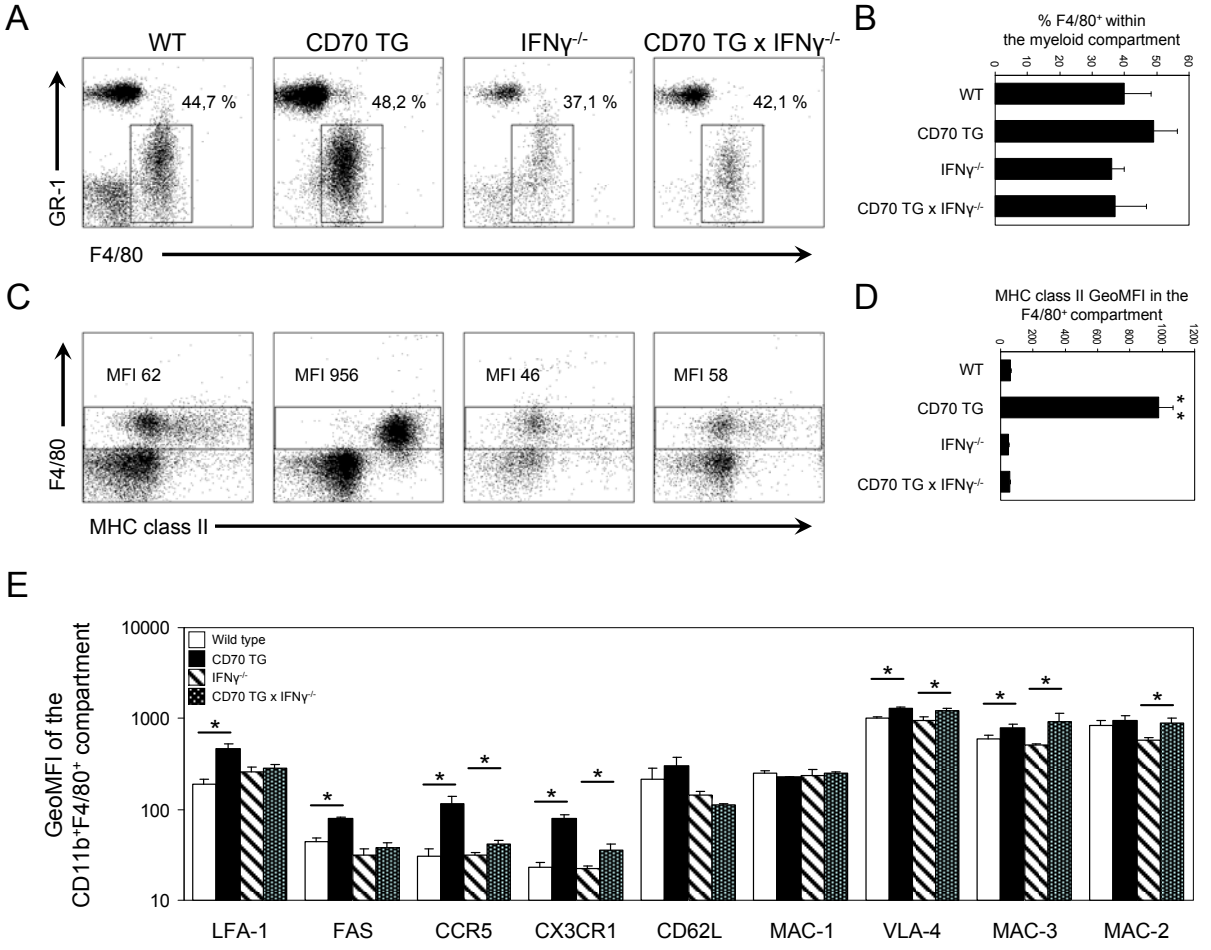

Figure 1. Increase in activated circulating blood monocytes in CD70 TG mice. Phenotypic analysis of peripheral blood in WT, CD70 TG, IFNγ$^{-/-}$ and CD70 TG x IFNγ$^{-/-}$ mice. (A) Representative staining and (B) average percentage of monocytes (F4/80^{hi}GR1^{-}) within the myeloid compartment (CD11b^{+}). (C) Representative staining and (D) average geometric mean fluorescence intensity (GeoMFI) of MHC class II expression on monocytes (CD11b^{+}F4/80^{+}). (E) (Ex-vivo analysis of) Integrins, FAS and chemokine receptor expression levels on monocytes (F4/80^{+}) in the myeloid compartment (CD11b^{+}) of WT (white bar), CD70 TG (black bar), IFNγ$^{-/-}$ (hatched bar) and CD70 TG x IFNγ$^{-/-}$ (dotted bar) mice. Error bars indicate the standard deviation of 3 mice per group. Asterisks denote a significant difference (* $p<0.05$, ** $p<0.01$) as determined by Student's *t*-test.

We also determined if the upregulation of MHC class II was accompanied by changes in membrane expression of other cell surface molecules that are regulated during monocyte activation [17-21] (Figure 1E). Expression of LFA-1 (CD11a), a member of the β2-integrin family, and the FAS receptor (CD95), a member of the tumor necrosis factor receptor superfamily, were strongly increased on monocytes from CD70 TG mice in an IFNγ-dependent manner, as were the chemokine receptors CCR5 (receptor for the inflammatory chemokines MIP-1α/β, RANTES and MCP-2) and CX3CR1 (the fractalkine receptor) [22, 23] (Figure 1E). The chemokine receptor for MCP-1, CCR2, was not differentially expressed (data not shown), nor was expression of CD62L. The β2-integrin family member MAC-1 (CD11b) also showed no differential expression. Interestingly, we found that certain molecules were upregulated in an IFNγ-independent manner, such as VLA-4 (CD49d), MAC-3 (LAMP-2, CD107b) [17] and to some extent MAC-2 (Galectin-3) [24]. These data show that both IFNγ dependent as well as IFNγ independent pathways alter the cell surface phenotype of monocytes in CD70TG mice and generate a circulating monocyte pool that has several key features of activation.

Monocytes from CD70 TG mice have normal migratory capacity *in vitro*

Given the changes in cell surface receptors necessary for monocyte homing (Figure 1E), we investigated if myeloid cells from CD70 TG mice would have an altered migratory capacity. First, we analyzed the ability of monocytes to transmigrate *in vitro*. CD70 TG derived monocytes showed a comparable chemotactic response to MCP-1 as WT monocytes (Fig. 2A). We also found that CD70 TG derived monocytes have an increased cytoskeletal staining with phalloidin than WT monocytes (data not shown), which reflects their increased activation and might indicate an increased cellular rigidity. However, decreasing the pore size of the transwells (3.0 μm instead of the standard 5.0 μm) did not show any differences either

in the chemotactic ability of monocytes from CD70 TG compared to WT mice (data not shown). These data indicate that CD70 TG derived monocytes have a normal capacity to transmigrate in response to the inflammatory chemokine MCP-1 in vitro and are not negatively affected by potential cytoskeletal changes.

CD70 TG mice do not accumulate monocytes during acute inflammation

To fully assess possible changes in the monocyte pool from CD70 TG mice, we analyzed their migratory capacity *in vivo*. To this end, sterile peritonitis was induced by i.p. injection of thioglycollate and the accumulation of myeloid cells in the peritoneal cavity was measured. The peak of granulocyte influx is generally reached 4 hours after thioglycollate injection, whereas the peak of monocyte/macrophage influx is reached 48 hours after peritonitis induction [25, 26]. CD70 TG mice show normal cell numbers in the peritoneal cavity prior to and 4 hours after peritonitis induction (Figure 2B). However, after 48 hours CD70 TG mice showed lower total cell numbers recruited to the peritoneal cavity and this decrease resulted from a diminishment in monocyte/macrophage accumulation (Figure 2B). As this accumulation is fully dependent on local production of MCP-1 [27], we tested MCP-1 production by peritoneal macrophages, but found that this was not affected in CD70TG mice (Figure 2C). In summary, these findings suggest that constitutive CD27-CD70 interactions directly and/or indirectly affect monocyte accumulation at the site of acute inflammatory responses.

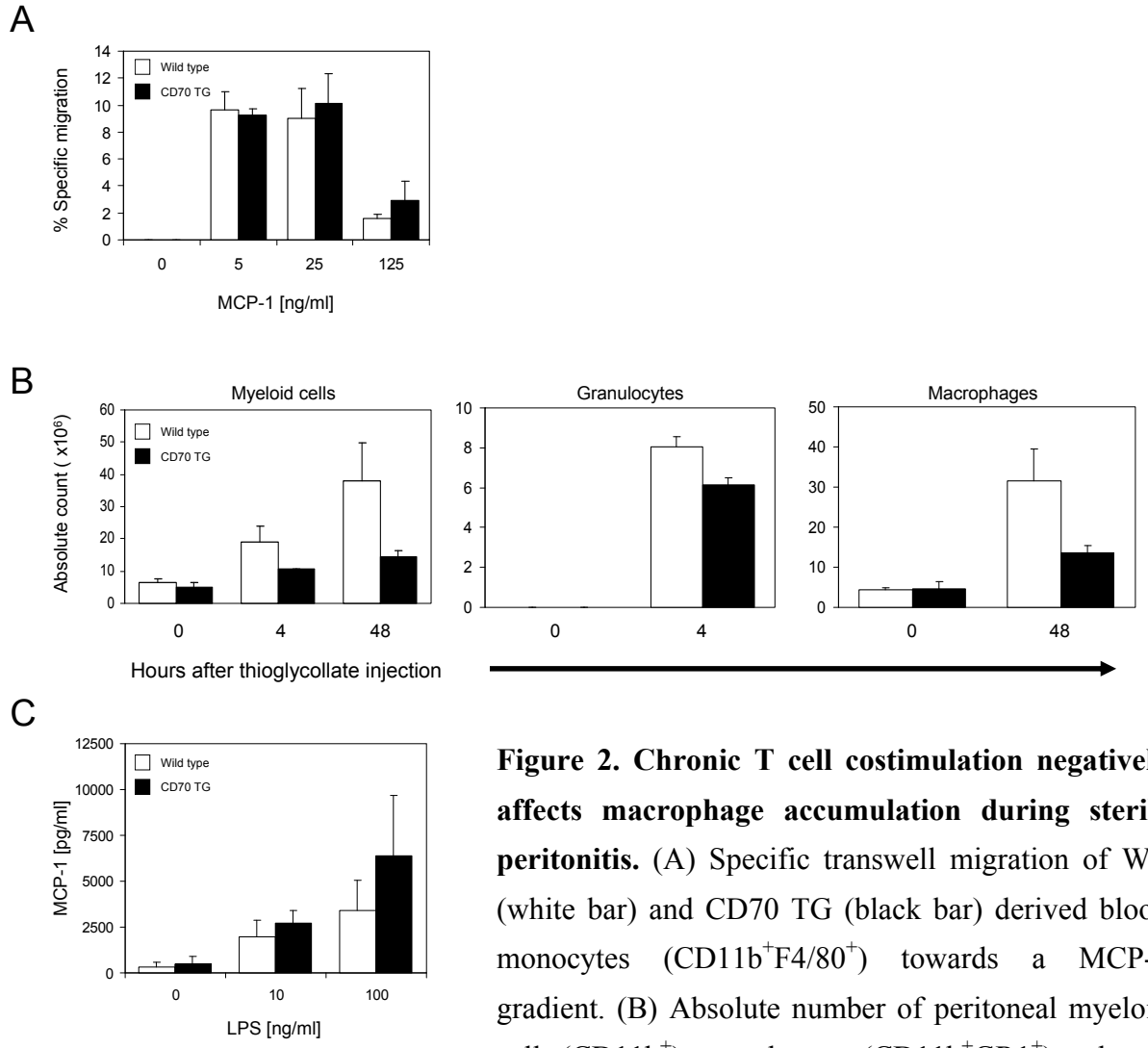

Figure 2. Chronic T cell costimulation negatively affects macrophage accumulation during sterile peritonitis. (A) Specific transwell migration of WT (white bar) and CD70 TG (black bar) derived blood monocytes ($CD11b^+F4/80^+$) towards a MCP-1 gradient. (B) Absolute number of peritoneal myeloid cells ($CD11b^+$), granulocytes ($CD11b^+GR1^+$) and macrophages ($CD11^+F4/80^+$) prior to, 4 hours and 48 hours after i.p thioglycollate injection. (C) The production of MCP-1 by WT (white bar) and CD70 TG (black bar) derived peritoneal macrophages after 24 hour culture with medium or LPS. Data are representative of two independent experiments of 3 mice per group. Asterisks denote a significant difference (* $p<0.05$) as determined by Student's *t*-test.

CD70 TG mice do not accumulate monocytes during chronic inflammation

To examine the effects of enhanced activation on monocyte accumulation during chonic inflammation *in vivo*, we investigated the formation of atherosclerotic plaque development in a well-established murine atherosclerosis model. We crossed CD70 TG mice with atherosclerosis-prone ApoE*3-Leiden mice, which develop atherosclerosis in a monocyte-

dependent fashion following a high cholesterol/fat-diet [28]. CD70 TG x ApoE*3-Leiden and ApoE*3-Leiden mice were analyzed after a 12, 16 or 20 week period of high cholesterol/fat-diet for the development of lesion formation in the aortic root. Representative sections of the aortic root are shown in figure 3A. Strikingly, CD70 TG x ApoE*3-Leiden mice developed hardly any atherosclerotic lesions, whereas the control ApoE*3-Leiden mice developed large atherosclerotic lesions (Figure 3A-B). We confirmed that also CD70 TG x ApoE*3-Leiden had the expected increase in serum cholesterol levels on a high cholesterol/fat-diet (Figure 3C). Furthermore, CD70 TG x ApoE*3-Leiden mice showed similar to CD70 TG mice a marked decrease of their B cells (Figure 3D) and consequently did not produce atheroprotective antibodies against oxidized LDL (ox-LDL) [28] (Figure 3E). In conclusion, these data suggest that enhanced activation of circulating monocytes results in a sustained protective effect against atherosclerotic lesion development, even in the absence of atheroprotective antibodies.

The enhanced maturation state of monocytes from CD70 TG mice does not influence their phagocytic capacity.

Next, it was examined if the enhanced activation state of circulating monocytes in CD70 TG mice was accompanied by changes in monocyte function. First the phagocytic capacity of these cells was measured *in vitro*, by culturing full blood from WT and CD70 TG mice in the presence of ox-LDL (the biological compound responsible for atherosclerotic lesion formation) or zymosan (heat-killed yeast particles) and analysis of the specific uptake of these compounds by monocytes. We found that monocytes from CD70 TG mice have a similar ability to phagocytose ox-LDL as well as zymosan compared to WT mice (Figure 4A-B).

Circulating ox-LDL levels correlate with lesion size, as uptake of ox-LDL by monocytes/macrophages [28] in atherosclerotic lesions is accompanied by foam cell formation[28].

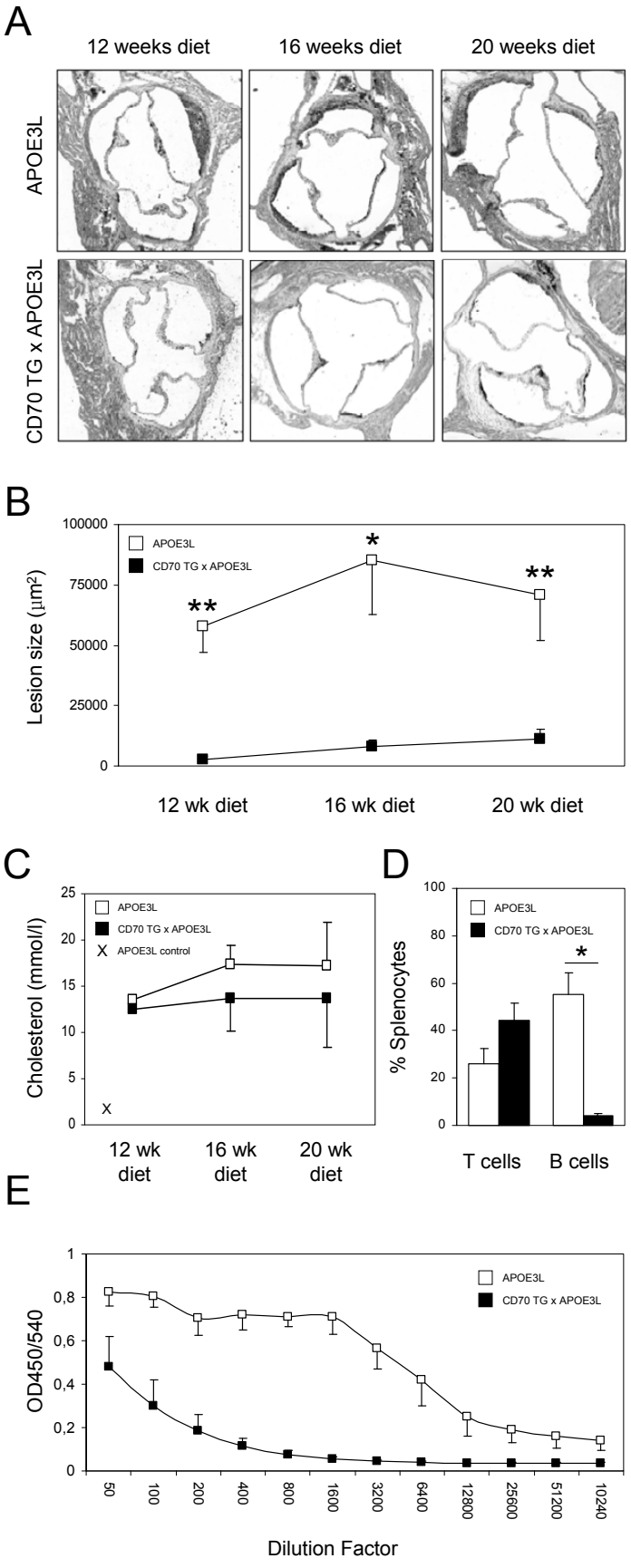

Figure 3. Constitutive CD70-CD27 signaling decreases lipid deposition during athero-sclerotic development.

(A) Representative lipid stainings of the aortic roots of ApoE*3-Leiden and CD70 TG x ApoE*3-Leiden mice after 12, 16 and 20 weeks of cholesterol rich diet. (B) Average lesion size in the aortic roots of ApoE*3-Leiden (white box) and CD70 TG x ApoE*3-Leiden (black box) mice. (C) Serum cholesterol levels in ApoE*3-Leiden (white box) and CD70 TG x ApoE*3-Leiden (black box) mice following the specified cholesterol rich diet. (D) Average percentage of splenic T and B cells in ApoE*3-Leiden (white bar) and CD70 TG x ApoE*3-Leiden (black bar) mice. (E) Serum levels of atheroprotective antibodies against oxidized LDL in ApoE*3-Leiden (white box) and CD70 TG x ApoE*3-Leiden (black box) mice. Error bars indicate the standard deviation of 8 to 10 mice per group. Asterisks denote a significant difference (* $p<0.05$, ** $p<0.01$) as determined by Student's *t*-test.

To determine if CD70 TG derived monocytes processed ox-LDL to a similar extent as their WT counterparts, we cultured WT and CD70 TG derived monocytes with ox-LDL for 5 days and assessed foam cell formation by oil-red O staining. The development of foamy macrophages was similar for WT and CD70 TG mice and was nearly seen for all macrophages in both cultures (Fig. 4C shows a representative picture). Thus, altered maturation state of monocytes in CD70 TG mice through constitutive CD27-CD70 interactions does not affect their function as measured by particle uptake and differentiation into foamy macrophages.

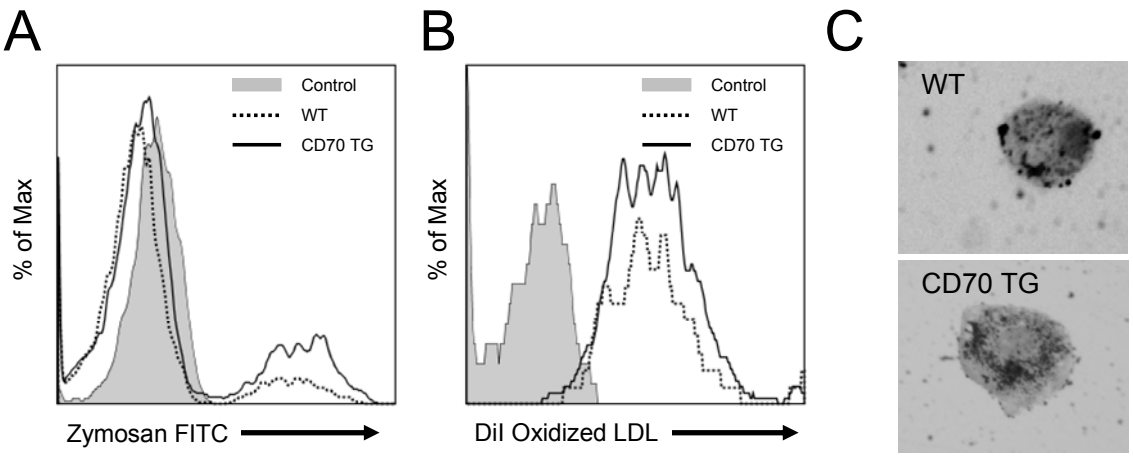

Figure 4. Normal phagocytic capacity of activated monocytes and macrophages in CD70 TG mice. Peripheral full blood was incubated in the absence or presence of DiI oxidized LDL or zymosan FITC. (A) Representative histogram for zymosan FITC and (B) DiI oxidized LDL phagocytosis from WT (dotted line) and CD70 TG (black line) derived monocytes. (C) Representative picture of foam cell formation following oxidized LDL uptake in WT and CD70 TG derived macrophages. Foam cell formation was determined by oil red O staining and shown to be uniform for the entire culture and comparable between WT and CD70 TG. Data are representative of two independent experiments of 3 mice per group.

Chronic CD27-CD70 signaling decreases cell death resistance of monocytes

Since the enhanced activation status and unaffected migratory potential in vitro appear to contradict the diminished accumulation of monocytes/macrophages at inflammatory sites in CD70TG mice, we considered the possibility that monocytes have an increased susceptibility for apoptosis during chronic immune activation, which would block their accumulation during inflammation. Indeed, our phenotypic analysis (Fig. 1E) revealed increased FAS expression on monocytes from CD70 TG mice. Therefore, we determined monocyte viability by measuring their mitochondrial membrane potential, as this is rapidly lost when cells go into apoptosis. The absolute number of circulating monocytes in CD70 TG mice was comparable to that of WT mice (5,2 x $10^5 \pm 2$ vs 6,4 x $10^5 \pm 4$ monocytes/ml) and no major differences were observed in cellular viability when measured directly *ex vivo* (Fig. 5 A-B). However, CD70 TG derived monocytes rapidly died upon overnight culture, which was dependent on IFNγ, as it was not seen in monocytes of CD70TG x IFNγ$^{-/-}$ mice (Fig. 5A-B). This survival defect was specific for monocytes, as it was not seen for granulocytes present in the same cultures (Fig. 5A-B). This indicates that CD70-driven immune activation and the resulting enhanced levels of IFNγ may suppress monocyte accumulation at inflammatory sites via enhanced susceptibility to cell death.

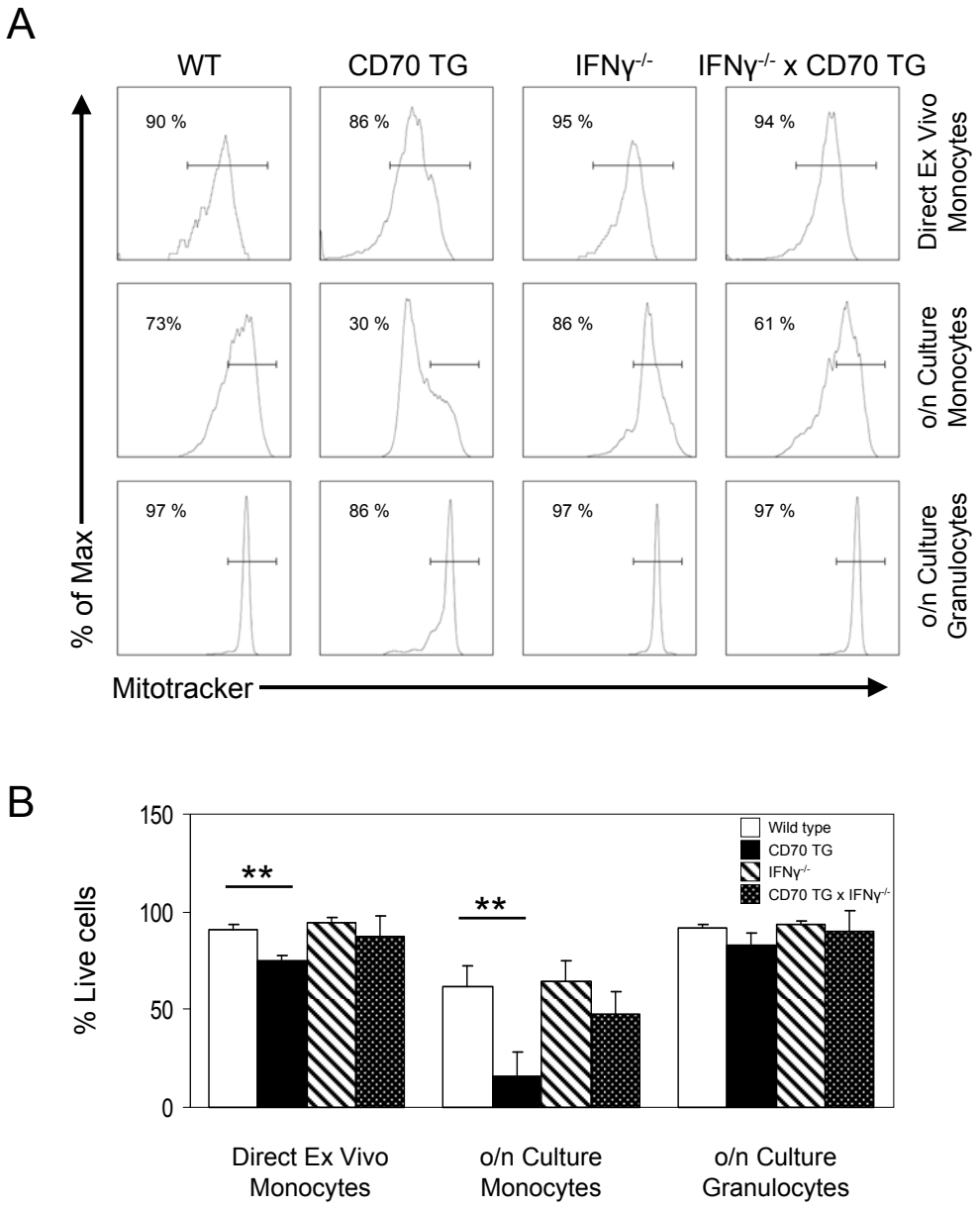

Figure 5. Monocytes from CD70 TG mice are more prone to apoptosis in an IFN-γ dependent manner.

(A) Representative staining for mitochondrial potential with mitotracker for monocytes ($CD11b^+F4/80^+$) and granulocytes ($CD11b^+F4/80^-$) of WT, CD70 TG, $IFN\gamma^{-/-}$ and $IFN\gamma^{-/-}$ x CD70 TG mice. (B) Average percentage of live monocytes ($mitotracker^+CD11b^+F4/80^+$) directly ex vivo and after an overnight culture; and granulocytes ($mitotracker^+CD11b^+F4/80^-$) after an overnight culture for WT (white bar), CD70 TG (black bar), $IFN\gamma^{-/-}$ (hatched bar) and CD70 TG x $IFN\gamma^{-/-}$ (dotted bar) mice. Error bars indicate the standard deviation of 3 mice per group. Asterisks denote a significant difference (** $p<0.01$) as determined by Student's *t*-test.

Discussion

Ligation of CD27 by its unique ligand CD70 has been shown to have a strong potentiating effect on T cell proliferation, survival and IFNγ production (reviewed in [1]). Moreover, previous studies have identified a direct effect on the colony forming potential and outgrowth of hematopoietic stem cells (HSC) and early progenitor cells [29]. Here, we provide evidence that constitutive ligation of CD70 to its receptor CD27 indirectly alters the monocyte pool predominantly via an IFNγ-dependent mechanism. Circulating monocytes display an activated phenotype and fail to accumulate at sites of inflammation most probably due to their enhanced propensity for apoptosis.

To resolve the issue of the effects of chronic costimulation on the function of monocytes and macrophages *in vivo*, we analyzed experimental models in which acute and chronic recruitment of monocytes can be analysed. The induction of sterile peritonitis via i.p injection of thioglycollate is generally accepted as an acute inflammatory model to investigate monocyte and granulocyte recruitment [26, 30]. We found a significant decrease of monocyte and macrophage accumulation in the peritoneum of CD70 TG mice, but we could not definitively settle the role of IFNγ, as the CD70 TG mice on an IFNγ-deficient background developed a lethal inflammatory response and could not be analyzed for peritoneal infiltrates (data not shown). In mice prone to atherosclerotic plaque formation, a model for chronic inflammation, we found that CD70 TG x ApoE*3-Leiden mice were less susceptible to diet induced atherosclerosis compared to ApoE*3-Leiden mice. Additional studies to clarify the role of IFNγ on monocyte migratory capacity and accumulation should be performed in this setting, but will be hampered by the fact that IFNγ also functions as a monocyte maturation factor in WT mice and is indispensable for the development of atherosclerotic lesions in this model [31].

We found that CD70 driven costimulation results in an IFNγ dependent loss of cells and of mitochondrial potential after a short period of *in vitro* culture. To resolve the mechanism of

cell death, we first investigated the FAS-FASL apoptotic pathway. Although FAS expression was upregulated on monocytes from CD70 TG mice in an IFNγ dependent manner (Figure 1E), we were not able to block the loss of mitochondrial potential *in vitro*, nor the monocyte/macrophage accumulation in the peritonitis model, using FASL blocking antibodies (data not shown). In addition, the pan-caspase inhibitor QVD did not rescue survival of CD70 TG monocytes (data not shown), which could suggest that this is a caspase-independent mechanism. Finally, since also the GSK inhibitor SB216763, that would interfere with the process of autophagy [32], did not rescue cell vitality (data not shown), the mode of monocyte death in CD70 TG mice remains elusive.

In human peripheral blood two distinct subsets of monocytes can be distinguished via CD14 and CD16 expression. $CD14^{++}CD16^{-}$ monocytes account for approximately 90% and $CD14^{+}CD16^{+}$ cells for the remainder of the monocyte population [33]. $CD14^{+}CD16^{+}$ monocytes have lower levels of CD11b, CD33 and CD64 and higher levels of MHC class II, VLA-4 and ICAM-1 [34], suggesting a more mature, activated phenotype than the $CD14^{++}CD16^{-}$ monocytes. In several pathological situations, including sepsis [35] and tuberculosis [36], a significant increase in human peripheral $CD14^{+}CD16^{+}$ blood monocytes is found. Moreover, HIV-infected individuals also show a marked increase in $CD14^{+}CD16^{+}$ blood monocytes [37, 38], which increases during disease progression [39, 40]. It thus appears that HIV-infected people and CD70 TG mice are strikingly similar with respect to the presence of an activated monocyte pool in the circulation. Likewise, both CD70 TG mice and HIV infected individuals are susceptible to opportunistic pulmonary infections such as *Pneumocystis carinii* pneumonia[14, 41]. Our data now demonstrate that chronic costimulation results in impaired monocyte accumulation at inflammatory sites and which could thereby contribute to in an increased susceptibility for opportunistic infections. This would suggest that specific

inhibition of IFNγ signaling pathways in lymphocytes could improve the innate immune response in patients with chronic active infection.

Materials and Methods

Mice

CD70 TG, IFNγ$^{-/-}$, CD70TG x IFNγ$^{-/-}$, ApoE*3-Leiden and wild-type mice, all on a C57Bl/6 background, were maintained at the animal department of the Academic Medical Center (Amsterdam, The Netherlands). For the induction of atherosclerosis, CD70 TG mice were backcrossed on a ApoE*3-Leiden-background. From these crosses, the female ApoE*3-Leiden^{+} littermates (WT and CD70 TG) mice were used and were fed a high cholesterol/fat diet (1% cholesterol, 18% fat, Purif Diet W, 4021.36, Hope Farms, Woerden, The Netherlands). Identification of mutant mice was performed by PCR analysis of tail DNA or by FACS analysis of peripheral blood cells. All mice used were 8-10 weeks of age at the onset of the experiment and were handled in accordance with institutional and national guidelines. All experimental protocols were approved by the Ethics Committee for Animal Experiments of the Academic Medical Centrum in Amsterdam.

Antibodies and conjugated reagents

The following monoclonal antibodies were obtained from Pharmingen: allophycocyanin-conjugated (APC) anti-CD11b (clone M1/70), peridinin chlorophyll protein-conjugated (PerCP) anti-B220 (clone RA3-6B2), Fluorescein isothiocyanate-conjugated (FITC) anti-CD3ε (clone 17A2), PerCp-conjugated anti-CD4 (clone L3T4), APC-conjugated anti-CD8 (clone Ly-2), phycoerythrin-conjugated (PE) anti-GR-1 (clone RB6-8C5), PE-conjugated FAS (clone Jo2) and PE- or APC- conjugated anti-CD62L (clone MEL-14). The following antibodies were purified from hybridoma supernatants and conjugated to FITC or biotin

according to standard procedures: biotinylated anti-MHC class II (clone M5-114) and FITC-conjugated CD70 (clone 3B9). For the detection of biotinylated antibodies, streptavidin-PE (Caltag Laboratories, CA), streptavidin-APC (Pharmingen) or streptavidin-conjugated PerCP-Cy5.5 (SAv-PerCP-cy5.5., Pharmingen) was used. Antibody used from eBioscience: FITC-conjugated anti-F4/80 (clone BM8). Non-conjugated antibodies used: anti-VLA-4 (clone PS/2, a kind gift from Dr. Reina Mebius), anti-MAC2 and anti-MAC-3 (both a kind gift from Dr. Reina Mebius), anti-CCR5 (clone MC-68, a kind gift from Dr. Matthias Mack [42]) and anti-LFA-1 (a kind gift from Dr. Yvette van Kooyk). Antibody used from Sanbio: PE-conjugated anti-CX3CR1 (clone 2A9-1). For the detection of non-conjugated antibodies a PE- or FITC-conjugated Donkey anti Rat (Jackson) or Goat anti-Rabbit (Southern Biotechnology Associates) was used. Intracellular stainings for FITC-conjugated phalloidin (Sigma) were performed subsequent to surface stainings followed by fixation and permeabilization (Becton Dickinson). Cells were then incubated for 30 min with phalloidin-FITC, thoroughly washed and analyzed by flow cytometry. When possible, Fc-receptor-mediated binding was blocked by preincubation with the 2.4G2 anti-FcγRII/III receptor (kind gift from Dr. Louis Boon, Bioceros, Utrecht, The Netherlands).

Flow cytometry

Single-cell suspensions from spleen were obtained by mincing through cell strainers. Blood (± 700 µl) was obtained by heart puncture and mixed with 10 µl heparine (5000 IE/ml, LEO Pharma bv). Erythrocytes were lysed with an ammonium chloride solution and cells were subsequently counted. Cells (5 x 10^5) were collected in 96-well V-bottomed plates in staining buffer (PBS with 0.5% bovine serum albumin) and stained for 30 min at 4˚C with antibodies in the presence of anti-CD16-CD32 (FcBlock, clone 2.4G2, PharMingen). Data acquisition was done with FACSCalibur and data were analyzed with Flowjo software (Tree Star).

Phagocytosis assay

Approximately, 700 µl blood was obtained by heart puncture and mixed with 10 µl heparin (5000 IE/ml). To determine the phagocytic capacity of blood monocytes, 100 µl blood was cultured for 3 hours at 37˚C in a total volume of 1 ml Iscove's modified dulbecco's medium (IMDM) + 10% FCS in the presence or absence of DiI-labeled oxidized LDL (5 µg/ml, Intracel) or FITC-conjugated zymosan. Next, cells were washed, erythrocytes were lysed with an ammonium chloride solution and leukocytes were subsequently stained for analysis by flow cytometry as described above.

Fluorescent zymosan was prepared as follows: unlabeled zymosan (a kind gift from Dr. Esther de Jong, AMC, The Netherlands) was resuspended in PBS at 25 mg/ml and heated for 30 min at 100°C. This suspension was pelleted by centrifugation and washed 2x with sterile PBS. Subsequently, the zymosan particles were resuspended at 5 mg/ml in PBS and incubated with 2,5 µg/ml fluoroscein-isothiocyanate (FITC; Sigma) for 45 minutes at RT. Finally, zymosan particles were washed 5x in sterile PBS, resuspended to a concentration of 5 mg/ml and stored at -20°C.

Foam cell formation

To determine foam cell formation, 100 µl blood was cultured in a total volume of 200 µl medium (IMDM 10% FCS) in the presence of 100 µg/ml ox-LDL for 5 days at 37°C (5% CO2). Foam cell formation was visualized by oil red O (Sigma) staining.

Chemotaxis assay

Blood was obtained by heart puncture (±700 µl blood in 10 µl Heparine, 5000 IE/ml) after which specific chemotaxis towards a MCP-1 gradient was determined via transwell assay. 120 µl full blood was loaded in triplicate into 5 µm pore size polycarbonate transwell inserts

(Costar, Corning). The lower well was loaded with 600 µl medium (IMDM, 10% FCS) with 0, 5, 25 or 125 ng/ml MCP-1 (R&D). After 4 hours incubation at 37°C (5% CO2) cells from the lower compartment were washed, erythrocytes were lysed with an ammonium chloride solution and leukocytes were subsequently stained for analysis by flow cytometry as described above. MCP1-specific migration was calculated as (the average amount of cells which migrated towards MCP-1) minus (the average amount of cells which migrated to medium) divided by the average amount of cells applied to the transwell.

MCP-1 production assay

Peritoneal cells were obtained by peritoneal lavage with 4 ml cold IMDM, 5% FCS. Peritoneal cells were counted and plated in a 6 well-plate at 6 x 10^5 cells per well in a total volume of 200 µl for 2 hours at 37°C, 5% CO_2. Non-adherent cells were removed by washing with medium and remaining cells were cultured for 24 hours in a total volume of 2 ml medium (IMDM, 10% FCS) with 0, 10 or 100 ng/ml LPS. Supernatants were collected and MCP-1 levels were determined by MCP-1 ELISA (Pharmingen; kindly provided by Dr. Tom van der Poll).

Thioglycollate-induced peritonitis

Mice were injected with 1 ml 3% (weight/volume) thioglycollate solution (T9032, Sigma) intraperitoneally. Four hours or 48 hours after injection, the total number of recruited peritoneal cells were collected by washing the peritoneum with 4 ml of sterile saline buffer. Peritoneal cells were counted and analyzed by flow cytometry as described above.

Serum cholesterol analysis

Mice underwent a fasting period of 4-12 hours after which blood was isolated. The concentrations of total cholesterol in the serum were determined according to the manufacturer's instructions (bioMerieux, France). A cholesterol calibrator (standardized serum; bioMerieux) was used as internal standard.

Measurement of oxidized LDL antibodies

The antibody titers were determined by an enzyme-linked immunosorbent assay. 96 wells plates were coated overnight at 4°C with oxidized LDL (10 µg/ml) (RP-047, Intracel) in coatings buffer (0.1 M NaH_4CO_3). Subsequently, plates were blocked with 5% BSA for 2 hours at room temperature. Sera from CD70 TG x ApoE*3-Leiden and ApoE*3-Leiden mice were added into duplicate wells in various dilutions for 2 hours at room temperature. The quantification of bound immunoglobulin was performed by incubating the plates with a biotin-conjugated goat anti mouse IgG (1034-08, SBA) followed by a 20 minute incubation at room temperature with streptavidin-conjugated horse radish peroxidase (P0397, DAKO). 50 µl substrate (10 ml NaAc, 100 µl Tetramethylbenzidine (6 mg/ml in DMSO, VWR), 10 µl 3% H_2O_2) was added and the enzyme reaction was terminated after approximately 25 minutes by the addition of 25 µl 2 N H_2SO_4. The optical density was measured at 450 and 540 nm using a microplate reader. Between the various steps the plate underwent extensive washing.

Histological analysis of hearts and aortas for atherosclerosis

Atherosclerotic lesion development was analyzed after completion of the high cholesterol/fat diet. Mice were anesthetized by i.p. injection of FFM mix (1 part hypnorm, 1 part dormicum, 2 parts H_2O). Hearts and aortas were perfused in situ with phosphate-buffered saline for 10 min via a cannula in the left ventricle. This was followed by a 10 min post-perfusion fixation

with 1% neutral-buffered formalin (Formal-Fix, Shandon Scientific UK) and subsequent storage of the hearts and aortas in formalin. Hearts were bisected at the level of the atria and the base of the heart was taken for analysis. Cryostat 7 µm cross-sections of the aortic root were made and stained with oil red O (Sigma). Quantification of the atherosclerotic lesion area in the sections was performed by Q-win analysis (Leica). The mean lesion area was calculated (in μm^2) from ten sections, starting at the appearance of the tricuspid valves.

Analysis of viable cells via mitochondrial activity

Cellular viability of the myeloid compartment was determined following incubation of 50 µl blood in a total volume of 200 µl IMDM with 200 nM MitoTracker Orange (Molecular Probes, Leiden, The Netherlands) for 30 minutes at 37°C. Cells were subsequently washed, stained on ice for cell surface molecules and analyzed by flow cytometry as described above.

Statistical analysis

Statistical analysis of the data was performed using an unpaired Student's *t*-test.

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Disclosures

The authors have no financial/commercial conflict of interests.

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Chapter 5

Protective CD8 T cell memory is impaired during chronic CD70-driven co-stimulation

Klaas P.J.M. van Gisbergen [*], Ronald W. van Olffen [*], Josine van Beek [†], Koenraad F. van der Sluijs [‡], Ramon Arens [§], Martijn A. Nolte [*] and René A.W. van Lier [*].

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[*] Department of Experimental Immunology and [‡] Department of Pulmonology, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

[†] Department of Immunopathology, Sanquin Research at CLB and Landsteiner Laboratory, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands.

[§] Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla CA 92037, USA.

Abstract

Chronic infection results in continuous formation and exhaustion of effector CD8 T cells and in failure of memory CD8 T cell development. Expression of CD70 and other molecules that provide co-stimulation to T cells is maintained during chronic infection. To analyze the impact of constitutive CD70-driven co-stimulation, we generated transgenic mice expressing CD70 specifically on T cells. We show that CD70 promoted accumulation of CD8 T cells with characteristics strikingly similar to exhausted effector CD8 T cells found during chronic infection. CD70 on T cells provided co-stimulation that enhanced primary CD8 T cell responses against influenza. In contrast, memory CD8 T cell maintenance and protection against secondary challenge with influenza was impaired. Interestingly, we found no effect on the formation and maintenance of either effector or memory CD4 T cells. We conclude that constitutive expression of CD70 is sufficient to deregulate the CD8 T cell differentiation pathway of acute infection reminiscent of events in chronic infection.

Introduction

CD8 T cells significantly contribute to immune responses that resolve acute viral infection. Activation of naïve CD8 T cells involves proliferation and differentiation into antigen-specific effector CD8 T cells. These effector CD8 T cells acquire effector functions such as the production of immune stimulatory IFN-γ and cytolytic agents that enables them to eliminate virally infected cells. The effector CD8 T cell population contracts upon antigen clearance, and the remaining CD8 T cells that survive in the absence of antigen provide enhanced protection against secondary challenge (1,2).

CD8 T cell differentiation is perturbed during chronic infection and this becomes apparent in exhaustion of effector function and in lack of memory development (3,4). Upon re-stimulation, virus-specific CD8 T cells in chronic lymphocytic choriomeningitis virus (LCMV) models display poor proliferation and cytotoxicity and low IL-2 and IFN-γ production (4). CD8 T cell exhaustion has been associated with up-regulated levels of inhibitory molecules such as PD-1 and IL-10 on CD8 T cells of HIV and hepatitis C virus patients as well as on CD8 T cells in experimental infection models with chronic LCMV (5-7). Blockade of PD-1 or IL-10R-mediated signals in chronic LCMV infection establishes pathogen clearance, showing that these inhibitory molecules are involved in development of CD8 T cell exhaustion (8-10). The underlying mechanism why memory CD8 T cells fail to develop during chronic infection, however, is less well understood. Low expression of IL-7Rα and IL-2/15Rβ on CD8 T cells during chronic infection indicates inefficient maintenance on homeostatic cytokines in the absence of antigen (3,11). Indeed, transfer of antigen-specific CD8 T cells of chronically infected animals to naïve animals results in disappearance of transferred CD8 T cells. This shows that removal of antigen is insufficient to restore memory formation (3).

The persistence of pathogens during chronic infection results in continual triggering of Toll-like receptors that constitutively up-regulate expression of co-stimulatory molecules and production of pro-inflammatory cytokines. One of the up-regulated pathways of co-stimulation is mediated through CD70 and CD27. CD70 is the unique ligand of the TNF receptor superfamily member CD27 that is expressed on naïve CD4 and CD8 T cells (12,13). CD70 induced triggering of CD27 enhances the proliferative capacity of T cells and the acquisition of effector functions, such as the production of IFN-γ (14-16). Primary and secondary CD8 T cell responses against influenza infection as well as secondary responses against acute LCMV infection are impaired in the absence of CD27, demonstrating that CD70 driven co-stimulation is important for in vivo immune responses (17,18). Expression of CD70 is restricted under homeostatic conditions but upon infection such as with influenza or acute LCMV it is found on mature DCs and activated B and T lymphocytes (17,19). In contrast to transient expression of CD70 in acute infection, constitutive expression of CD70 occurs in chronic HIV-1 infection and chronic autoimmune disease (20-22). The constitutive CD70 expression is primarily detected on T cells (20-22). To dissect the effects of constitutive expression of CD70 specifically on T cells, we generated transgenic (Tg) mice expressing CD70 under a T-cell specific promoter. We found that constitutive expression of CD70 on T cells drives antigen-dependent formation of CD8 T cells that strikingly resemble the phenotype of effector CD8 T cells during chronic infection. In particular, CD70-driven co-stimulation resulted in enhanced primary CD8 T cell responses, but impaired memory CD8 T cell responses against acute influenza infection. This demonstrates that constitutive expression of CD70 de-regulates CD8 T cell differentiation compatible with events in chronic infection and identifies CD70 as a target for intervention in HIV-1 and other chronic diseases.

Results

CD70 on T cells regulates peripheral T cell homeostasis

To study the impact of persistent CD70 expression on T cells as described in chronic infections (22), we generated Tg mice that express CD70 under control of the human CD2 promoter (Fig. 1 A). This resulted in expression of CD70 protein on T cells, but not on other cell types (Fig. 1 B). Because the transgene expression of CD70 was low on heterozygous Tg T cells, we generated homozygous Tg mice, displaying T cell-specific CD70 expression at higher levels than heterozygous Tg mice (Fig. 1 B). The levels of CD70 on T cells of homozygous Tg mice were comparable to expression of CD70 on T cells during chronic infection (22). This prompted us to use homozygous Tg mice throughout the study.

The CD70 transgene was expressed early during development of T cells within the thymus, and was present on all thymocyte subsets (unpublished data). In WT mice, CD27 is expressed on thymocytes as well as on T cells (12). In contrast, CD70 Tg mice did not have expression of CD27 on thymocytes, indicating that CD70 induced triggering and shedding of CD27 within the thymus (unpublished data). Interestingly, this did not induce apparent changes in thymocyte development, and thymocyte subsets were similar in size in WT and CD70 Tg mice (Fig. 1 C).

The CD70 transgene was detected on T cells in spleen, peripheral lymph nodes (pLNs) and BM of CD70 Tg mice (unpublished data). The levels of CD27 were reduced on T cells within these tissues compared to WT mice (unpublished data), indicating that the CD70 transgene has engaged its ligand. We analyzed whether CD70 on T cells impacted the formation of effector memory (EM) T cells similar to CD70 on DCs and B cells (14,25). Therefore, the profile of CD44 and CD62L expression was determined on CD4 and CD8 T cells. CD44 and CD62L characterize distinct T cell populations in mice, and $CD44^{low}CD62L^{high}$ T cells are defined as naïve T cells, $CD44^{high}CD62L^{high}$ T cells as central memory (CM) T cells and

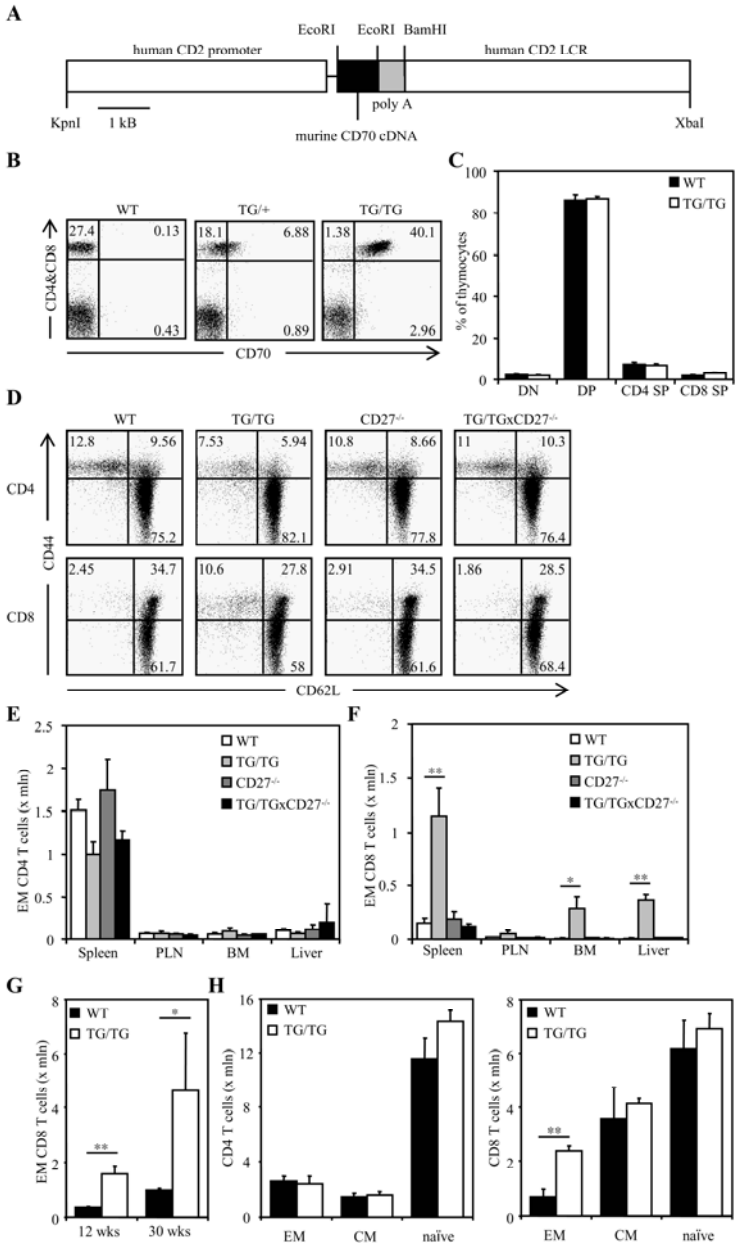

Figure 1. CD70 on T cells induces effector CD8 T cell formation. (A) The schematic representation depicts the construct used to generate the CD70 Tg mice. The cDNA of murine CD70 was cloned into the EcoRI site of a plasmid containing the human CD2 promoter and the human CD2 locus control region (LCR). Approximately 12 kb of the plasmid containing the CD2 promoter elements and the CD70 cDNA was isolated using digestion with KpnI and XbaI. (B) The expression of CD70 was analyzed on T cells within the blood of WT and heterozygous and homozygous CD70 Tg animals. (C) The composition of thymocyte subsets was determined as a percentage of the total thymocyte population in WT and CD70 Tg mice (DN: double negative, DP: double positive, SP: single positive). (D) The expression of CD44 and CD62L was analyzed on CD4 T cells (top row) and CD8 T cells (bottom row) from spleen of WT, CD70 Tg, CD27$^{-/-}$ and CD70 Tg x CD27$^{-/-}$ mice to analyze the percentage of naïve, central memory (CM) and effector memory (EM) T cells. (E, F) The absolute number of (E) EM CD4 T cells and (F) EM CD8 T cells in spleen, pLNs, BM and liver of WT, CD70 Tg, CD27$^{-/-}$ and CD70 Tg x CD27$^{-/-}$ mice was determined. (G) The absolute number of EM CD8 T cells, as determined by low expression of CD62L and high expression of CD44, was followed in time within the spleen of WT and CD70 Tg mice. (H) The absolute number of EM, CM, and naïve CD4 T cells (left panel) and CD8 T cells (right panel) was determined within the spleen of WT and CD70 Tg animals through FACS analysis of CD44 and CD62L expression. CM denotes central memory T cells and EM denotes effector memory T cells. Results shown apply to mice that were 8 wks of age unless stated otherwise. Error bars indicate standard deviation of 3 individual mice. ∗ denotes $p<0.05$ and ∗∗ denotes $p<0.005$. Results are representative of at least 3 separate experiments.

$CD44^{high}CD62L^{low}$ T cells as EM T cells (26). CD70 Tg mice had higher percentages as well as absolute numbers of EM T cells within the CD8 compartment, but not within the CD4 compartment of the spleen (Fig. 1, D-H). The absolute number of EM CD8 T cells, in contrast to that of EM CD4 T cells, was also increased within the liver and BM, but not the pLNs (Fig. 1, E and F). We observed that the absolute number of EM CD8 T cells within the spleen of CD70 Tg animals steadily increased with age, as occurred in WT animals (Fig. 1G). In contrast to EM CD8 T cells, the size of all CD4 T cell populations and of naïve and CM CD8 T cell populations were not changed in CD70 Tg mice compared to WT mice (Fig. 1H). Crossing the CD70 Tg mice onto $CD27^{-/-}$ background completely reversed the memory T cell phenotype to WT and $CD27^{-/-}$ levels (Fig. 1, D-F), showing that EM CD8 T cell formation is mediated by CD70-CD27 signaling. Thus, CD70 expression is functional on T cells and induces increased EM differentiation of CD8 T cells.

Constitutive CD70 induces CD8 T cells that resemble exhausted effector CD8 T cells

We analyzed the phenotype of EM CD8 T cells in CD70 Tg mice to examine how CD8 T cell differentiation under constitutive CD70 co-stimulation related to that of chronic infection. We observed that EM CD8 T cells of CD70 Tg mice had reduced levels of IL-7Rα and enhanced levels of CD69 and PD-1 compared to those of WT mice (Fig. 2, A and B). This was not observed on other CD4 and CD8 T cell populations (unpublished data). The low expression of IL-7Rα indicates maintenance of EM CD8 T cells independent of the homeostatic cytokine IL-7 (27), while high expression of CD69 and PD-1 indicate recent stimulation on antigen (28). Antigen-driven proliferation displays a much higher turn-over as compared to cytokine-driven homeostatic proliferation of memory CD8 T cells (29). Therefore, to establish whether T cell proliferation was enhanced during constitutive CD70 co-stimulation, we analyzed the expression of Ki-67, which is expressed by cycling cells. The expression of Ki-67 was up-

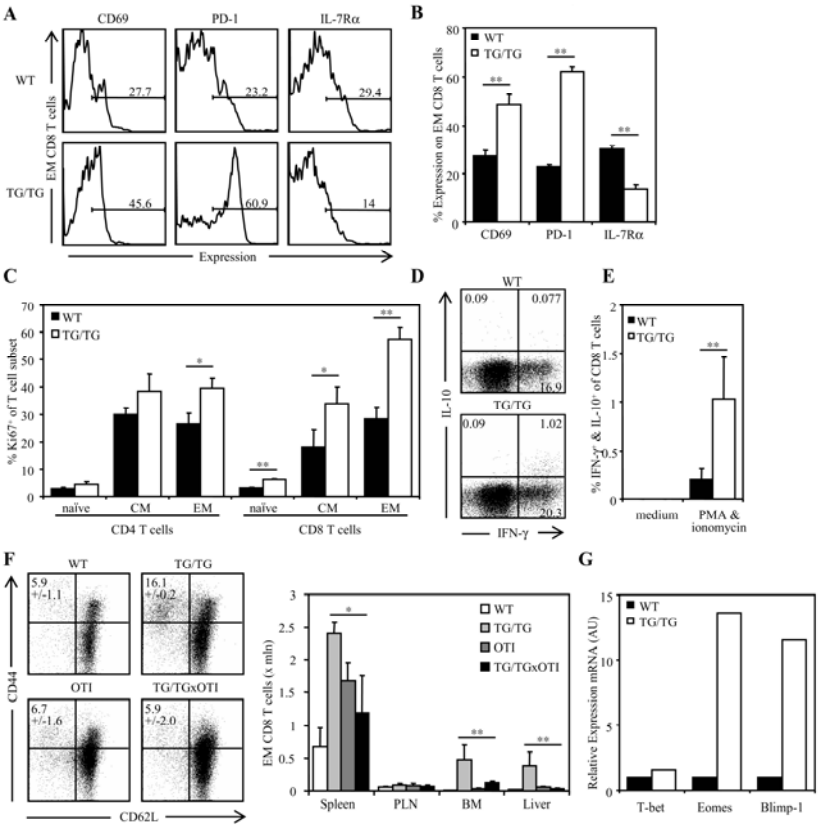

Figure 2. CD8 T cells under constitutive CD70 triggering display an exhausted phenotype. (A) Histograms show the expression of CD69, PD-1 and IL-7Rα on EM CD8 T cells of spleen of WT (top row) and CD70 Tg animals (bottom row). (B) The percentage of EM CD8 T cells that express CD69, PD-1 and IL-7Rα within spleen of WT and CD70 Tg animals was determined. (C) The percentage of CD4 and CD8 T cell subsets of spleen of WT and CD70 Tg that undergo cell division was analyzed using intracellular staining for Ki-67. CM denotes central memory T cells and EM denotes effector memory T cells. (D) Dotplots show intracellular staining for IFN-γ and IL-10 on splenocytes gated for CD8 T cells of WT (left panel) and CD70 Tg mice (right panel) that had been stimulated for 5 hrs with PMA and ionomycin. (E) The percentage of CD8 T cells of spleen of WT and CD70 Tg that co-produce IFN-γ and IL-10 upon 5 hrs of PMA and ionomycin stimulation was determined. (F) The expression of CD44 and CD62L was analyzed on CD8 T cells of spleen from WT, CD70 Tg, OTI and CD70 Tg x OTI mice (left panel). Insets in upper left corner represent average percentage of EM CD8 T cells +/- standard deviation. The absolute number of $CD44^{+}CD62L^{-}$ EM CD8 T cells in spleen, pLNs, BM and liver of WT, CD70 Tg, OTI and CD70 Tg x OTI mice was examined (right panel). (G) $CD8^{+}CD44^{+}CD62L^{-}$ splenocytes were sorted to obtain EM CD8 T cells. The expression levels of T-bet, Eomes and Blimp-1 were analyzed in EM CD8 T cells of WT and CD70 Tg mice using quantitative PCR. Results shown apply to mice that were 8 wks of age. Error bars indicate standard deviation of 3 individual mice. ∗ denotes $p<0.05$ and ∗∗ denotes $p<0.005$. Experiments were performed at least 3 times with identical results.

regulated in EM CD8 T cells of CD70 Tg mice compared to WT mice (Fig. 2 C). EM CD4 T cells and naïve and CM CD8 T cells of CD70 Tg mice also had elevated expression levels of Ki-67, but to a lesser extent (Fig. 2 C). This indicates that naïve, CM and EM CD8 T cell populations may all contribute to the generation of EM phenotype CD8 T cells in CD70 Tg mice. The unaltered Ki-67 levels of naïve and CM CD4 T cells and the marginally increased Ki-67 levels of EM CD4 T cells in CD70 Tg mice correspond with the absence of CD70-driven EM CD4 T cell formation. The expression profile of EM CD8 T cells of CD70 Tg mice is reminiscent of that of pathogen-specific CD8 T cells in chronic LCMV or HIV-1 infection: i.e. low levels of IL-7Rα (30), high levels of Ki-67 (31,32), and high levels of activation-induced molecules including CD69 (31) and PD-1 (5,6). Thus, co-stimulation through CD70 enhances the formation of CD8 T cells that phenotypically resemble antigen-dependent and rapidly proliferating effector CD8 T cells in chronic infection.

Up-regulation of inhibitory molecules is another distinctive feature of CD8 T cells of chronic infections (5-10). We observed up-regulation of the inhibitory molecule PD-1 on EM CD8 T cells of CD70 Tg mice (Fig. 2, A and B). Therefore, we also analyzed WT and CD70 Tg CD8 T cells for the intracellular expression of the inhibitory cytokine IL-10 after short-term PMA and ionomycin stimulation (Fig. 2, D and E). CD8 T cells of both CD70 Tg and WT animals produced IFN-γ, but CD8 T cells from CD70 Tg animals uniquely co-produced IL-10 (Fig. 2, D and E). Although EM CD8 T cell numbers were increased, we did not observe enhanced IFN-γ production in CD8 T cells of CD70 Tg mice compared to WT mice (Fig 2, D and E). This shows that CD8 T cells that are continually stimulated through CD70 have up-regulated levels of inhibitory molecules that in chronic infection have been shown to induce CD8 T cell exhaustion (8-10).

To examine whether the constitutive CD70-driven activation of CD8 T cells was indeed antigen-dependent, we generated OTI Tg mice co-expressing the CD70 transgene. The CD8 T

cells of OTI Tg mice contain a Tg TCR that specifically recognizes the MHC class I H-2K^b restricted ovalbumin peptide $OVA_{257-264}$ SIINFEKL (33), an antigen that they normally do not encounter. We found that the enhanced EM phenotype of the CD8 T cell compartment of CD70 Tg mice was dependent on TCR triggering, as shown by comparable percentages and absolute numbers of EM CD8 T cells of OTI Tg mice and CD70 x OTI Tg mice (Fig. 2. F). Higher levels of EM CD8 T cells were present within spleen, BM and liver but not the pLNs of CD70 Tg mice compared to CD70 x OTI Tg mice (Fig. 2F). Thus, recognition of environmental antigens is required for CD70 to drive formation of CD8 T cells with an EM phenotype.

We next analyzed whether transcription factors involved in CD8 T cell development were differentially expressed in EM CD8 T cells during constitutive CD70-driven co-stimulation. We observed that, in contrast to T-bet, Eomes and Blimp-1 were strongly up-regulated in EM CD8 T cells of CD70 Tg mice compared to those of WT mice (Fig. 2 G), as has been previously described for exhausted effector CD8 T cells (34). Taken together, our data shows that constitutive signaling through CD70 and CD27 accelerates antigen-driven formation of CD8 T cells that acquire a phenotype similar to exhausted CD8 T cells.

Poly-functional cytokine responses are impaired under CD70 driven co-stimulation

Exhausted CD8 T cells in chronic infection produce low levels of cytokines such as IFN-γ, IL-2 and TNF-α and display poor cytotoxicity upon re-stimulation (4). Poly-functional analysis of CD8 T cells in HIV-1 patients has shown that in particular the ability to produce multiple cytokines such as IFN-γ, TNF-α and IL-2 simultaneously is impaired (35). Reduction of the poly-functional CD8 T cell response correlates with poorer effector function of CD8 T cells on a per cell basis (35). Since we found up-regulation of the inhibitory molecules PD-1 and IL-10 on CD8 T cells of CD70 Tg animals, we examined the poly-functional T cell

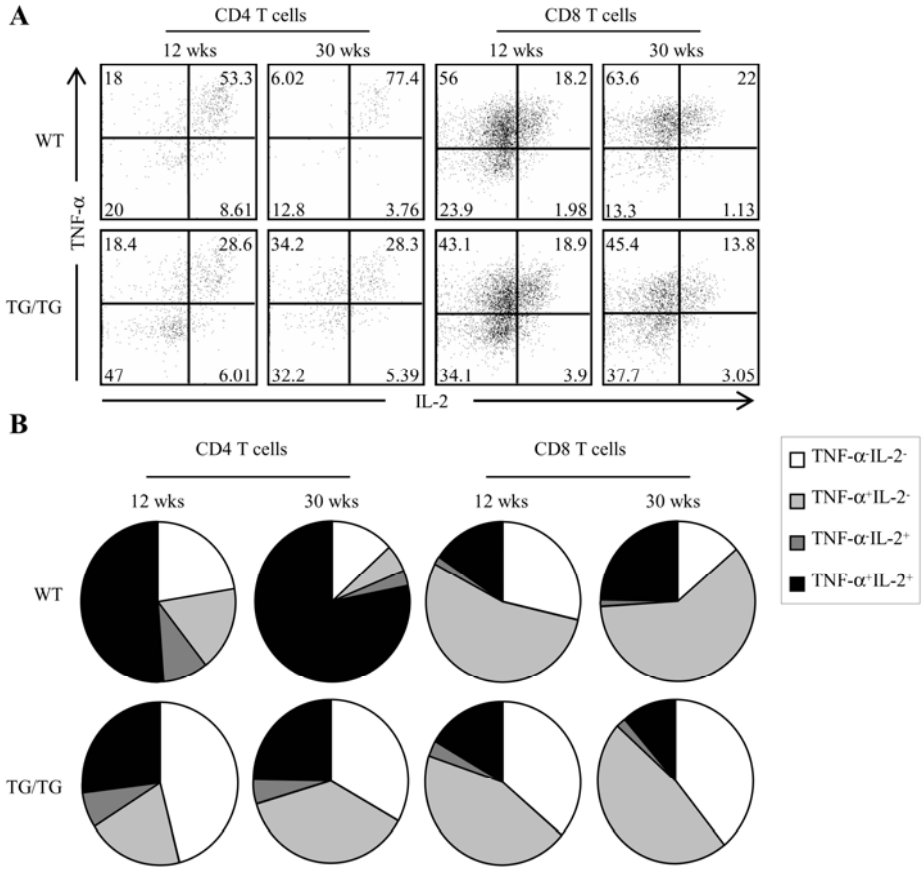

Figure 3. Constitutive CD70-driven co-stimulation compromises the poly-functional cytokine response of CD4 and CD8 T cells. The intracellular expression of IFN-γ, TNF-α and IL-2 was analyzed in CD4 T cells and CD8 T cells of spleen from WT and CD70 Tg animals upon 5 hr PMA and ionomycin stimulation. (A) Dotplots were gated on IFN-γ producing CD4 T cells or CD8 T cells and display the intracellular expression of TNF-α and IL-2 of representative WT and CD70 Tg mice of 12 and 30 wks of age. (B) Pie charts show the average percentage of IFN-γ-producing CD4 or CD8 T cells of WT and CD70 Tg mice that express only TNF-α, only IL-2, both TNF-α and IL-2 or neither TNF-α and IL-2. Graphs display results of 2 independent experiments with 3-5 mice per group. The reduction of TNF-α and IL-2 expression in CD70 Tg compared to WT mice are significant for CD4 T cells at 12 wks ($p<0.005$) and 30 wks ($p<0.005$) and for CD8 T cells at 30 wks ($p<0.05$).

response in WT and CD70 Tg mice. We observed that the IFN-γ-producing CD4 and CD8 T cell populations of CD70 Tg mice were less poly-functional than WT mice upon stimulation with PMA and ionomycin (Fig. 3, A and B). In particular, the ability of CD4 and CD8 T cells to co-produce TNF-α and IL-2 together with IFN-γ was hampered (Fig. 3, A and B). The effect was more pronounced within the CD4 T cell population and increased with age within both the CD4 and CD8 T cell population (Fig. 3, A and B). This shows that constitutive expression of CD70 on T cells induces exhaustion in CD4 and CD8 T cells.

CD70 enhances effector CD8 T cell responses against influenza

Stimulation through CD70 on APCs quantitatively and qualitatively enhances CD8 T cell responses against acute viral infection (18,36). We were interested whether constitutive CD70 on T cells was also able to enhance CD8 T cell responses. For this purpose, WT and CD70 Tg mice were intranasally infected with the influenza virus A/PR8/34 and as a measure of disease the body weight of the mice was monitored. The decrease in body weight upon influenza infection was less severe and resolved at earlier time points in CD70 Tg mice than in WT mice (Fig. 4 A). In addition, the viral loads of CD70 Tg mice were reduced compared to those of WT mice at day 10 (Fig. 4 B), but not at day 7 or 14 (unpublished data). To measure the magnitude of CD8 T cell responses, we employed tetramer staining of peripheral blood cells. Within the blood influenza-specific CD8 T cells peak at higher levels in CD70 Tg mice than in WT mice, but levels of influenza-specific CD8 T cells of CD70 Tg mice return to WT levels when infection is resolved (Fig. 4 C). At the peak of the CD8 T cell response against influenza CD70 Tg mice also contained higher numbers of tetramer$^+$ CD8 T cells than WT mice within the spleen, and mediastinal LN (mLN), but not within the lungs (Fig. 4 D). Moreover, higher numbers of CD8 T cells in spleen of CD70 Tg mice than of WT mice produced IFN-γ upon peptide re-stimulation (Fig. 4 E). Production of granzyme B and IFN-γ

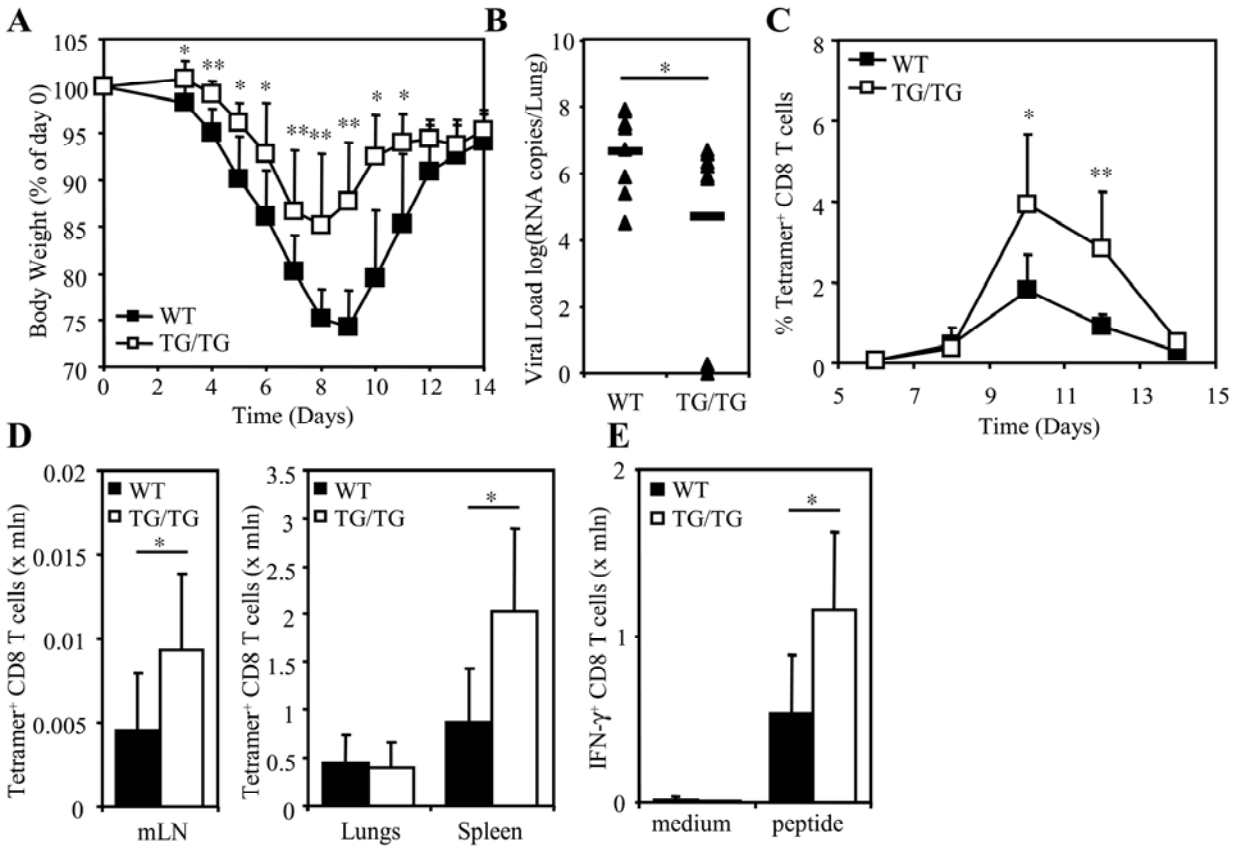

Figure 4. Enhanced primary CD8 T cell responses develop against influenza in CD70 Tg mice. WT and CD70 Tg mice were intranasally infected with the influenza virus A/PR8/34. (A) The body weight of WT and CD70 Tg mice was followed in time after primary influenza infection. (B) Viral loads within the lungs of WT and CD70 Tg mice were examined upon primary influenza infection at day 10. (C) The percentage of tetramer$^+$ CD8 T cells within the blood of influenza infected WT and CD70 Tg mice was followed in time. (D) The absolute numbers of tetramer$^+$ CD8 T cells were determined within lungs, spleen and mLN of WT versus CD70 Tg mice at day 10 of primary influenza infection. (E) The absolute numbers of IFN-γ producing CD8 T cells were determined in WT and CD70 Tg spleen of influenza-infected mice upon 5 hr re-stimulation with influenza-specific peptide and IL-2. Results shown apply to mice that were 12 wks of age at the start of the experiment. Error bar indicates standard deviation of 8 individual mice. ∗ denotes $p<0.05$ and ∗∗ denotes $p<0.005$. Comparable experiments with similar results were performed 3 times.

upon peptide re-stimulation was not different between lung-derived CD8 T cells of WT and CD70 Tg animals corroborating the tetramer analysis of the lungs (unpublished data). We have no direct evidence for involvement of CD8 T cells in the increased anti-viral response of CD70 Tg mice, although the enhanced CD8 T cell response in CD70 Tg mice correlates with improved clinical performance and viral clearance. As the influx of granulocytes, monocytes or macrophages within the lungs was not enhanced and antibody responses were not improved in CD70 Tg mice (unpublished data), this strongly suggests that the increased anti-viral response is due to the enhanced CD8 T cell response.

CD70 did not induce further up-regulation of PD-1 expression on tetramer$^+$ CD8 T cells, and did not trigger IL-10 production by CD8 T cells upon peptide re-stimulation (unpublished data). Analysis of co-production of IFN-γ, TNF-α and IL-2 upon peptide re-stimulation did not reveal differences in the poly-functional response between WT and CD70 Tg CD8 T cells of spleen and lungs (unpublished data). Taken together, this shows that constitutive CD70 on T cells provides co-stimulation, and does not induce T cell exhaustion in a setting of acute infection.

Constitutive CD70 induces waning of CD8 T cell memory against influenza

Differentiation pathways of effector and memory CD8 T cells separate early after infection. At the peak of the CD8 T cell response KLRG-1 identifies short-lived effector CD8 T cells (SLECs) and IL-7Rα identifies memory precursor effector CD8 T cells (MPECs) (27,37). Therefore, we analyzed these fractions within influenza-specific CD8 T cells at day 10 after primary influenza infection within the blood. We found that constitutive expression of CD70 enhanced the generation of SLECs as well as MPECs (Fig. 5 A). This indicates that CD70 has a positive effect on memory formation through the generation of larger numbers of memory precursors.

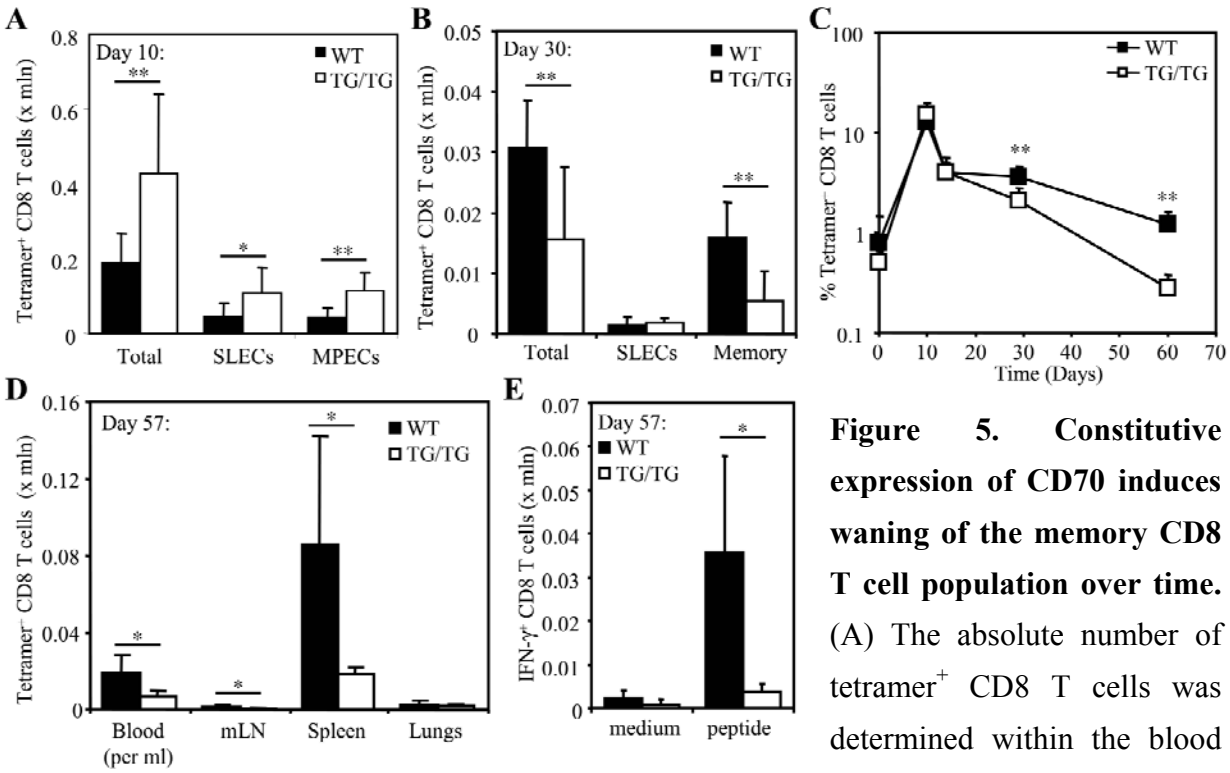

Figure 5. Constitutive expression of CD70 induces waning of the memory CD8 T cell population over time. (A) The absolute number of tetramer$^+$ CD8 T cells was determined within the blood at day 10 of primary influenza infection. Based on expression of IL-7Rα and KLRG1 the absolute number of IL-7Rα^{low}KLRG1^{high} short-lived effector cells (SLECs) and IL-7Rα^{high}KLRG1^{low} memory precursor effector cells (MPECs) was determined within the tetramer$^+$ CD8 T cell population. (B) Absolute numbers of total, IL-7Rα^{low}KLRG1^{high} short-lived effector cell (SLEC) phenotype and IL-7Rα^{high}KLRG1^{low} memory phenotype tetramer$^+$ CD8 T cells are shown at day 30 after primary infection with HKx31. (C) Long-term follow up is shown of the percentage of tetramer$^+$ CD8 T cells within the blood after primary influenza infection with HKx31 in WT and CD70 Tg mice. (D) Absolute numbers of tetramer$^+$ CD8 T cells were determined within blood, mLN, spleen and lungs of WT and CD70 Tg mice at day 57 after primary infection with HKx31. (E) Absolute numbers of spleen-derived CD8 T cells that produce IFN-γ upon 5 hr re-stimulation with peptide and IL-2 were determined in WT and CD70 Tg mice that had been infected with HKx31 for 57 days. Results shown apply to mice that were 12 wks of age at the start of the experiment. Error bars indicate standard deviation of 5-8 individual mice. ∗ denotes $p<0.05$ and ∗∗ denotes $p<0.005$. Comparable experiments with similar results were performed 3 times.

The development of memory is a cardinal feature of CD8 T cell responses against acute infection and transient CD70-driven co-stimulation has been shown to enhance memory CD8 T cell responses against influenza and acute LCMV infection (17,18). To examine the effect of constitutive CD70 co-stimulation on memory CD8 T cell responses, we did a long-term follow-up of tetramer$^+$ CD8 T cells after influenza infection. Remarkably, at day 30 CD70 Tg mice contained less total and less memory phenotype influenza-specific CD8 T cells than WT mice within the blood (Fig. 5 B). The number of SLECs at this time-point is very low, which corresponds with viral clearance in both WT and CD70 Tg mice (Fig. 5B). Follow-up of tetramer$^+$ CD8 T cells within the blood of WT and CD70 Tg mice beyond 30 days revealed a steady decline in the number of influenza-specific memory CD8 T cells that was much more pronounced in CD70 Tg mice (Fig. 5 C). Around day 60 percentages and absolute numbers of influenza-specific memory CD8 T cells within the blood were about 3 to 5-fold lower in CD70 Tg mice compared to WT mice (Fig. 5, C and D). Within all other tissues examined such as the lungs, mLN and spleen, we detected only very low numbers of influenza-specific memory CD8 T cells within CD70 Tg mice (Fig. 5 D). WT animals contained significantly more influenza-specific CD8 T cells within the mLN and in particular within the spleen than CD70 Tg animals (Fig. 5 D). Also peptide re-stimulation revealed strongly decreased numbers of IFN-γ-producing influenza-specific CD8 T cells in the spleen of CD70 Tg mice compared to WT mice (Fig. 5 E). Thus, despite higher primary effector CD8 T cell responses and higher levels of MPECs, maintenance of memory CD8 T cells under constitutive CD70 co-stimulation is severely compromised.

Recall of influenza-specific CD8 T cells is reduced under constitutive CD70

Reduced maintenance of influenza-specific memory under constitutive CD70-driven co-stimulation may result in compromised secondary responses upon re-challenge with influenza

virus. Therefore, WT and CD70 Tg mice were sequentially infected with the influenza virus strains A/PR8/34 and HKx31. The use of serologically distinct virus strains excludes interference by influenza-specific antibodies (38). CD70 Tg animals had more pronounced weight loss and higher viral loads at day 8 upon secondary influenza infection than WT animals (Fig. 6, A and B). Although CD70 Tg mice underwent more severe disease, similar to WT mice they were able to recover and cleared the influenza virus by day 12 (Fig. 6 B). This indicates that in stark contrast to primary CD8 T cell responses, secondary CD8 T cell responses are impaired through constitutive CD70 triggering. Indeed, percentages of influenza-specific CD8 T cells within the blood were reduced in CD70 Tg mice compared to WT mice early but not late in the secondary response (Fig. 6 C). Enumeration of influenza-specific CD8 T cells by tetramer staining at day 8 after re-challenge also showed a severe reduction in absolute numbers within blood and spleen, but not within lungs of CD70 Tg mice compared to WT mice (Fig. 6 D). This difference was not apparent or strongly reduced in all compartments at day 12 after re-challenge (Fig. 6 D). This shows that recall CD8 T cell responses are delayed in CD70 Tg mice, which reflects the reduced memory maintenance in these mice. The numbers of IFN-γ-producing CD8 T cells upon peptide re-stimulation were also reduced within the spleen of CD70 Tg mice at day 8 (Fig. 6 E). Within the lungs we did not detect differences in cytokine or granzyme B producing CD8 T cells, reflecting the numbers of tetramer$^+$ CD8 T cells at this site (unpublished data). Thus, maintenance of memory CD8 T cells is impaired, and therefore, secondary CD8 T cell responses are delayed but not abolished under constitutive CD70-driven co-stimulation.

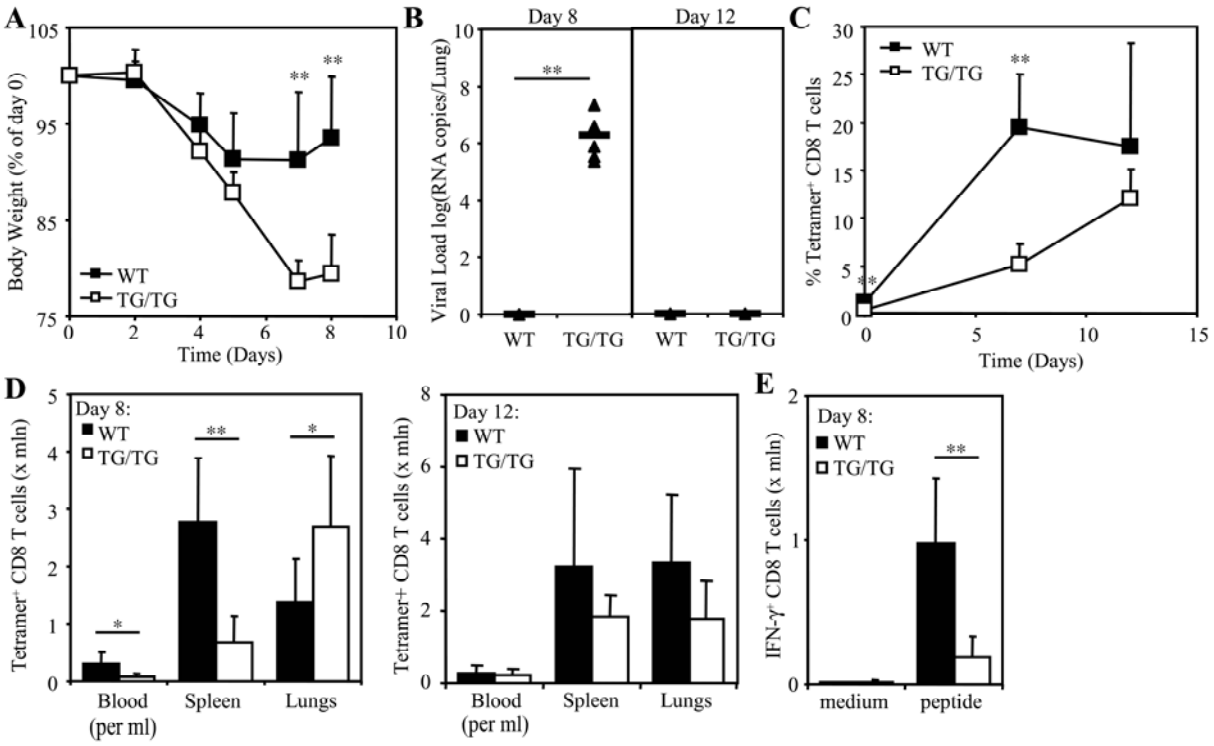

Figure 6. Reduced memory CD8 T cell responses develop against influenza in CD70 Tg mice. WT and CD70 Tg mice were intranasally infected with influenza virus HKx31 and 51 or 61 days later with the serologically distinct influenza virus A/PR8/34. (A) The body weight of WT and CD70 Tg mice was followed in time after the secondary influenza infection. (B) Viral loads within the lungs of WT and CD70 Tg mice were examined at day 8 and 12 after the secondary influenza infection. (C) The percentage of tetramer$^+$ CD8 T cells was determined within the blood of WT and CD70 Tg mice at the indicated time-points after secondary influenza infection. (D) The absolute numbers of tetramer$^+$ CD8 T cells were determined within blood, spleen and lungs of WT versus CD70 Tg mice at day 8 (left panel) and 12 (right panel) of the secondary influenza infection. (E) The absolute numbers of IFN-γ producing CD8 T cells were determined upon 5 hr re-stimulation with influenza peptide and IL-2 in WT and CD70 TG spleen of mice that had undergone a secondary influenza infection for 8 days. Results shown apply to mice that were 12 wks of age at the start of the experiment. Error bars indicate standard deviation of 5-8 individual mice. ∗ denotes $p<0.05$ and ∗∗ denotes $p<0.005$. Comparable experiments with similar results were performed 3 times.

Constitutive CD70 reduces CD4 T cell memory but does not impair CD4 T cell help

In the absence of CD4 T cell help primary CD8 T cell responses are normal, but secondary CD8 T cell responses are severely compromised (39). To examine whether CD70 impaired CD4 T cell help, we analyzed the CD4 T cell response against influenza. Spleens of WT and CD70 Tg animals contained equal numbers of influenza-specific CD4 T cells at the peak of the response (Fig. 7, A and B). At late time-points after infection the percentage of influenza-specific CD4 T cells in spleens of CD70 Tg mice had declined about 4-fold below those of WT mice (Fig. 7, A and B). This shows that CD4 and CD8 T cells respond similarly in that they are unable to maintain their memory population, but that CD70 co-stimulation does not induce helpless CD8 T cell responses through elimination of CD4 T cells.

CD4 T cell help includes signaling through CD40L and IL-2 (40-43). Therefore, we analyzed expression of these molecules on CD4 T cells of WT and CD70 Tg mice. CD4 T cells under constitutive CD70 driven co-stimulation up-regulated IL-2 and CD40L upon re-stimulation with PMA and ionomycin, although levels of CD40L were higher and levels of IL-2 were lower compared to WT mice (Fig. 7, C and D). The lower levels of IL-2 may represent functional exhaustion of EM-type CD4 T cells in CD70 Tg mice. However, this was not observed in influenza-specific CD4 T cells of CD70 Tg mice at the peak of the response (Fig. 7, A and E). Thus, we have no evidence that constitutive triggering through CD70 impairs CD4 T cell help.

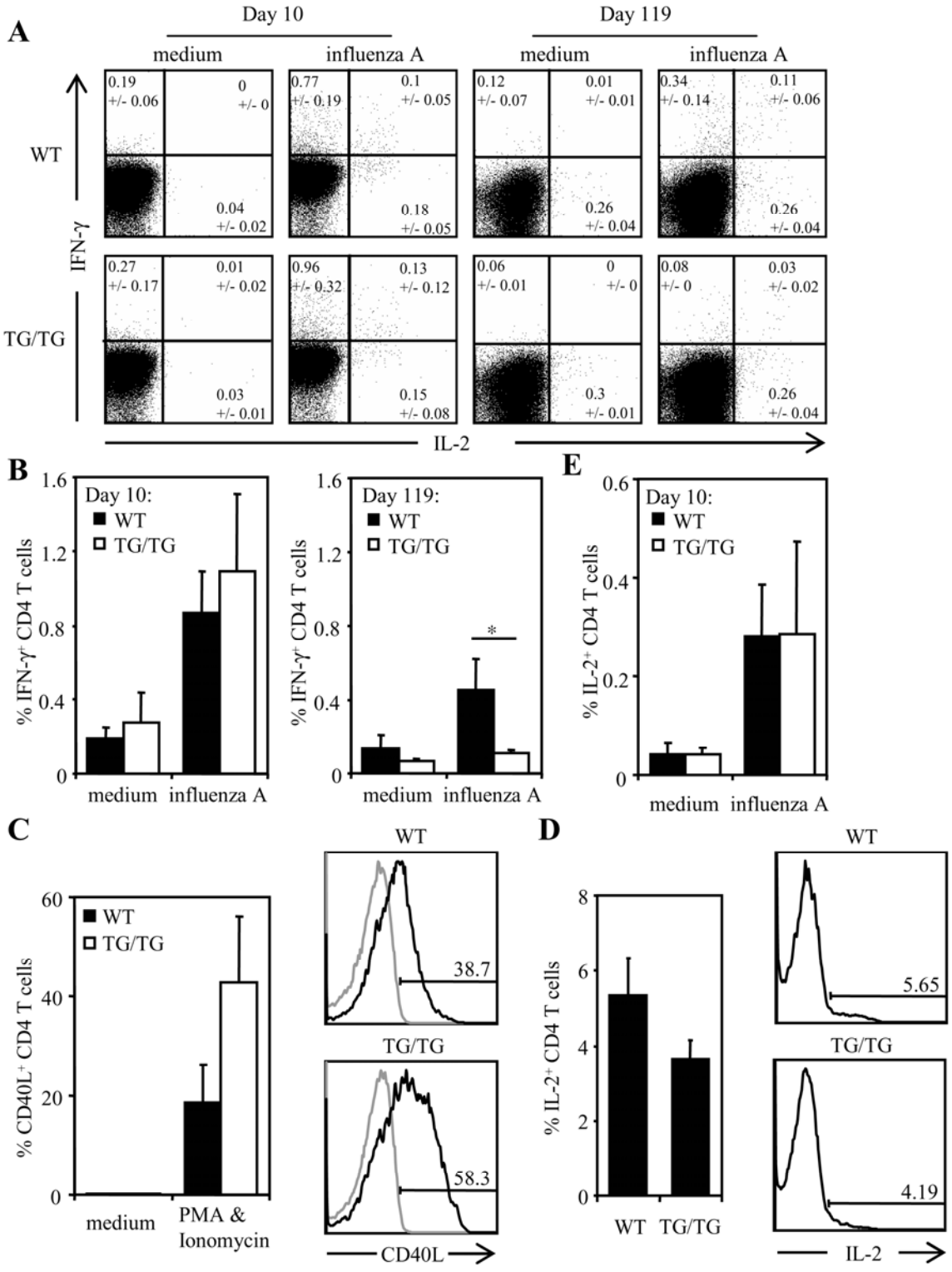

Figure 7. Normal primary but reduced memory CD4 T cell responses develop in CD70 Tg mice. (A) Dotplots display intracellular IFN-γ and IL-2 expression of CD4 T cells upon 5 hr re-stimulation of splenocytes with influenza virus at day 10 and 119 after primary influenza infection. Insets represent average percentage of cells within quadrant +/- standard deviation.

Figure 7 Continued. (B) The percentage of IFN-γ producing CD4 T cells of total CD4 T cells is shown upon 5 hr re-stimulation of splenocytes of WT and CD70 Tg animals with influenza virus. Splenocytes were isolated after 10 (left panel) or 119 days (right panel) of primary influenza infection. (C) CD40L expression was examined on CD4 T cells of WT and CD70 Tg mice after 5 hr stimulation with PMA and ionomycin (left panel). Representative histograms of individual WT and CD70 Tg mice display CD40L expression under medium (gray line) and PMA and ionomycin conditions (black line) on CD4 T cells (right panel). (D) IL-2 expression was analyzed on CD4 T cells of WT and CD70 Tg mice by intracellular cytokine staining after PMA and ionomycin stimulation (left panel). Representative histograms of individual WT and CD70 Tg mice show IL-2 expression in CD4 T cells upon PMA and ionomycin re-stimulation (right panel). (E) IL-2 expression of CD4 T cells was determined after 5 hr re-stimulation with influenza virus of splenocytes from WT and CD70 Tg mice that had been infected with influenza virus for 10 days. Results shown apply to mice that were 12 wks of age at the start of the experiment. Error bars indicate standard deviation of 3-8 mice. ∗ denotes $p<0.05$. Experiments were repeated at least once with similar results.

Discussion

In the present study we have investigated the impact of constitutive expression of the co-stimulatory molecule CD70 on T cells. CD70 on APCs acts as a co-stimulatory molecule and induces EM CD8 T cell formation with enhanced effector function (14,15). We have observed that CD70 on T cells is functional as well and induced co-stimulation that resulted in EM CD8 T cell differentiation and in enhanced primary CD8 T cell responses against influenza. T cells are non-APCs that in mice do not present antigens to other T cells. This indicates that CD8 T cells acquire TCR stimulation separate from CD70-driven co-stimulation and that this similar to TCR and CD70-triggering by the same APC induces EM CD8 T cell differentiation. Indeed, in-trans co-stimulation of CD8 T cells by CD70 has been observed by others using soluble CD70 Ig constructs (36). On APCs co-delivery of CD70 and MHC class II molecules to the immunological synapse with T cells occurs, underlining the hypothesis

that co-expression of MHC class II and CD70 is required for CD4 T cell activation (44). We did not find that CD70 on T cells significantly enhanced EM formation of CD4 T cells. Constitutive expression of T cell specific CD70 did not enhance numbers of EM CD4 T cells within spleen, BM and at peripheral sites such as the liver. Although CD70 on T cells enhanced influenza-specific CD8 T cell responses, it did not enhance virus-specific CD4 T cell responses against primary influenza infection. In contrast, CD70 on B cells and DCs mediates differentiation of naïve CD4 T cells into Th1-type T cells that produce IFN-γ (14,16). Possibly, CD4 T cell activation requires a single APC to provide TCR stimulation and CD70 co-stimulation.

The size and activation state of other leukocyte populations are affected in the CD70 Tg mice as has been described for CD70 Tg mice that express CD70 on B cells (14). Specifically, the levels of B cells but not of other APCs such as monocytes and macrophages are decreased in CD70 Tg mice. Remaining B cells, monocytes and macrophages in CD70 Tg mice express enhanced levels of MHC class II, indicating an elevated activation state ((14) and unpublished data). This is the indirect consequence of enhanced IFN-γ signaling in CD70 Tg mice ((14) and unpublished data). Importantly, formation of EM CD8 T cells is not altered in the absence of IFN-γ, indicating that enhanced EM CD8 T cell formation occurs directly through CD70-driven co-stimulation rather than indirectly through enhanced activation of APCs ((14) and unpublished data).

Little is known on how co-stimulation through CD70 and CD27 affects the differentiation pathway of naïve CD8 T cells into effector and memory cells. Here, we showed that introduction of CD70 enhances the formation of effector memory phenotype CD8 T cells. Based on further analysis using molecules that are expressed on effector cells such as CD69 and PD-1 and molecules that are present on memory cells such as IL-7Rα, it can be argued that these cells are effector rather than effector memory CD8 T cells. Antigen is required for

the formation and maintenance of effector CD8 T cells (29). We observed that CD70 required antigen to induce CD8 T cell differentiation. Antigen-driven proliferation of CD8 T cells is tightly linked with differentiation into effector-phenotype T cells. We do not know whether CD70 on T cells induces naïve, CM or EM CD8 T cells to differentiate into effector CD8 T cells. However, analysis of Ki-67 expression showing elevated proliferation of naïve, CM and EM CD8 T cells may indicate that all of these populations are stimulated to generate effector CD8 T cells under constitutive expression of CD70.

We showed that EM CD8 T cells under constitutive CD70-driven co-stimulation have a phenotype remarkably similar to antigen-specific CD8 T cells in HIV-1 infection and other chronic infections. EM phenotype CD8 T cells that develop during constitutive CD70 triggering expressed inhibitory molecules that are involved in the induction of T cell exhaustion in chronic infection such as PD-1 and IL-10. These molecules functionally impair effector CD8 T cells in chronic infection, resulting in low production of IL-2 and IFN-γ and poor cytotoxicity, and this prevents pathogen clearance (8-10). The level of cytokine production on a per cell basis and the number of cytokines, importantly IFN-γ, TNF-α, and IL-2, co-produced by individual cells are major determinants of the strength of CD8 T cell responses (45). Comparison of IFN-γ-producing CD4 and CD8 T cells of CD70 Tg mice with those of WT mice revealed reduced ability to co-produce TNF-α and IL-2 indicative of functional exhaustion. The induction of functional impairment of T cells likely requires the presence of persistent antigen. In the absence of persistent antigen, such as during influenza responses, we did not observe that constitutive CD70-driven co-stimulation resulted in CD8 T cell exhaustion. Introduction of CD70 in acute influenza infection did not induce PD-1 and IL-10 up-regulation (unpublished data). Moreover, poly-functional analysis of the spleen and lung-resident influenza-specific CD8 T cells did not reveal functional exhaustion during primary and secondary responses (unpublished data).

Survival of CD8 T cells in chronic infection depends upon continuous stimulation through persistent antigen (29). Antigen-dependent survival of CD8 T cells in chronic infection likely requires contribution of signals from inflammatory cytokines and co-stimulatory molecules. We hypothesize that CD70 provides such signals and enables antigen-dependent maintenance of CD8 T cells and that this developmental pathway may ultimately result in T cell exhaustion. We reported previously that CD70 drives progressive effector T cell formation that eventually results in depletion of T cells (46). Progressive accumulation of EM CD8 T cells also occurred in CD70 Tg mice with CD70 on T cells, although not as dramatic as in CD70 Tg mice with CD70 on B cells. This may reflect expression levels of CD70, which are higher in B cell CD70 Tg mice or cell-specific expression of the CD70 transgene. Taken together with our current results, this provides a strong argument that exhaustion and depletion of T cells that prevent viral clearance in HIV-1 infection and other chronic infections result from CD70-driven immune activation.

We have shown that constitutive signaling through CD70 and CD27 is detrimental for long-term CD8 T cell-dependent immunity as it prevents formation of memory CD8 T cells. In striking resemblance, the antigen-specific T cell population in chronic infection such as with LCMV does not contain a memory T cell subset that survives independently of antigen (3). The reason for the lack of memory T cells in chronic infection is unknown and this may result from impaired development or from elimination of memory CD8 T cells. We did not observe that CD70 induced preferential effector cell differentiation or impaired memory development during acute infection with influenza. Rather CD70 enhanced formation of memory precursors early in the primary response. We were also unable to find evidence that CD70 impaired CD4 T cell responses that provide essential help for memory CD8 T cell responses against viruses including influenza (39,47). CD4 T cell removal in chronic LCMV infection results in exacerbation of disease (4), indicating that CD4 T cell help is functional during

chronic infection as well. This argues against defective development of memory CD8 T cells through CD70 signaling and in chronic infection. Indeed, some evidence indicates that elimination of memory CD8 T cells underlies the memory defect. Although FASL and FAS normally do not mediate T cell apoptosis upon acute infection, this pathway of T cell apoptosis was observed upon acute influenza infection in CD70 Tg mice (48), and recently, it has been proposed that FASL and FAS contribute to apoptosis of antigen-specific T cells in chronic infection (49-51). It will be interesting to analyze whether FASL and FAS also remove memory T cells upon triggering through CD70 and in chronic infection. However, it needs to be mentioned that blockade of FASL and FAS-dependent apoptosis has also been shown as a mechanism of CD70 to enhance memory CD8 T cell responses (52).

Impairment of CD8 T cell memory through CD70 signaling is in striking contrast to earlier findings that demonstrated that CD70 enhanced memory CD8 T cell responses. Ablation of CD70-driven co-stimulation using $CD27^{-/-}$ mice resulted in reduced primary and secondary CD8 T cell responses against influenza and reduced secondary responses against acute LCMV (17,18). A major difference with these studies is the expression of CD70 that in acute LCMV and influenza infection is found transiently on low percentages of APCs and T cells (17,19). Signaling through CD70 and CD27 is regulated through expression of CD70, and thus, differences in the expression level of CD70 and in the window of CD70 expression may influence the outcome of immune responses. Constitutive high levels of CD70 expression such as in CD70 Tg animals and in chronic infection may result in over-stimulation of CD4 and CD8 T cells and consequently immuno-pathology. Indeed, in striking contrast to acute LCMV, $CD27^{-/-}$ mice are protected against chronic LCMV infection (53). The secretion of copious amounts of IFN-γ and TNF-α by CD4 T cells that was attributed to CD70-driven co-stimulation resulted in disruption of the production of neutralizing antibodies (53). Also, memory CD8 T cell development during chronic LCMV was restored in the absence of CD27

signaling, but this was considered secondary to the development of a neutralizing antibody response (53). Our experimental setup prevents that neutralizing antibodies contribute to the secondary response against influenza, demonstrating that CD70-induced immuno-pathology includes impairment of memory CD8 T cell formation. Thus, under high and constitutive expression levels such as occur in chronic infection, CD70 impairs rather than enhances memory CD8 T cell responses.

In conclusion, CD70 is functional as a co-stimulatory molecule on T cells, and enhances effector CD8 T cell mediated-immune responses, but abrogates long-term protection through impairment of CD8 T cell memory. Peptide immunization in the presence of soluble CD70 results in strong primary and secondary responses (36). These adjuvant properties of CD70 have fueled the idea that co-stimulation through CD70 and CD27 can be harnessed as a strategy to break tolerance in the treatment of tumors (54). However, defective maintenance of long-term memory after constitutive CD70-driven co-stimulation warrants caution regarding the strength and duration of the therapeutic use of CD70 in vaccination strategies. Moreover, our results indicate that treatment of chronic infection or chronic auto-immune disease may benefit from blockade rather than activation of the co-stimulatory CD70-CD27 axis.

Materials and Methods

Generation of Tg mice.

To generate CD70 Tg mice that express CD70 specifically on T cells a construct was created containing the cDNA of murine CD70 under the control of the human CD2 promoter (Fig. 1 A). The CD70 cDNA was isolated by digestion with EcoRI from pcDNA3 plasmid (23), and then cloned into the EcoRI site of the hCD2 promoter plasmid (kindly provided by Dr. R. Meuwissen, NKI, Amsterdam). The hCD2 promoter plasmid also contains the locus control

region (LCR) of the human CD2 gene that confers T cell specific, copy-dependent and position-independent gene expression in transgenic mice. After linearization and removal of plasmid sequences by digestion with KpnI and XbaI the hCD2-mCD70 construct was injected into the pronuclei of fertilized oocytes of C57Bl/6 mice. A founder was identified by Southern blot analysis of tail DNA and mated with C57Bl/6 mice to obtain heterozygous CD70 Tg mice. These mice were backcrossed to create homozygous CD70 Tg mice.

C57Bl/6/J, OTI Tg (from Jackson Lab.), $CD27^{-/-}$ (18) and homozygous CD70 Tg mice and homozygous CD70 Tg mice crossed with OTI Tg or $CD27^{-/-}$ mice were maintained at specific-pathogen free conditions at the animal department of the AMC (Amsterdam, Netherlands). Screening of the mice for CD70 Tg and OTI Tg expression was performed by flow cytometry of leukocytes from tail vein blood using anti-CD70 antibodies (3B9) and anti-Vβ5 antibodies (MR9-4), respectively. CD27 genotype of the mice was screened using PCR on genomic DNA as described previously (18). Mice were used at 8-12 wks of age unless stated otherwise and within individual experiments mice were strictly age-matched. All animal experiments were performed according institutional and national guidelines.

Antibodies.

The following monoclonal antibodies from eBioscience were used: anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD27 (LG.3A10), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD70 (FR70), anti-B220 (RA3-6B2), anti-PD-1 (RMP1-30), anti-IL-7Rα (A7R34), anti-KLRG1 (2F1) and anti-CD40L (MR1). Anti-IFN-γ (XMG1.2), anti-IL-2 (JES6-5H4), anti-IL-10 (JES5-16E3), anti-TNF-α (MP6-XT22) and anti-Ki-67 (B56) were purchased from BD Biosciences.

Flow cytometry.

Single cell suspensions were obtained from spleen, pLNs, lungs, liver and BM by grinding tissue over nylon filters (BD biosciences). Contaminating red blood cells were removed from these preparations using erylysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA). Absolute cell counts were determined by an automated cell counter (CasyCounter, Innovatis). Cells were stained with the indicated fluorochrome-conjugated or biotinylated primary antibodies in the presence of anti-CD16/CD32 block (2.4G2, kind gift of Louis Boon, Biosource BV) for 30 min at 4°C in PBS containing 0.5% BSA. In the case of biotinylated primary antibodies, cells were incubated with fluorochrome-conjugated streptavidin (eBioscience) for 30 min at 4°C in PBS containing 0.5% BSA. For staining of the nuclear antigen Ki-67 cells were fixed and permeabilized for 30 min at 4°C with fixation and permeabilization buffer (eBioscience). Expression was measured using Calibur or Canto flow cytometers (BD Biosciences).

Intracellular cytokine staining.

Splenocytes were stimulated with 10 ng/ml PMA (Sigma) and 1 μM ionomycin (Sigma) for 1 hr. Then, 10 μg/ml brefeldin A (Sigma) was added to prevent cytokine release and after 4 hrs cells were harvested and stained with antibodies against CD4 and CD8. Next, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and labeled for intracellular cytokines using specified antibodies.

Quantitative PCR.

RNA was extracted using the Invisorb Spin Cell RNA Mini Kit (Invitek), cDNA was synthesized using Superscript Reverse Transcriptase II (Invitrogen) and poly T oligos (Invitrogen), and quantitative real-time RT-PCR was performed on a Lightcycler (Roche).

Transcription levels were obtained using the Lightcycler FastStart DNA Master SYBR Green reagent kit (Roche) and the following primer sets for 18S (forward: 5'-TCAAGAACGAAAGTCGGAGG-3', reverse: 5'-GGACATCTAAGGGCATCACA-3'), T-bet (forward: 5'-CAACAACCCCTTTGCCAAAG-3', reverse: 5'-TCCCCCAAGCA GTTGACAGT-3'), Eomes (forward: 5'-TGGA CTACCATGGACATCCAGAA-3', reverse: 5'-TTCTCTTGCAAGCGCTGTTGT-3') and Blimp-1 (forward: 5'-CCTCATCCCATGC TCAATCCA-3', reverse: 5'-GGACTACTCTCGT CCTTCATGCT-3'). Values are represented relative to that of 18S, with the lowest experimental value standardized at 1.

Influenza infection.

Mice were intranasally infected with 10x $TCID_{50}$ of the H1N1 influenza A virus A/PR8/34 for analysis of primary immune responses. Heterotypic infection with 100x $TCID_{50}$ of the H3N2 influenza A virus HKx31 and 10x $TCID_{50}$ of A/PR8/34 was performed to examine secondary responses. At fixed time intervals body weights of the infected mice were obtained as a measure of disease and blood samples were drawn from the tail vein to determine levels of influenza-specific CD8 T cells. At the indicated days after infection mice were sacrificed, and blood, spleen, mLN and lungs were collected for analysis. Viral loads within the lungs were quantified using qPCR as described (24). Influenza-specific CD8 T cells were enumerated using anti-CD8 antibodies and PE- or APC-conjugated tetramers of $H\text{-}2D^b$ containing the influenza-derived peptide $NP_{366\text{-}374}$ ASNENMETM.

Statistical analysis.

Figures represent means and error bars denote standard deviation. Student's T-test was used to analyze for statistical significance. $P < 0.05$ was considered statistically significant.

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Disclosures:

The authors have no financial conflict of interest.

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Chapter 6

Function of CD27 in helper T cell differentiation.

Ronald W. van Olffen[1*], Sten Libregts[1*], Koen van der Sluijs[1],

Rene A.W. van Lier[1] and Martijn A. Nolte[1].

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[1]Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands.

*These authors contributed equally to this work.

Abstract

Differentiation of naïve $CD4^+$ T cells to functional effector T-helper (T_H) cells is driven by both costimulatory molecules and cytokines. Although polarizing cytokines can induce the differentiation into a particular T_H-subset, certain costimulatory molecules also seem to affect this polarization process. We have previously found that CD70-transgenic (CD70TG) mice develop large numbers of IFN-γ-producing $CD4^+$ T cells and we therefore questioned whether CD27 triggering provides an instructive signal for T_H1 differentiation or rather supports T_H cell formation in general. Although CD70TG mice on a T_H1-prone C57Bl/6J background develop more T_H1 cells, we found that this phenotype is lost when CD70TG mice are backcrossed on a T_H2-prone Balb/c background, but is not replaced with more T_H2 cells. Furthermore, CD70-overexpression is not sufficient to drive T_H17 cell formation, nor does it affect the generation of $FoxP3^+$ regulatory T cells. Using an *in vitro* setting, we found that CD27-triggering does not provide instructive signals for a specific T_H cell subset, but rather supports the formation of IFN-γ-producing as well as IL-13-producing $CD4^+$ T cells depending on the cytokine milieu and genetic background, while inhibiting T_H17 formation. Induction of allergic airway inflammation in CD70TG Balb/c mice further illustrates that CD27 plays a supportive role in T_H1 differentiation, without modulating the classical T_H2 response. This supportive role of CD27 in T_H cell polarization could not be attributed to a specific modulation of transcription factor expression levels. In summary, this study indicates that CD27 signaling supports T_H1 differentiation, permits T_H2 formation, but inhibits T_H17 formation.

Introduction

Recognition of a MHC-peptide complex via the TCR is the first signal required for effector T cell formation, as it initiates T cell activation and clonal expansion. Subsequent to MHC-peptide binding, T cells depend on a second signal for their survival and proliferation, which is provided in the form of costimulatory molecules. For the final differentiation and polarization to effector cells, activated T cells require a third signal, which is provided by polarizing cytokines (reviewed in (1)). Thus, naïve T cells rely on a triad of signals for their activation and differentiation into an effector population. The large number of molecules that have been implicated in this process either instruct, support or permit the formation of a specific effector T cell population.

Within the CD4 T cell population, a large variety of helper T (T_H) cell subsets has been identified, such as T_H1, T_H2, T_H17, T_H3, T_R1 and T_{Regs}, which have been attributed a specific function in the immune system. Classically, these T_H subsets can be distinguished by their cytokine production and/or transcription factor expression. As such, T_H1 cells are characterized by the ability to produce high levels of IFN-γ and TNF-α, thereby supporting cell-mediated immunity (reviewed in (2)). On the other hand, humoral immunity is linked to T_H2 formation and increased secretion of IL-4, IL-5, IL-6, IL-10 and IL-13 (reviewed in (2). Protective anti-bacterial immunity as well as development of autoimmunity is generally linked to an increase in IL-17 producing T_H17 cells (3;4). Next to these effector T cell subsets, two inducible regulatory T cell subsets can be identified by their production of IL-10 and TGF-β, which are respectively referred to as T_R1 and T_H3 cells (5-7). Finally, naturally occurring regulatory T cells (T_{Regs}) are not characterized by their cytokine production, but are generally distinguished by their expression of the transcription factor FoxP3 (8;9). The function of these T_{Reg} subsets is to regulate inflammatory responses and to prevent the induction of autoimmunity.

The differentiation of naïve $CD4^+$ T cells towards these different helper T cell lineages is classically driven by polarizing cytokines, which affect the expression and/or function of instructive transcription factors. T_H1 polarization occurs subsequent to the production of IL-12 by antigen presenting cells (APCs), which results in the upregulation of the transcription factor T_{Bet} in T cells (10). In contrast, T_H2 formation is enhanced following IL-4 signalling and through the upregulation of the transcription factor GATA-3(11). Commitment of a T cell to the T_H17 lineage is induced by the transcription factor RORγt. In mice, this transcription factor is upregulated by the combination of TGF-β and IL-6, whereas in humans the combination of TGF-β and IL-1-β is necessary (4;12-15). Regulatory T cells are induced by increased levels of TGF-β and retinoic acid and result in the upregulation of the transcription factor FoxP3 (16-19).

Although polarizing cytokines clearly fulfill a key function in T_H cell formation, costimulatory molecules may also play an important role in T cell differentiation and polarization. Several lines of evidence suggest that engagement of the TNFR superfamily member CD27 by its ligand CD70 enhances T_H1 cell development. Whereas CD70 is only transiently expressed on APCs and lymphocytes during immune activation (20-24), we have previously shown that constitutive expression of CD70 on B cells induces a strong increase in the numbers of IFN-γ producing $CD4^+$ and $CD8^+$ T cells, thereby enhancing T cell mediated immunity (25-27). CD27 ligation on human T cells not only drives proliferation of $CD4^+$ T cells, but also T_H1 polarization via upregulation of IL-12Rβ2 and T_{Bet} (28). Moreover, human TNFα-induced $CD70^+$ DCs can evoke T_H1, but also T_H17 responses, although it was not shown whether these responses are indeed dependent on CD27-engagement (29). Finally, CD27 ligation in mice can induce a T_H1-type gene expression profile in $CD4^+$ T cells (30), and can under certain conditions promote T_H1 cell formation independently of IL-12 (31).

This would suggest that CD27, as a typical "signal 2", can directly induce T_H1 cell differentiation without the need for the classical "signal 3".

Based on these observations, we questioned whether triggering through CD27 provides instructive signals for T_H1 differentiation, or that it rather supports the formation of T_H1 cells. As the genetic background of mice has been associated with a predisposition to T_H cell polarization and disease development (32-35), we decided to approach our question by backcrossing CD70TG mice from a T_H1-prone C57Bl/6J to a T_H2-prone Balb/c background. Our data indicate that the strong T_H1 skewing observed in CD70TG mice is highly dependent on the genetic background, as it does not induce T_H1, nor T_H2 skewing on a Balb/c background. Importantly, CD27 ligation during the induction of allergic airway inflammation (AAI), a typical T_H2 response, enhanced the generation of T_H1 cells without affecting the formation of T_H2 cells. Together with *in vitro* polarization studies, our data indicate that CD27 does not instruct, but rather supports the formation of T_H1 cells, both *in vitro* and *in vivo*.

Results

Strain specific enhancement of T_H1 polarization via CD27 ligation under homeostatic conditions

Ensuing T cell activation, the genetic background predisposes polarization to a specific helper T cell subset (32;38). To determine whether CD27 ligation differentially affects helper T cell polarization depending on the genetic background, we backcrossed the CD70TG C57Bl/6J mice on a Balb/c background and studied the *in vivo* effects of CD70-driven costimulation. By direct stimulation of WT and CD70TG splenocytes with PMA/ionomycin and brefeldin A, we confirmed that CD70TG C57Bl/6J mice have increased percentages of IFN-γ production by $CD4^+$ T cells and decreased production of the T_H2 cytokines IL-5 and IL-13 by $CD4^+$ T

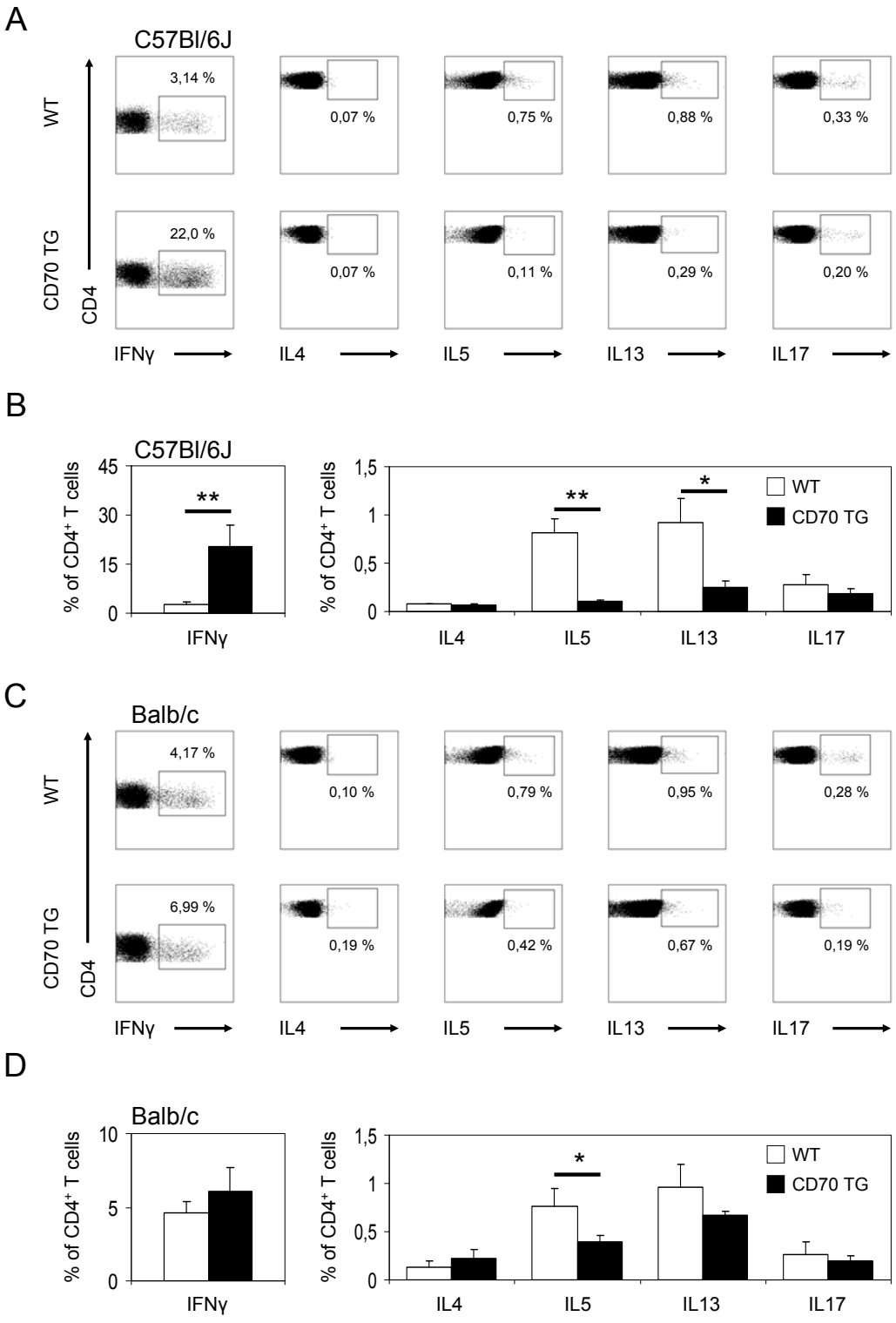

Figure 1. T_H profile of CD70 TG mice on different genetic backgrounds. Wild type (wt) and CD70 TG C57Bl/6J and Balb/c mice were analyzed for their intrinsic cytokine production capacity directly ex vivo. A representative staining on a (A) C57Bl/6J and (C) Balb/c background for IFN-γ, IL-4, IL-5, IL-13 and IL-17 production by $CD4^+$ T cells in WT and CD70 TG mice after stimulation with PMA/ionomycin is shown. The percentage of cytokine production by WT or CD70 TG derived $CD4^+$ T cells on a (B) C57Bl/6J (average of 4 mice ± SD) or (D) Balb/c (average of 3 mice ± SD). background. Asterisks denote significant differences (* $p<0.05$; ** $p<0.005$).

cells (Fig. 1A-B). No significant changes were identified for the cytokines IL-4 and IL-17 (Fig. 1A-B). This indicates that CD27 ligation enhances T_H1 lineage commitment on a C57Bl/6J background (25). In contrast, we found that CD70TG Balb/c mice showed no significant difference in the production of the T_H1 cytokine IFN-γ by $CD4^+$ T cells (Fig. 1C-D). Importantly, constitutive CD27 triggering in Balb/c mice did not enhance T_H2 differentiation either (Fig. 1C-D). These data indicate that CD27 triggering enhances T_H1 differentiation in a strain-dependent manner.

CD27 ligation does not affect the regulatory T cell compartment

Since $CD4^+$ T_{Regs} also express CD27 (39;40), we assessed whether enhanced CD27 triggering by CD70 could affect the formation and/or activation of this distinct T cell subset. This is particularly important because T_{Regs} might also influence T_H cell formation. $CD4^+$ T_{Regs} are characterized by their expression of the transcription factor FoxP3, high levels of CD25 and can be distinguished into two subsets based on CD103 and CD62L expression (8;9;41). CD70TG mice showed no significant difference in the percentage and absolute numbers of $CD4^+$ T_{Regs} for both the C57Bl/6J (Fig. 2A-B) and Balb/c (Fig. 2E-F) mouse strain. The expression of CD27 on T_{Regs} was significantly downregulated in CD70TG mice, indicative of an interaction with CD70 (Fig. 2C/G). However, this did not correlate with an increase in the activation state of these cells, as T_{Regs} in CD70TG C57Bl/6J mice showed a small increase in CD103 expression, but no difference was observed for CD62L and CD25 expression (Fig. 2C-D). In addition, T_{Regs} in CD70TG Balb/c mice did not differ in CD25, CD103 and CD62L expression (Fig. 2G-H). These data indicate that although CD27 is expressed on $CD4^+$ T_{Regs}, enhanced ligation through CD70 does not affect the number nor the activation status of these cells.

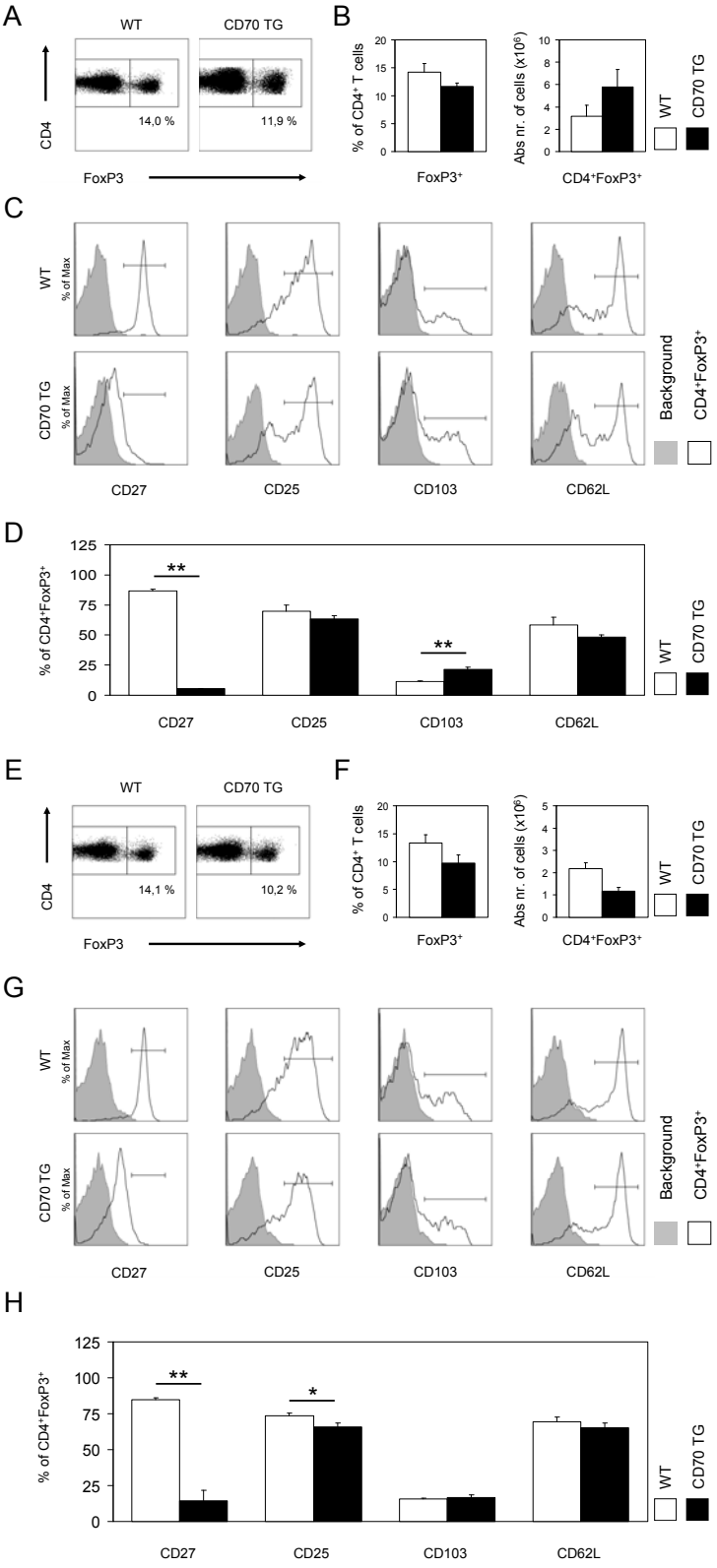

Figure 2. CD70 driven costimulation does not affect regulatory T cell development.

The regulatory T cell compartment was analysed for WT and CD70 TG C57Bl6J (A-D) and Balb/c (E-H) mice. (A,E) Representative staining for FoxP3 within the splenic $CD4^+$ T cell population. (B, F) The percentages and absolute numbers of splenic derived regulatory T cells in mice. (C, G) Representation and (D, H) average expression of the percentage of $CD4^+FoxP3^+$ T cells that express CD27, CD25, CD103 and CD62L, based on gate in respective histograms. Asterisks denote significant differences (* $p<0.05$; ** $p<0.005$).

Strain specific enhancement of IFN-γ producing cells via CD27 ligation under non-polarizing conditions

To determine if CD27 ligation provides an instructive, supportive or permissive signal for T_H cell polarization, we performed T cell stimulation assays under specific polarizing conditions using naïve WT T cells and providing CD27 triggering by the addition of either WT or CD70TG B cells. These experiments revealed that CD27 ligation enhanced the formation of IFN-γ producing T cells under non-polarizing (T_H0) conditions for C57Bl/6J derived cells (Fig. 3A), but only marginally affected IFN-γ production for Balb/c derived cells (Fig 3B). CD27 ligation did not enhance nor inhibit the specific formation of cytokine producing cells under T_H1 and T_H2 polarizing conditions (Fig 3A).

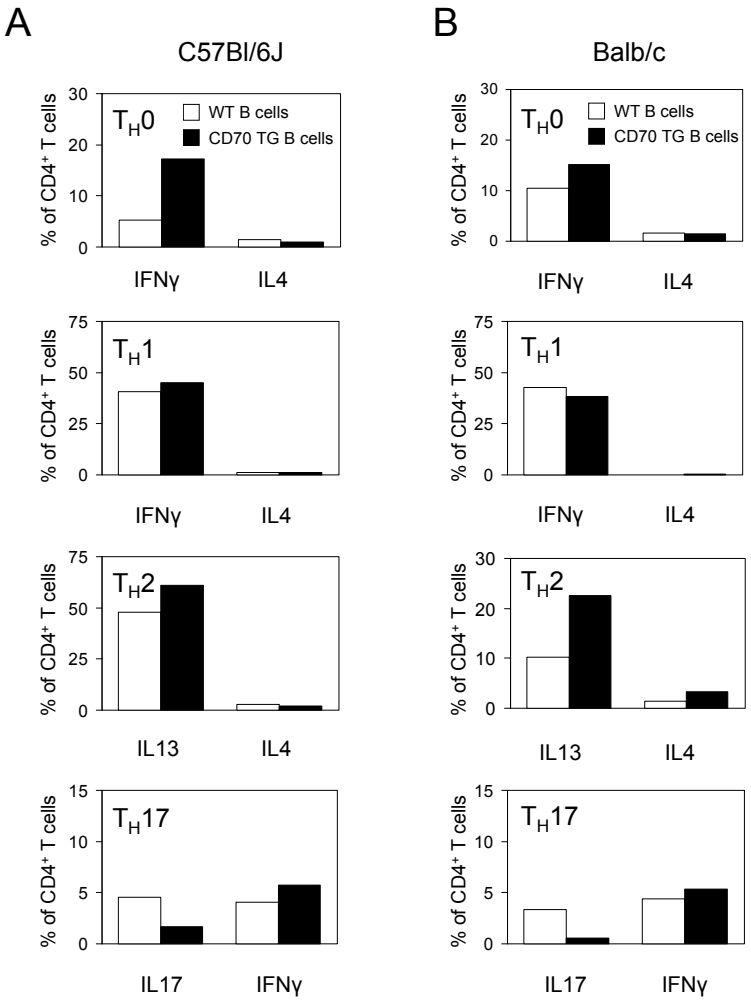

Figure 3. Genetic differences on T cell polarization following CD70 driven T cell costimulation *in vitro*. T cell polarization assays were performed using WT derived naïve $CD4^+$ T cells and WT or CD70 TG derived B cells. T cells were cultured under T_H0 (non-polarizing), T_H1 or T_H17 polarizing conditions for a period of 3 days or under T_H2 polarizing conditions for 7 days. (A) C57Bl/6J or (B) Balb/c polarized cells were studied for their capacity to produce T_H1, T_H2 or T_H17 associated cytokines upon PMA-ionomycin stimulation after the specified polarization.

Moreover, CD70 driven costimulation did not affect T cell polarization under T_H1 conditions in experiments using cells obtained from Balb/c mice (Fig 3B). CD27 ligation enhanced the formation of IL-13 producing cells under T_H2 polarizing conditions (Fig 3B), but the induction of IL-13 was less efficient for Balb/c than C57Bl/6J cells. Interestingly, CD27 triggering did inhibit the formation of IL-17 producing cells on both genetic backgrounds (Fig 3A&B). These data indicate that CD27 ligation *in vitro* enhances IFN-γ production under non-polarizing conditions, does not have a major effect on T_H1 or T_H2 induction under polarizing conditions, but does inhibit T_H17 induction.

CD70 driven costimulation does not inhibit T_H2 polarization during allergic airway inflammation

As these experiments indicate that CD27 triggering does not induce an instructive signal for T_H1 development, but does affect T_H2 differentiation to some extent, we further examined the impact of CD27 triggering on T_H2 differentiation *in vivo*. Therefore, we used the allergic airway inflammation (AAI) model, which induces T_H2 cells that can amplify allergic inflammation via the production of cytokines, chemokines and enhancing IgE production (reviewed in (42)) . Although the pathogenesis of AAI also includes the recruitment of other T cell subsets into the lung, T_H2 cells play an essential role in the inflammatory response (reviewed in (43)). In addition, blockade of the T_H2 cytokines IL-4 and IL-13 by antibodies or neutralizing fusion proteins, respectively, resulted in reversing and/or preventing allergen-induced airway hyperresponsiveness during sensitization and challenge phases (44-47). We found that both WT and CD70TG Balb/c mice had a significant infiltration of $CD4^+$ T cells in the lung and thus allowed us to investigate the effect of CD27 ligation on T_H cell polarization (Fig. 4A). WT mice showed increased numbers of IL-4, IL-13 and IFN-γ producing $CD4^+$ T cells in the lung of OVA challenged mice compared to PBS-treated mice (Fig. 4B).

Importantly, CD70TG mice showed normal numbers of IL-4 and IL-13 producing $CD4^+$ T cells, but a significant increase of IFN-γ producing $CD4^+$ T cells compared to challenged WT mice (Fig. 4B). This indicates that even in a T_H2 inflammation model, CD27 stimulation *in vivo* enhances T_H1 cell formation while permitting T_H2 cell polarization.

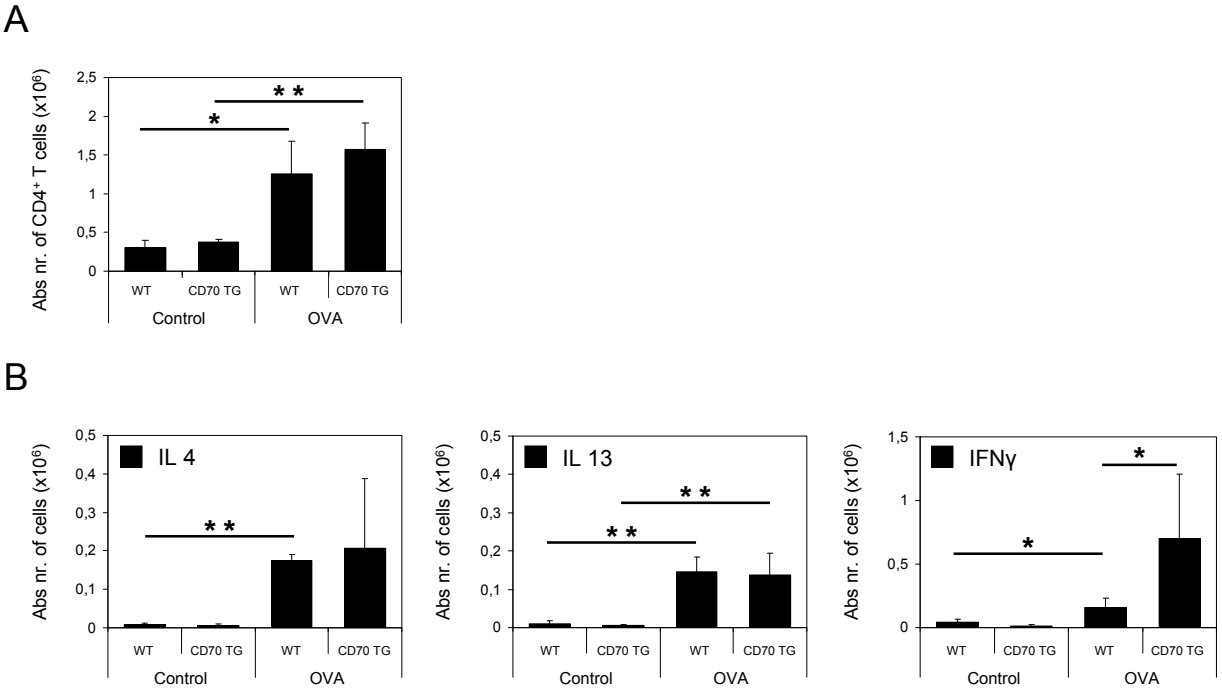

Figure 4. CD70 TG mice show parallel T_H1 and T_H2 polarization during allergic asthma model.

Allergic airway inflammation (AAI) was induced in WT and CD70 TG Balb/c mice to specifically promote T_H2 polarization. (A) Absolute total number of infiltrated pulmonary $CD4^+$ T cells on day 32 of AAI induction. (B) The absolute number of pulmonary $CD4^+$ T cells which produce IL-4, IL-13 and IFN-γ in WT (average of 8 mice ± SD) and CD70 TG Balb/c (average of 7 mice ± SD) mice. Asterisks denote significant differences (* $p<0.05$; ** $p<0.005$).

CD70 driven transcription factor regulation

To investigate how CD27 ligation affects $CD4^+$ T cell differentiation on a molecular level, we examined the impact of CD70-driven costimulation on transcription factor expression, as changes on this level would be expected if CD27 ligation would provide instructive signals

for T_H cell formation, The transcription factor T_{Bet} is the main transcription factor associated with T_H1 polarization. T_{Bet} induces the expression of IL-12Rβ2, thereby allowing cells to differentiate to T_H1 cells following IL-12 signaling (10;48;49). We found that T_{Bet} mRNA is induced under T_H0 polarizing conditions compared to naïve T cells, maintained under T_H1 polarizing conditions, but downregulated under T_H2 or T_H17 conditions. Importantly, CD27 ligation did not affect mRNA expression of T_{Bet} under these conditions (Fig. 5A). GATA3 is the central transcription factor responsible for T_H2 polarization (11), and is essential for the cytokine profile associated with T_H2 polarized cells (11;50;51). We found that GATA3 transcript levels remained similar to levels found in naïve T cells under T_H0 conditions, were downregulated under T_H1 and T_H17 conditions, and were upregulated under T_H2 conditions. However, CD27 triggering did not affect the GATA3 transcription expression levels under any conditions (Fig. 5B). The transcription factor RORγt, which is important for T_H17 lineage commitment (15), was only found under T_H17 conditions and was not regulated by CD27 stimulation (Fig. 5C). The transcription factors TWIST and FOG have been implicated in a negative feedback loop for T_H1 and T_H2 polarization, respectively (52-54). We found that both factors were downmodulated with respect to naïve T cells under all polarizing conditions and that CD27 ligation did not affect the respective transcription factor expression levels (Fig. 5D-E). Finally, we investigated the transcription factor Blimp-1. Expression of Blimp-1 has been associated with increased T_H2 polarization through active repression of T_H1 associated genes, such as T_{Bet} and IFN-γ (55). We found that Blimp-1 expression remained similar to naïve T cells in cells cultured under T_H1 polarizing conditions and was downregulated under all other conditions. However, enhanced signalling through CD27 did not influence Blimp-1 expression in any culture conditions (Fig. 5F).

Thus, we conclude that CD27 triggering does not influence the expression of instructive transcription factors, which is in line with the notion that CD27 supports, but does not instruct the formation of IFN-γ producing $CD4^+$ T cell following T cell activation .

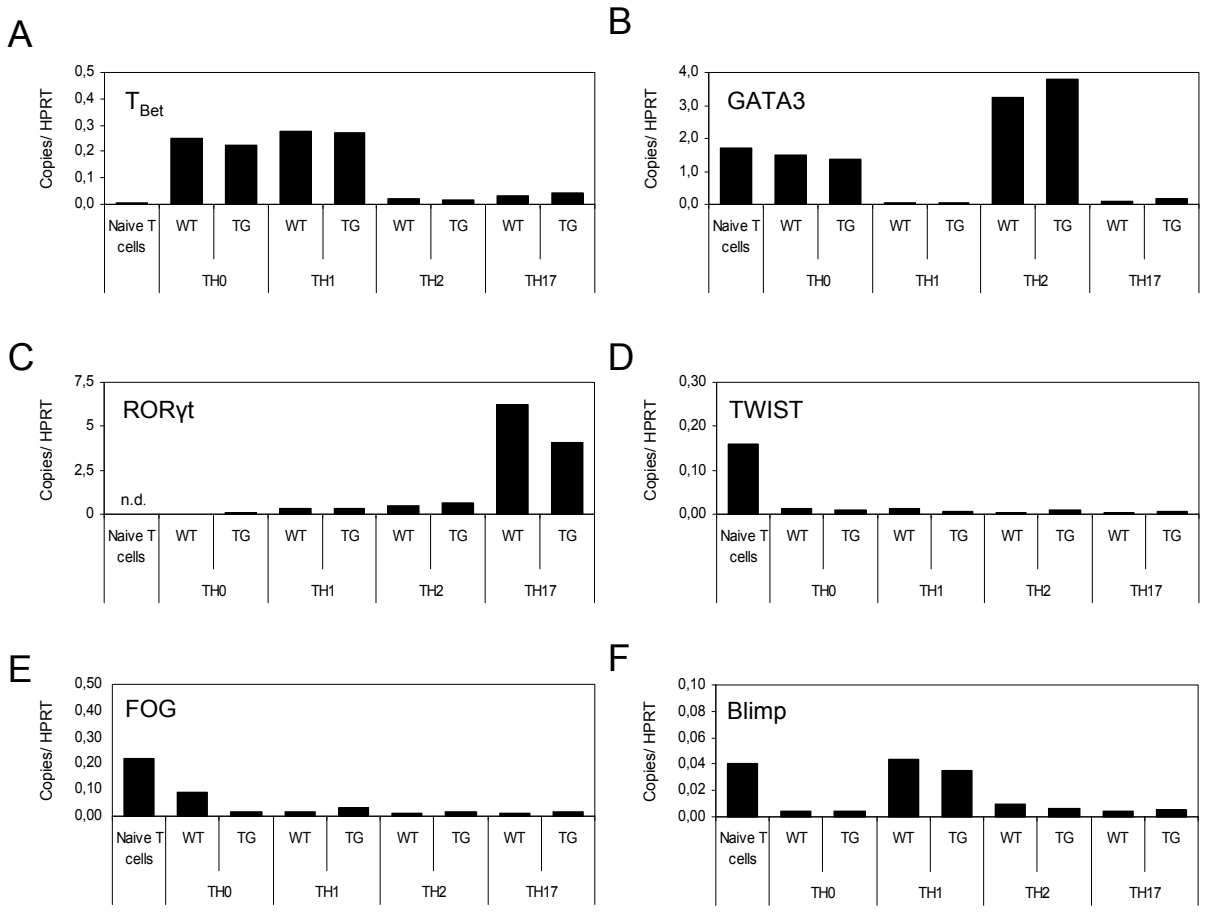

Figure 5. Enhanced CD70 driven IFN-γ production is not transcriptionally regulated.

Naïve $CD4^+$ T cells were stimulated under specific T_H0, T_H1, T_H2 and T_H17 polarizing conditions in the presence of WT or CD70 TG C57Bl/6J derived B cells. Transcript levels encoding (A) T_{Bet}, (B) GATA3, (C) RORγt, (D) TWIST, (E) FOG and (F) Blimp were analysed by qPCR, normalized with HPRT for each condition and are depicted as the number of copies of the respective transcript per HPRT transcript.

Discussion

In the present study, we show that although CD70-overexpression strongly promotes the formation of IFN-γ producing $CD4^+$ T cells, costimulation through CD27 does not induce differentiation of T_H1 cells per se. Instead, CD27 seems to support T_H1 cell formation, but this is dependent on the culture conditions and genetic background of the mice. The latter has been associated with a predisposition towards T_H1 or T_H2 polarization, i.e. C57Bl/6J mice are more prone towards T_H1 cell development, whereas Balb/c mice are more T_H2 prone. This variance in polarization can be attributed to a difference in a dominant genetic locus between the different genetic backgrounds, but could also be related to differences in chromatin remodeling subsequent to receptor signaling (56). In this respect, it has been suggested that Balb/c mice are less capable of sustaining responsiveness to IL-12 compared to C57Bl/6J mice, thereby decreasing their T_H1 polarizing capacity (57;58). Although it is unknown which genetic differences are responsible for the distinct phenotypes of C57Bl/6J and Balb/c CD70TG mice, it could be that these differences are associated with the enhanced availability of the T_H1 locus in C57Bl/6J mice. As there is some IFN-γ production during AAI even in Balb/c mice, this would suggest that the T_H1 locus is accessible during these conditions and that this might be the reason why CD27 ligation is capable of enhancing the formation of IFN-γ producing $CD4^+$ T cells (Fig. 4).

Whereas CD70 clearly stimulates T_H1 cell development, it has also been shown that TNFα-induced $CD70^+$ DCs can induce Th17 responses in humans (29). Our data clearly indicate that CD27 triggering in murine T cells inhibit formation of T_H17 cells *in vitro*, but it is not yet clear what the underlying mechanism of this inhibition is. Our transcription factor analysis would argue against a direct effect of CD27 ligation on the expression of RORγt in this setting (Fig. 5C). Instead, it is more likely that the CD27-mediated increase in IFNγ production affects T_H17 cell formation, as it has been well documented that loss of IFNγ secretion

promotes T_H17 formation, whereas IFNγ negatively can directly inhibit formation of the T_H17 lineage (reviewed in (59)). Nevertheless, our data raise the question if CD70 overexpression, which enhances anti-viral immunity (27), would actually inhibit anti-bacterial responses? We have not yet been able to addressed this question, but we have previously shown that absence of CD27 at least does not affect the outcome of an infection with Mycobacterium tuberculosis (60). Whether the number of IL-17 producing cells was affected in these CD27-deficient mice during this infection was not investigated in this study.

As T cell immunity can be seen as a balance between activation and regulation, we postulated that CD27 signalling could influence T_{Reg} numbers and/or their activation state. Importantly, other TNFR superfamily members have been shown to influence T_{Reg} numbers and/or function. GITR is capable of promoting T_{Reg} proliferation without impairing its regulatory function (Unpublished observation and (61)), whereas OX40 inhibits the induction of regulatory T cells from effector T cells (62). We found that T_{Regs} express CD27, and that transgenic overexpression of CD70 resulted in a significant reduction of membrane bound CD27, indicative for an interaction with CD70. However, both the activation state and absolute numbers of regulatory T cells were not affected in CD70TG mice on both the C57Bl/6J and Balb/c background. Thus, these data suggest that regulatory T cells are not accountable for the differences observed in helper T cell formation between C57Bl/6J and Balb/c mice.

Classically, the generation of IFN-γ producing T_H1 polarized cells occurs following the upregulation and activation of the transcription factor T_{Bet}. T_{Bet} activation results in an upregulation of IL-12Rβ2, and the subsequent formation of active IL-12R. In addition, T_{Bet} plays an important role in chromatin remodeling (63), thus allowing transcription of T_H1 dependent genes. Steinman *et al*. (31) showed that CD27 ligation could induce formation of IFN-γ producing $CD4^+$ T cells in an IL-12 independent manner, suggesting that CD27

signalling can promote helper T cell polarization independently of the classical polarization via the cytokine environment. These observations are congruent with our results showing that CD27 ligation enhances formation of IFN-γ producing $CD4^+$ T cells under non-polarizing conditions *in vitro* (Fig. 3). However, we also show that the T_H1 polarizing effects of CD27 are not due to changes of T_{Bet} expression levels (Fig. 5), though the effects of CD27 ligation on the function of T_{Bet} are not known. A possible mechanism for the observed effects could be the specific modulation of proliferation and/or survival via CD70 driven costimulation. CD27 has been described to enhance expansion of TCR stimulated $CD8^+$ T cells in an IL-2 independent manner without affecting differentiation and cytokine production (64). In addition, it has also been shown that CD27 ligation can promote expression of the anti-apoptotic protein Bcl-Xl in human $CD4^+$ T cells (28). Therefore, it remains possible that CD27 triggering stimulates proliferation and/or survival of T_H1 polarized cells, thereby inducing the accumulation of IFN-γ producing T cells. In line with this, ligation of OX40, another member of the TNFR superfamily, can also induce proliferation and survival of CD4 T cells and thereby enhance the pool of T_H1 (65;66). However, the difference with CD27 is that this costimulatory effect of OX40 is not specific for T_H1 cells, as it can also enhance the pool of T_H2 cells, depending on the model (67-69).

In conclusion, these data indicate that CD27 signalling specifically enhances the pool of IFN-γ producing $CD4^+$ T cells, not by providing instructive polarizing signals, but most likely by the combination of sensitizing these cells for IL-12 mediated signaling and by acting on the proliferation and/or survival of these cells.

Methods

Mice

CD70TG mice were generated on a C57Bl/6J background and bred in the animal department of the Academic Medical Center (Amsterdam, The Netherlands) under specific-pathogen-free conditions (25). To generate CD70TG Balb/c mice, mice were backcrossed 10x with WT Balb/c mice (Harlan). WT mice were obtained from CD70TG C57Bl/6J or Balb/c littermates. Mice were used at 6-12 weeks of age, age- and sex-matched within experiments and were handled in accordance with institutional and national guidelines.

Cell staining and flow cytometry

Single-cell suspensions were obtained by mincing the specified organs through 40 μm cell strainers (Becton Dickinson). Erythrocytes were lysed with an ammonium chloride solution and cells were subsequently counted using an automated cell counter (Casy, Schärfe system). Cells (5×10^5- 5×10^6) were collected in staining buffer (PBS with 0.5% bovine serum albumin (Sigma)) and stained for 30 min at 4˚C with antibodies in the presence of anti-CD16-CD32 (FcBlock, clone 2.4G2; kind gift from Dr. Louis Boon, Bioceros, The Netherlands). The following monoclonal antibodies were obtained from Pharmingen: allophycocyanin-conjugated (APC) anti-B220 (clone RA3-6B2); peridinin chlorophyll protein-conjugated (PerCP) anti-CD3ε (clone 145-2C11); Fluorescein isothiocyanate-conjugated (FITC) anti-CD3ε (clone 17A2); PE- or PerCp-conjugated anti-CD4 (clone L3T4); PerCp-, FITC or APC-conjugated anti-CD8 (clone Ly-2); PE- or APC- conjugated anti-CD62L (clone MEL-14). Antibodies used from eBioscience: PE-conjugated anti-FoxP3 (clone NRRF-30); FITC-conjugated anti-CD44 (clone IM7); FITC-conjugated anti-CD27 (clone LG.7F9). Intracellular stainings for FoxP3 were performed using Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience), according to the manufactures protocol. Data were collected on a

FACSCalibur or FACSCanto (Becton Dickinson) and were analyzed with FlowJo software (Treestar, Inc.).

T cell stimulation assays

Direct ex vivo cytokine production. Splenocytes were plated at 1 x 10^6 cells/well in a 96-well round-bottom plate and stimulated for 2 hours with 1 ng/ml PMA and 1 μM ionomycin. After 2 hours the protein-secretion inhibitor Brefeldin A was added at 1 μg /ml final concentration (Sigma). Hereafter, cells were stained for CD4 and CD8 followed by fixation and permeabilization. Cells were then incubated for 30 min with fluorescent labelled antibodies against either IFN-γ, IL-17, IL-4, IL-5, IL-13 (eBioscience/BD).

T_H cell polarization. Naïve ($CD44^-CD62L^+$) $CD4^+$ T cells and B ($B220^+$) cells were electronically gated and sorted using a FACSAria cell sorter (Becton Dickinson). The purity of cells sorted using this method was consistently > 96%. Sort purified naïve $CD4^+$ T cells from WT mice were then stimulated for 3 days under T_H0, T_H1 or T_H17 polarizing conditions, or 7 days under T_H2 polarizing conditions, in the presence of WT or CD70TG derived B cells in a 1:1 ratio. All T cell polarization conditions included plate-bound αCD3 (clone 145-2C11, 5 μg/ml), soluble αCD28 (clone PV-1, 1 μg/ml) (both a kind gift from Dr. Louis Boon, Bioceros, The Netherlands) and soluble IL-2 (25 ng/ml) (Invitrogen). For T_H1 polarization 10 ng/ml IL-12 (R&D Systems) and 5 μg/ml αIL-4 (clone 11B11, a kind gift from Louis Boon, Bioceros) was added. For T_H2 polarization 50 ng/ml IL-4 (R&D Systems), 5 μg/ml αIL-12 (clone c17.8) and 20 μg/ml αIFN-γ (clone XMG 1.2, both mAbs were a kind gift from Louis Boon, Bioceros) was added. For T_H17 polarization, 3 ng/ml TGF-β (R&D Systems) and 20 ng/ml IL-6 (Peprotech) was included. Following stimulation, cells were stimulated with 1 μM ionomycin, 1 ng/ml PMA and 1 μg/ml Brefeldin A for 5 hours. Cells were then stained for

CD4 and CD8 followed by fixation and permeabilization and stained for IL-4, IL-5, IFN-γ, IL-17, IL-10 and IL-13 as described above.

Allergic Airway inflammation

Wild type and CD70TG Balb/c mice were sensitized to OVA by i.p injection of 20 μg OVA (Fluka, Switzerland) in a 200 μl alumimun potassium sulfate suspension (Sigma-Aldrich, Germany) on day 0 and 14 (36). Mice were then challenged on day 28, 29 and 30 by i.n. administration of 100 μg OVA in 50 μl of PBS. Control sensitization was performed with aluminum potassium sulfate in PBS and control challenge was performed with PBS alone. All mice were sacrificed on day 32 and serum, spleen and lung was collected.

Quantative real-time PCR (qPCR) analysis

RNA was isolated from naïve T cells and polarized cells (as described above) using TRIzol (Invitrogen), and cDNA was prepared by reverse transcription of 0,5 μg RNA. The resulting cDNA was subjected to qPCR analysis with the LightCycler System (Roche Diagnotics) in microcappilary tubes with a QuantiTect SYBR Green PCR kit solution (Qiagen). HPRT was used as a reference. Relative changes were calculated by the $2^{-\Delta\Delta CT}$ method (37). The primers used to detect mRNA transcripts are as follows: mTBet, 5'-CAACAACCCC TTTGCCAAAG-3' (forward) and 5'-TCCCCCAAGCAGTTGACAGT-3' (reverse); mGATA3, 5'-AGAACCGGCCCCTTATCAA-3' (forward) and 5'-AGTTCGCGC AGGATGTCC-3' (reverse); mRORγt, 5'-TGTCCTGG GCTACCCTACTG-3' (forward) and 5'-GTGCAGGAGTAGGCCACATT-3' (reverse); mTWIST, 5'-CGCACGCAGTCGCTGAA CG-3' (forward) and 5'-GACGCGGACATGGACCAGG-3' (reverse); mFOG, 5'-TCCCCTG AGAGAGAAGAACCG-3' (forward) and 5'-GCAGCATCCTTAGCCAGCA-3' (reverse); mBlimp-1, 5'-GACGGGGGTACTTCTGTTCA-3' (forward) and 5'-GGCATTCTTGGGA

ACTGTGT-3' (reverse); mHPRT, 5'-TGAAGAGCTACTGTAATGATCAGTCAAC-3' (forward) and 5'-AGCAAGCTTGCAACCTTAACCA-3' (reverse).

Statistical analysis

Statistical analysis of the data was performed using the unpaired Student's *t*-test. Asterisks denote significant differences (* $p<0.05$, ** $p<0.005$).

Acknowledgements

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Chapter 7

Timing and tuning of CD27-CD70 interactions: The impact of signal strength in setting the balance between adaptive responses and immunopathology.

Ronald W. van Olffen, Martijn A. Nolte, Klaas P.J.M. van Gisbergen,

and René A.W. van Lier.

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Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands.

Summary

After binding its natural ligand CD70, CD27, a TRAF-binding member of the tumor necrosis factor (TNF) receptor family, regulates cellular activity in subsets of T, B and NK cells and hematopoietic progenitor cells. In normal immune responses, CD27 signaling appears to be limited predominantly by the restricted expression of CD70, which is only transiently expressed by cells of the immune system upon activation. Studies performed in CD27-deficient and CD70-transgenic mice have defined a non-redundant role of this receptor-ligand pair in shaping adaptive T cell responses. Moreover, adjuvant properties of CD70 have been exploited for the design of anti-cancer vaccins. However, continuous CD27-CD70 interactions may cause immune dysregulation and immunopathology in conditions of chronic immune activation such as during persistent virus infection and autoimmune disease. We conclude that optimal tuning of CD27-CD70 interaction is crucial for the regulation of the cellular immune response. We provide a detailed comparison of costimulation through CD27 with its closely related family members 4-1BB, CD30, HVEM, OX40 and GITR and we argue that these receptors do not have a unique function *per se*, but that rather the timing, context and intensity of these costimulatory signals determines the functional consequence of their activity.

Dimensions of costimulation

The ability of T cells to detect virtually any pathogenic invader is granted by its extraordinarily diverse receptor repertoire, which allows the T cell pool to recognize a vast number of peptides upon presentation by major histocompatibility complex (MHC) molecules. Still, signaling through the TCR (also referred to as "signal 1") is not sufficient for adequate T cell activation, as costimulatory molecules provide indispensable signals for proliferation, survival and differentiation ("signal 2"). In fact, naïve T cells that only receive "signal 1" without "signal 2" are rendered anergic or die through apoptosis. The integration of "signals 1 and 2" is required for full T cell activation and the strength of these signals shapes the size of the ensuing T cell pool. Moreover, full differentiation into effector T cells is generally dependent on a third signal, which is supplied by the antigen-presenting cell (APC) in soluble form (as a cytokine, e.g. IL-12) and provides instructive signals for the type of effector T cell that is required.

This "three signal" concept depicts a model for the activation of naïve T cells and the subsequent formation of effector T cells. Yet, the immune system provides a plethora of diverse costimulatory molecules and these various types of "signal 2" all contribute in their own unique manner to the quality of the T cell response. Indeed the term "signal 2" reduces the complexity of the intricate regulatory circuits that control tailored T cell expansion and differentiation. Costimulatory signals can act on particular aspects of T cell activation, such as survival, cell-cycle progression, and differentiation to either effector or memory cell. The function of a particular costimulatory molecule is strongly related to the timing of its action, since early costimulatory signals are functionally distinct from those that act late during the T cell response. It is for this reason that the expression of each costimulatory molecule and/or its ligand is tightly regulated and dependent on the activation status of the cell.

The best-characterized costimulatory receptor is CD28, a member of the immunoglobulin superfamily of costimulatory molecules, which is already expressed on naïve T cells and can therefore play an important role in the initial phase of T cell stimulation. Upon interaction with one of its two ligands CD80 and CD86, which are rapidly expressed on APCs upon activation, CD28 induces upregulation of survival genes in the naïve T cell, facilitates its cell-cycle progression and enhances the production of IL-2 (reviewed in (1)). Other important costimulatory molecules on T cells are members of the TNF-R superfamily, such as CD27, 4-1BB, CD30, herpes virus entry mediator (HVEM), OX40 and glucocorticoid-induced TNFR family related gene (GITR). As new insight into the function of CD27 has been gained during recent years, we will focus our attention on this unique receptor in regulating immune responses and compare its function with its closely related family members.

Molecular aspects of CD27 evoked signals

CD27 is expressed as a disulphide-linked dimer and upon interaction with its unique ligand CD70 a truncated form of CD27 is released, most probably by a membrane-linked protease (2). Increase in soluble CD27 has been documented in situations of immune activation such as in autoimmune disease and during viral infection (reviewed in (3)). CD70, the only CD27 ligand that has been identified, is a membrane expressed homotrimeric type II membrane molecule. Analogous to other members of the TNF-R family, the first event after ligand engagement is most likely the trimerization of CD27, which forms the first step in the iniation of intracellular signaling. In line with this notion, it has been demonstrated that the intracellular tail of CD27 couples to TNFR receptor-associated factor (TRAF)-2 and TRAF5 (4;5) and deficiency of TRAF5 impairs CD27-mediated co-stimulation (6). Ligation of CD27 by CD70 induces strong ubiquination of TRAF and the activation of both canonical and non-canonical NF-κB pathways (7) (see also *Figure 1*). Additionally, CD27 has been shown to

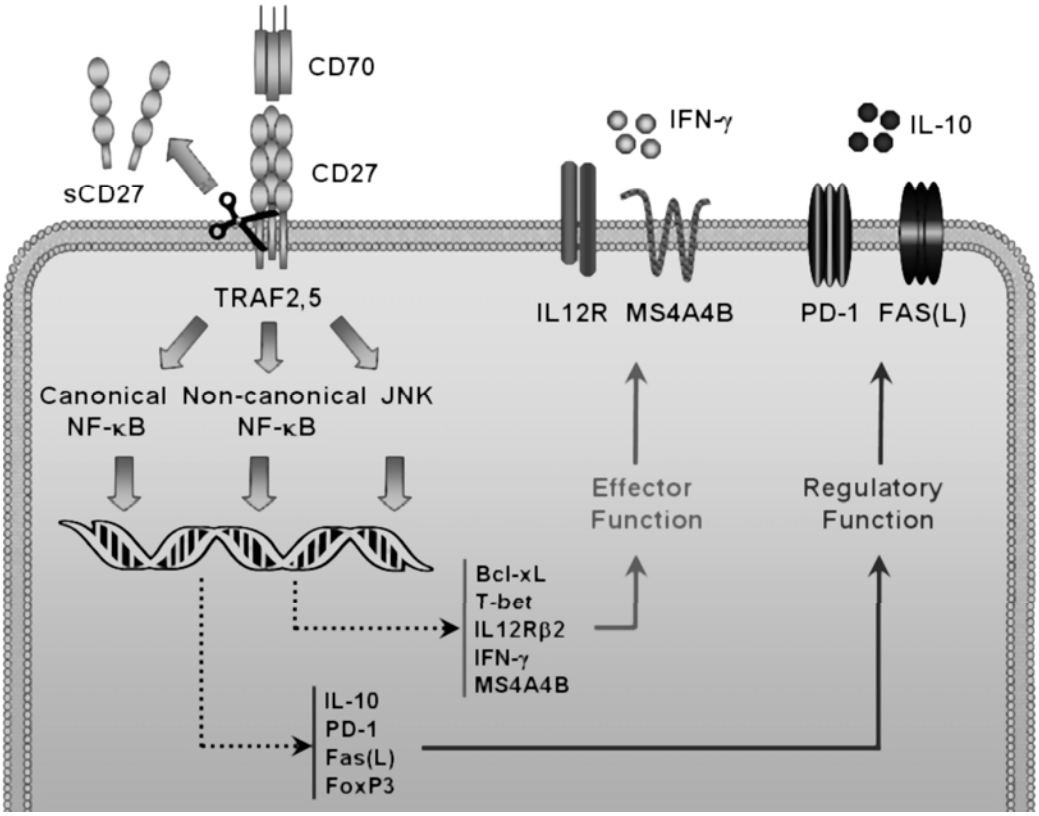

Figure 1: CD27 signalling induces multiple intracellular signalling pathways that contribute to immune regulation. CD70-induced triggering through CD27 on T cells is mediated by TRAF2 and TRAF5, which leads to the activation of canonical and non-canonical NF-κB pathways, as well as the JNK pathway. Either directly or indirectly, these signaling events can induce molecules involved in pro-inflammatory effector functions of T cells (in green) and anti-inflammatory regulatory functions (in red).

result in the initiation of the jun-N-terminal kinase (JNK) signaling cascade (4;5). Finally, it has been reported that CD27, as well as GITR, can bind Siva-1, an intracellar mediator of apoptosis (8;9), but the role of this interaction for the function of CD27 has yet to be resolved as ligation of CD27 generally does not limit but rather contributes to the expansion of activated lymphocytes (see below).

With the aim to identify genes that are induced as a consequence of CD27 triggering, it has recently been shown by means of an elegant *in vivo* immunization model in which transcriptomes of WT and CD27 deficient murine T cells are compared, that CD27 signaling contributes to the establishment of a T-helper-1 (Th1) type gene expression profile in CD4 T cells (10). Interestingly, MS4A4B, a tetraspan surface molecule homologous to CD20 that has previously been implicated in the induction of Th1 responses (11), is strongly induced as a consequence of CD27 signaling (10). These murine data indicate that CD27 costimulated responses induce Th1, which is in line with data obtained in the human sytem showing that CD27-CD70 interactions sensitize naive CD4 T cells for IL-12-induced Th1 cell development (12). In addition, CD27 ligation on these cells induces the upregulation of anti-apoptotic Bcl-xL, an established target of NF-κB signaling (12).

The interpretation of the *in vivo* consequences of CD27-CD70 interaction is complicated by the fact that CD70 can function as signal transducing receptor itself. Stimulation of CD70 with an agonistic anti-CD70 antibody initiates a signaling cascade that regulates expansion and differentiation of both murine and human T and B cells (13-15). Although the AKT/PKB pathway may play a role in these functional responses, the membrane proximal signaling events remain to be defined. Intracellular tails of murine and human CD70 show a low level of sequence homology and CD70-associated molecules have not been defined until now.

Expression of CD27 and CD70

Expression characteristics of both CD27 and CD70 are highly similar between mouse and man. The receptor CD27 is expressed on early thymocytes, as well as on naïve CD4 and CD8 T cells. Upon T cell activation, the expression of CD27 is increased, but it is downregulated when T cells have undergone several rounds of division and differentiate towards effector cells (16;17). Analysis in various infection models in mice and men has revealed that effector

CD8 T cells indeed have low to no expression of CD27 (18-21). Interestingly, central memory T cells, which reside in secondary lymphoid organs do express CD27, which indicates that expression of this receptor is highly dynamic (20).

Expression of CD70 is highly restricted and activation dependent, as it is only transiently expressed on activated T cells, B cells and dendritic cells (DCs) (22-26). Antigen receptor stimulation on T and B cells and Toll-like receptor triggering on both B cells and DCs induces CD70 expression, which is further enhanced by CD40 triggering (24;27). Depending on the cell type and culture conditions, CD70 expression on human lymphocytes can be enhanced by cytokines such as IL-1α, IL-12, GM-CSF and TNFα, while IL-4 and IL-10 can decrease CD70 expression (24;28). Provision of high doses of IL-2 *in vitro* or *in vivo* induces CD70 expression on human T cells, which results in a concomitant loss of cell surface expression of CD27 on CD8 T cells (29).

Impact of the CD27 costimulatory signal on T cell function

Although CD27 is highly expressed on thymocytes and naïve T cells, these cells do not seem to depend on CD27 for their generation and maintenance, as CD27-deficient mice have normal T cell development in the thymus and similar numbers of naïve T cells in secondary lymphoid organs as WT controls (30). However, deletion of CD27 does hamper the generation of T cell immunity, since CD27-deficient mice infected with influenza virus have a reduced number of CD4 and CD8 effector T cells that infiltrate the lung. The formation of memory T cells also depends on CD27, since a memory T cell response to a secondary challenge with influenza is greatly reduced in these mice. Importantly, CD27 is not required for entry into cell cycle upon T cell activation and neither for the differentiation towards interferon-gamma (IFN-γ) producing or cytolytic effector T cells (30). Instead, CD27 promotes survival of activated T cells throughout successive rounds of division and thereby

contributes to the accumulation of effector T cells (31). It has also been found that CD27 on CD8 T cells can induce proliferation in the absence of IL-2, which does not lead to effector cell differentiation (32). These studies indicate that CD27-triggering itself is not required, nor sufficient to induce effector cell formation, but that CD27 contributes to the formation of the effector cell pool by inducing proliferation and survival. Moreover, adoptive transfer experiments with WT or CD27-deficient ovalbumin (OVA)-specific CD4 T cells into mice that are subsequently immunized intranasally with OVA show that CD27 expressed on CD4 T cells promotes both the primary CD8 T cell response and the secondary expansion of memory CD8 T cells. In this model, CD27 instructs CD4 T cells to provide help to effector/memory CD8 T cells by inducing a Th1-type gene expression profile and a subsequent increase in the frequency of IL-2 and IFN-γ producing effector CD4 T cells (10). Since IL-2 is important to program memory CD8 T cells for secondary expansion (33), this might indicate that CD27-induced IL-2 production is the mechanism through which CD27 on CD4 T cells helps the memory CD8 T cells response, but this hypothesis remains to be confirmed.

Apart from studies with CD27-deficient mice, a lot of insight in the role of CD27-CD70 interactions has been gained by the *in vivo* use of blocking anti-CD70 antibodies. Several studies have shown that CD70 expression can be rapidly induced on DCs by stimulation of CD40 or Toll-like receptors (34-36) and in particular when these stimuli are combined (27). Subsequent *in vivo* blockade of CD70 is sufficient to inhibit priming of CD8 T cells (27;34-36). Treatment with anti-CD70 is even able to inhibit priming of splenic CD8 T cell responses upon infection with vaccinia virus, Listeria monocytogenes and vesicular stomatitis virus (37). Blockade of CD70 during the late, but not the early phase of a primary response to influenza virus prevents apoptosis of antigen-specific CD8 T cells and decreases the quality of the memory CD8 T cell response (38). Moreover, anti-CD70 can also block the priming of CD4 and CD8 T cells localized in the gut mucosa that respond to oral infection with Listeria

monocytogenes. It was found that the priming of these T cells depends on a unique population of APCs in the lamina propria that expresses high levels of CD70 (39). In the spleen, CD70 plays an important role on a specific DC subset that expresses the uptake receptor CD205 (40). When antigen is targeted specifically to this DC subset with an anti-CD205 antibody, priming and IFN-γ production of cognate CD4 T cells is independent of IL-12 and fully depends on CD70 expression by this DC subset. When the same antigen is targeted to a distinct, DCIR2-expressing DC subset, Th1 differentiation does not depend on CD70, but rather on IL-12 production (40). This indicates that CD70 expression is even differentially regulated between APC subsets and can play an instructive role in CD4 T cell differentiation. Importantly, these findings imply that signaling through CD27 ("signal 2") is in particular circumstances sufficient to induce differentiation to effector T cells and can thus overcome the requirement for "signal 3".

The aforementioned studies indicate that the CD27-CD70 axis plays an important role for the priming of T cells in a variety of immunization and infection models. Interestingly, the role of CD27 in protection against infection with lymphocytic choriomeningitis virus (LCMV) appears to be slightly different. Using CD27-deficient mice, it was shown that CD27 is not required during a primary infection with regards to effector cell formation and viral elimination (41), which was confirmed in WT mice using blocking antibodies against CD70 (37). However, CD27 is critically important for clonal expansion of memory cells and protection upon re-infection (41). It was found that LCMV-specific memory cytotoxic T lymphocytes (CTLs) retain CD27 expression, which depends on CD70 expressed by polyclonally activated B cells during the retraction phase (41) and on help provided by CD4 T cells during the primary infection (42). Ligation of CD27 on these memory CTLs during restimulation strongly enhances autocrine IL-2 production and thereby the secondary expansion (42). Whether a similar mechanism applies for the memory response during

influenza virus infection is not yet known and would be interesting to investigate, since retained CD27 expression can be found on memory CTLs induced by LCMV infection, but not by infection with vaccinia virus or immunization with tumor cells (41). This could suggest that the expression as well as the function of CD27 depends on the type of immune response. Alternatively, it could be that LCMV rather takes advantage of the CD27-CD70 axis, since CD27 signaling on CD4 T cells during LCMV-infection also induces immunopathology and suppression of neutralizing antibodies due to the enhanced production of IFN-γ and TNF-α by CD4 T cells. In fact, blockade of CD27 signaling is sufficient to eliminate an otherwise persistent strain of LCMV (43). These data indicate that CD27 can serve as a potent costimulatory molecule for T cell activation, but that its activity should be carefully maintained to prevent collateral damage (44).

Induction of strong CD27 activity *in vivo*

The potency of costimulation through CD27 also becomes evident in mice that constitutively express CD70 on all B cells: these CD19-CD70TG mice develop increased numbers of effector T cells, both in the CD4 and CD8 T cell compartment (45). The advantage of this enhanced signaling through CD27 is that these mice develop augmented CD8 T cell responses to influenza virus infection and are protected against a lethal dose of poorly immunogenic EL4 tumor cells (46). Antigen-specific CD8 T cells in these mice expand more rapidly, produce more IFN-γ and have a greater cytotoxic potential on a per cell basis. Thus, although CD27 triggering might not be necessary for effector CD8 T cell differentiation (30), constitutive stimulation of CD27 clearly potentiates this differentiation pathway. Still, these CD19-CD70TG mice also demonstrate the downside of this mechanism of enhanced effector T cell differentiation for the immune system, since the increased conversion of naïve T cells into effector cells culminates in the depletion of naïve T cells from lymphoid organs (47).

Moreover, also the differentiation and maintenance of B cells is severely compromised in these mice. Although part of the B cell differentiation defect is caused by CD27 stimulation on stem- and/or progenitor cells in the bone marrow (48), the major part of this B cell depletion is caused by IFN-γ production by effector T cells (45). The loss of B cells itself has severe implications for the architecture and function of the spleen, as it results in the gradual depletion of the splenic marginal zone (49). As a result of this dramatic phenotype, CD19-CD70TG mice die around 6-8 months of age from opportunistic lung infections with Pneumocystis carinii (47). This clearly indicates that continuing CD27-CD70 interactions, which can occur during chronic human immunodeficiency virus (HIV)-1 infection and chronic autoimmune diseases (50-52), not only enhance effector T cell formation, but can also exhaust both the T cell and the B cell pool and thereby contribute to the morbidity and mortalilty.

To further examine the role of CD27 triggering in the direct context of antigen presentation, the group of Dr. Janny Borst has recently described the phenotype of a transgenic mouse model in which CD70 is constitutively expressed on DCs. These CD11c-CD70TG mice have a phenotype that is comparable to the CD19-CD70TG mice, in that they also show a rapid CD27-mediated conversion of naïve CD4 and CD8 T cells to effector cells and develop a concomitant immunopathology (53). Interestingly, intravenous administration of MHC-I restricted peptide in the absence of adjuvants, which induces deletional tolerance in WT mice, induces high numbers of functional, long-lived effector CD8 T cells in CD11c-CD70TG mice, and to a lesser extent in CD19-CD70TG mice (53). Similar effects are seen in WT mice when, together with TCR stimulation, CD27 is triggered through a soluble recombinant form of CD70 (sCD70) (54). These data indicate that upon TCR triggering, costimulation through CD27 is sufficient to promote strong CTL responses *in vivo*. In fact, CD27-driven immunity by CD70 expression on DCs is even strong enough to break a pre-exisiting state of LCMV-

specific CD8 T cell tolerance (53). To what extent these mice are still able to maintain tolerance to self-antigens and prevent autoimmunity remains to be addressed.

Since not only APCs, but also T cells can transiently express CD70 upon activation, we recently developed a transgenic mouse line in which T cells constitutively express CD70 (55). This allows us to address whether T cell activation can also be mediated by CD70 provided by surrounding (activated) T cells. The first interesting finding in these CD2-CD70TG mice is that CD27-triggering during thymic development does not affect T cell differentiation. Secondly, CD2-CD70TG mice also generate more effector T cells over time, but the naïve T cell pool is not lost during aging of the mice. This is a striking difference with the CD19-CD70TG and CD11c-CD70TG mice, which suggests that CD70 expression on T cells differently affects the surrounding T cell population than CD70 expression on APCs. Whether this is related to the finding that the intracellular routing and surface expression of CD70 is differently regulated in cells that do not express MHC class II (56), such as murine T cells, remains to be examined. Thirdly, splenomegaly and B cell depletion in CD2-CD70TG mice is mild compared to CD19-CD70TG and CD11c-CD70TG mice and we find that CD8 T cells affect humoral immune responses. A fourth important observation in CD2-CD70TG mice is that CD70 expression by T cells does enhance primary CD8 T cell responses against acute influenza infection, but severely impairs memory CD8 T cell responses (55). Detailed analysis after the primary response indicates that antigen-specific CTLs in these mice have an exhausted phenotype and are unable to be maintained as memory cells. The combined findings in CD27-deficient and CD70 transgenic mice thus show that not only inhibition of CD27 activity, but also its constitutive triggering severely impairs memory CD8 T cells. To what extent this detrimental impact on memory formation is caused by the cell type that expresses CD70 is currently under investigation.

Regulation of CD27 signals

The potency of *in vivo* CD27 signaling necessitates negative feedback mechanisms that limit prolonged signaling and/or antagonize the strong cellular responses. One part of the regulation relies on the strict control of CD70 expression that under physiological conditions only occurs in the early phase of immune responses. CD27 appears to be the only receptor for CD70, which makes it unlikely that a negative signal transducer, in analogy to the CD28-CTLA4 system, balances signaling. At the receptor level, prolonged stimulation of T cells either *in vitro* or *in vivo* leads to reduced receptor expression, which is not only regulated at the level of transcription, but also by proteolytic cleavage of the transmembrane molecule followed by shedding of the extracellar part of the molecule (2;26) (see *Figure 1*).

At the cellular level, T cells chronically stimulated via CD70 produce the anti-inflammatory cytokine IL-10 and upregulate PD-1 (55), an inhibitory receptor belonging to the CD28 family. Production of IL-10 by effector T cells is thought to prevent inflammatory pathology in persistent infection (57), while the high expression of PD-1 likely downregulates cellular activation after binding its specific ligands. It can therefore be expected that a blocking PD-1L antibody will even further potentiate immune activation in the CD70 transgenic mice described above, but might thus also increase immune pathology. Additionally, CD70 induces the upregulation of Fas (CD95) expression and sensitivity (58) as well as FasL expression (55). The strong exacerbation of the immune activation phenotype in CD70 transgenic mice deficient for Fas demonstrates that the extrinsic apoptosis pathways contribute to the control of the activated T cell pool, which increases in size as a consequence of strong CD27-CD70 interactions (58). However, as CD27 ligation also results in the upregulation of Bcl-xL (12), it is likely that also intrinsic apoptosis pathways are modulated by CD27-CD70 interactions (see *Figure 1*).

Finally, at the population level recent observations using either CD70-expressing B cell non-Hodgkins lymphoma's (59) or chronic lymphocytic B cell leukemias (60) have shown that regulatory T cells can develop *in vitro* as a consequence of CD27 costimulation. Moreover, it has been argued that proliferating memory T cells can differentiate into regulatory T cells (61), which might imply that immune activation provides its own negative feedback mechanism on a population level in order to restore immune homeostasis. However, it is still unsettled if CD27 plays an important role for regulatory T cells *in vivo*, as in none of the CD70 transgenic mice described above, nor in CD27 deficient animals, (either on a C57Bl/6 or Balb/c background) conspicuous changes in numbers of regulatory $CD4^+$ T cells have been found (see *Figure 2A*). Although the expression of CD27 on regulatory T cells is downregulated in CD19-CD70TG mice due to continuous interactions with its ligand (chapter 6 of this thesis), no significant changes could be observed in numbers of naïve ($CD62L^+$) and effector ($CD62L^-$) regulatory T cells (see *Figure 2B*) (62). Expression of CD103 and CTLA4, which are expressed on highly active regulatory T cells,was also not differentially regulated in these mice, but we did find a significantly higher expression of GITR in CD70 TG mice (see *Figure 2C*). Interestingly, GITR expression on regulatory T cells was highest in mice with the strongest B cell depletion, i.e. CD19-CD70TG mice (see Figure 2C), which might indicate that GITRL expression on B cells normally suppresses surface expression of GITR on T cells. Conclusively, these data suggest that CD27-triggering does not affect the number nor the activation status of regulatory T cells, which is in sharp contrast to naïve and effector/memory T cells.

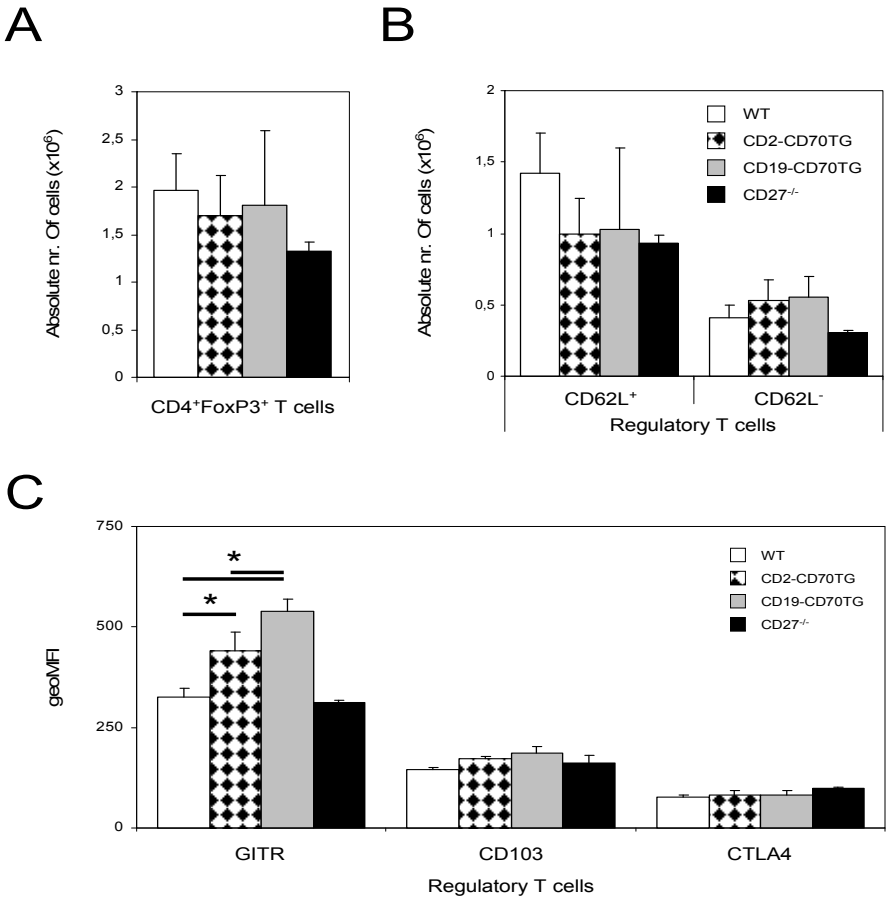

Figure 2: Numbers of regulatory T cells in genetically modified mice. The total number of (A) splenic regulatory ($FoxP3^+$) T cells and (B) the number of naïve ($CD62L^+$) and effector ($CD62L^+$) regulatory T cells in WT, CD2-CD70TG, CD19-CD70TG and $CD27^{-/-}$ mice on a C57Bl/6J background (average of 3 mice ± SD).

Costimulation through CD27: how important and unique is it?

Collectively, these studies demonstrate that CD27 can play an important role in the formation of the effector T cell pool by enhancing proliferation and survival of activated T cells. The finding that CD27-deficient mice have a diminished formation of effector and memory cells suggests that CD27 has a non-redundant function in this aspect of T cell biology. However, since CD27 only functions upon interaction with CD70, it can also be inferred that the degree of CD70 expression rather than the presence of the receptor determines the CD27-dependency of the ensuing T cell response. In other words: inflammatory responses that do not induce the

expression of CD70 are thus not dependent on CD27 and might thrive on other costimulatory signals. This leads to the important question to what extent CD27 has a specific and unique function or whether closely related members of the TNF-R superfamily can do the same job if they are expressed? To address this question, we will compare the role of CD27 with its closely related family members of the TNF-R superfamily.

In an elegant review, Michael Croft has suggested a model in which the function of CD27, 4-1BB, CD30, OX40 and HVEM is dependent on the timing of their maximal expression and that of their respective ligands (63). In the proposed model, HVEM and CD27 play an important role in the early phase of the T cell response, next to CD28, because they are expressed on naïve T cells. Since HVEM expresion is rapidly downregulated and CD27 expression is upregulated upon T cell activation, it was suggested that HVEM and its ligand LIGHT (which stands for "lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes") have their most important function during initial activation, while CD27-CD70 interactions mainly stimulate the subsequent phase of clonal expansion by stimulating survival and proliferation. In contrast, expression of OX40, 4-1BB and CD30 and their respective ligands is induced on proliferating T cells and activated APCs and it is therefore most likely that these molecules play a role during later stages of the response. Both 4-1BB and OX40 are able to prevent apoptosis by inducing the expression of anti-apoptotic molecules such as Bcl-xL and Bfl1 and thereby contribute to the size of the effector pool (64-66). It is not yet known whether CD30 can act in a similar manner, but parallels with OX40 and 4-1BB have been suggested (63;67). Thus, rather than having specialized and distinctive functions, the unique contribution of each receptor would actually rely on the timing of its expression and that of its ligand. This would be in line with the rather common signal transduction pathway of these molecules, since all five receptors signal through association with various TRAF family members, which

successively activate the transcription factors NF-κB and in most cases AP-1 (reviewed in (63;68)). Activation of these rather general pro-inflammatory transcription factors can induce a plethora of pro-inflammatory genes, of which many are involved in cell cycle and survival. This supports the notion that not the receptor as such provides unique signals, but that the timing and context of its signaling is most important for its particular function.

Convincing evidence on the actual contribution of these costimulatory molecules to a T cell response has been obtained in mice that lack one of the receptors (see *Table 1*; for a detailed overview on the expression of these molecules, see reference (68)). Similar to CD27, mice lacking expression of 4-1BB, OX40 or their ligands have defective primary and secondary T cell responses against several viruses (69-77). Important differences in these models are that 4-1BB typically regulates the CD8 T cell response, while OX40 governs the CD4 T cell response. Interestingly, 4-1BB-deficient mice generate a more enhanced effector CD4 T cell response to a protein antigen, which suggests that 4-1BB might also have a negative regulatory role on T cells (78). In this respect, it is interesting to note that both OX40 and 4-1BB can play an important role on regulatory T cells, possibly in both positive and negative manners (reviewed in (79)). Furthermore, experiments performed with mice deficient in both 4-1BBL and OX40L confirm that 4-1BB and OX40 act independently and nonredundantly to facilitate robust CD8 and CD4 recall responses, respectively (80). Still, other reports indicate that this specialization is not absolute, as OX40 can also contribute to CD8 T cell responses under certain circumstances (81-83). Mice double-deficient for OX40 and CD30 show that these receptors can act synergistically, which is important for the survival of memory CD4 T cells in germinal center responses (84) and effector CD4 T cells during Salmonella infection (67). Studies in mice deficient for either CD30 or CD30L indicate that CD30 is required for adequate effector CD4 T cell responses during mycobacterial infections (85;86) and in the generation of long-lived memory CD8 T cells following infection with Listeria

Table 1 Immunological consequences of gene targeting the TNFR superfamily members

Molecule	Expression	KO	TG	Phenotype	Refs
CD27	T & B cells NK cells Progenitors	Full KO	-	Reduced CD4 and CD8 T cell expansion and memory formation to influenza; normal primary T cell response, but reduced memory response to LCMV.	(30;41)
		-	-	-	
CD70	T & B cells DCs Macrophages	-	-	-	
		-	B cell-TG	Enhanced CD4 and CD8 effector T cell formation; improved clearance of influenza and tumor; splenomegaly and IFNγ dependent B cell depletion; premature death due to opportunistic infection.	(45-47)
		-	DC-TG	Enhanced CD4 and CD8 effector T cell formation; lack of CD8 deletional tolerance; splenomegaly and B cell depletion.	(53)
		-	T cell-TG	Enhanced CD8 effector T cell formation, but impaired maintenance of memory CD8 T cells; only minor splenomegaly and B cell depletion.	(55)
4-1BB	T & B cells NK cells DCs	Full KO	-	Reduced CD8 T cell response to VSV; enhanced CD4 T cell response to protein immunization.	(72;78)
		-	T cell-TG	Enhanced T cell proliferation *in vitro* and increased CHS.	(108)
4-1BBL	B cells DCs Macrophages	Full KO	-	Reduced CD8 T cell expansion and memory formation upon acute infections; normal CD8 T cell numbers but impaired function during chronic infection.	(70;73;74;76)
		-	B cell-TG	Normal T cell response, but reduced APC function during allogeneic stimulation; splenomegaly, B cell depletion and reduced IgG responses.	(107)
CD30	T cells	Full KO	-	Reduced T cell expansion and IFN-γ production by CD4 T cells upon mycobacterium infection; contradictory reports on thymic negative selection.	(85;115;116)
		-	T cell-TG	Enhanced thymocyte apoptosis upon stimulation; splenomegaly.	(114)
CD30L	T & B cells Macrophage	Full KO		Reduced CD8 memory T cell formation upon Listeria infection; reduced CD4 effector T cell formation upon mycobacterium infection.	(86;87)
		-	-	-	
HVEM	T & B cells DCs Macrophages	Full KO	-	Enhanced T cell activation and cytokine production.	(96)
		-	Soluble form	Resistant to infection with HSV-1, but not pseudorabies virus.	(120)
Light	T cells DCs	Full KO	-	Reduced CD8 T cell expansion and effector cell formation to SEB or peptide; reduced CD8 T cell proliferation and CD4 T cell IL-2 production in MLR.	(88;100)
		-	T cell-TG	Enhanced CD4 and CD8 effector T cell formation; splenomegaly, autoantibodies and inflammation of several organs; reduced thymic output.	(117) (118;119)
OX40	T & B cells DCs	Full KO	-	Reduced CD4 T cell proliferation and effector cell formation to infection with influenza and LCMV, but not with L. major, N. brasiliensis or TMEV; reduced formation of effector and memory CD8 T cells to VACV infection.	(71;75;83)
		-	-	-	
OX40L	T & B cells DCs Macrophages	Full KO	-	Reduced CD4 T cell proliferation and effector cell formation to protein immunization; reduced CD8 T cell response to allogeneic stimulation.	(69;77)
		-	DC-TG	Increased CD4 accumulation in B cell follicles.	(112)
		-	T cell-TG	Enhanced CD4 T cell responses, inflammation of lung and intestine; more severe EAE; enhanced Th2 response and impaired clearance of L. major.	(109) (110;111)
		-	All cell-TG	Increased CD4 T cell numbers, enhanced CHS and allogeneic response.	(113)
GITR	T cells	Full KO	-	Reduced regulatory CD4 T cell numbers; reduced effector T cell activity and disease intensity during experimental colitis or arthritis; enhanced CD4 effector T cell formation upon Candida infection.	(101;105) (103;106)
		-	-	-	
GITRL	B cells	-	-		
		-	B cell-TG	Enhanced *in vivo* effector and regulatory CD4 T cell proliferation, delayed disease induction in EAE model.	(146)

Expression profile of costimulatory members of the TNF-R superfamily and the phenotype of genetically mutated mice, in which these respective molecules have either been deleted (KO) or transgenically expressed (TG).

monocytogenes (87). Furthermore, in a variety of models with HVEM- or LIGHT-deficient mice it has been shown that the LIGHT-HVEM costimulatory system can play an activating role in both CD4 and CD8 T cell responses (88-92), although expression of LIGHT is dispensable for T cell responses during influenza virus infection (93). Apart from its function as an activating receptor through binding LIGHT, HVEM can also act as a ligand for the inhibitory receptor B and T lymphocyte attenuator (BTLA) and thereby deliver inhibitory signals to T cells (94-96). Another complicating factor in the study of LIGHT-HVEM interactions is that LIGHT can also bind the lymphotoxin (LT)-β receptor and decay receptor 3, while HVEM can also bind soluble lymphotoxin α3 (97-99). The importance of this was revealed in a study using mice deficient for both LIGHT and LTβ, which not only confirmed the costimulatory function of LIGHT in T cell activation, but also revealed a cooperative role for LIGHT and LTβ in lymphoid organogenesis (100). It is thus possible that changes in LIGHT-HVEM interaction also affect the function of other binding partners of these molecules. The last receptor worth mentioning in this series is GITR, which is expressed on activated CD4 and CD8 T cells as well as on regulatory CD4 T cells. Studies using GITR-deficient mice show that costimulation through GITR enhances T cell proliferation and that the absence of GITR is protective in several inflammatory disease models, which is attributed to an impaired effector function of T cells (101-104). This can indeed be a direct effect of less costimulatory signals for the activated T cells, but it can also be an indirect effect due to the fact that GITR-deficient non-regulatory T cells can not escape suppression by regulatory T cells and are therefore less functional than WT controls (105). On the other hand, GITR-deficient mice are less susceptible to infection with Candida albicans and develop more protective effecor Th1 cells, which is attributed to the finding that GITR on regulatory T cells can inhibit IL-12 production by DCs and thereby affect Th1 differentiation (106).

In summary, deletion of one of these costimulatory receptors or their ligands generally leads to a mild phenotype in mice and hampers but does not prevent the formation of a functional pool of effector and memory T cells. The consequence of such a deficiency for the course of the T cell response depends on the cell type that is affected, the degree by which expression of the ligand and/or receptor is induced in a particular model and whether other costimulatory molecules are expressed that can take over the function of the deleted molecule.

The impact of constitutive costimulation

A more serious phenotype is generally observed when the costimulatory ligands (and in some cases also the receptor) are overexpressed and constitutively provide costimulation to T cells (*Table 1*). Similar to CD70, overexpression of 4-1BBL on APCs, using the MHC class II I-Eα promoter, induces a profound splenomegaly, B cell depletion in both primary and secondary lymphoid organs and a concomittant reduction of IgG responses upon immunization (107). This phenotype is quite similar to CD19-CD70TG mice (45), but it is not yet known whether the phenotype of 4-1BBL-TG mice is equally dependent on the increased IFN-γ production by effector T cells. Overexpression of 4-1BB on T cells does not induce gross phenotypic changes in lymphoid organs, but it does enhance proliferative responses of CD4 T cells *in vitro* and it induces an elevated contact hypersensitivity (CHS) response *in vivo* (108). In contrast, mice that overexpress OX40L on T cells develop a severe autoimmune phenotype, as they develop massive splenomegaly, interstitial pneumonia, inflammatory bowel disease and produce anti-DNA auto-antibodies (109). These mice display a more severe disease intensity during experimental autoimmune encephalomyelitis (EAE) (110) and are more susceptible to infection with Leishmania major, which is accompanied by an excessive Th2 response (111). Moreover, these mice have hyperproliferative CD4 T cells, accumulate high numbers of effector memory CD4, but not CD8 T cells and do not loose their B cells (109) as seen in

CD70TG and 4-1BBL-TG mice (45;107). OX40L-overexpression on dendritic cells induces an accumulation of activated CD4 T cells in B cell areas following immunization, but this does not influence antibody development (112). Finally, mice that overexpress OX40L on all cell types, using a β-actin promoter, have increased numbers of CD4 T cells, which are more responsive in an allogeneic setting and induce an enhanced CHS response (113). Whether these two transgenic models develop an equally severe phenotype as the T cell-specific OX40L-transgenic mice is not described in these papers.

Whereas overexpression of CD70 or OX40L on T cells does not seem to affect T cell selection in the thymus, constitutive expression of CD30 on T cells induces apoptosis of thymocytes, but only after deliberate stimulation (114). This study suggests that CD30 is important in thymic negative selection, which correlates with findings in CD30-deficient mice (115), but which is opposed in another study (116). CD30-overexpression also increases the size of the spleen and mesenteric lymph nodes (114), but the quality of T cell responses in these mice has not been described. Furthermore, overexpression of LIGHT on T cells is sufficient to increase apoptosis of double-positive thymocytes and it is suggested that LIGHT plays a role in negative selection of T cells in the thymus (117). A large proportion of the T cells that make it into the periphery in these mice differentiate into IFN-γ producing effector T cells, which elicit a dramatic phenotype, including splenomegaly and lymphadenopathy, severe intestinal inflammation, glomerulonephritis and destruction of the reproductive organs (118;119). Adoptive transfer of LIGHT-transgenic T cells to RAG-deficient mice is sufficient to induce intestinal inflammation, which is dependent on expression of LTβ receptor in the recipient and HVEM on the donor T cells (96). Whether the rest of the severe phenotype of LIGHT-transgenic mice also depends on both receptors is not clear. Finally, transgenic mice that overexpress a soluble form of HVEM-Ig are resistent to infection with herpes simplex vius type 1 (HSV-1), but not pseudorabies virus (120). HSV-1 uses HVEM as a receptor for

entry into the cell (hence its name) and the transgenic soluble form of HVEM can bind the virus and thereby block its infectivity. Whether HVEM-overexpression also affects anti-viral T cell responses, eg. by increased signaling through BTLA, is not described.

The above described models clearly show that constitutive signaling through one of these costimulatory molecules is generally sufficient to induce a severe phenotype with immunopathology. We recently found that the GITR-GITRL pathway forms an exception to this rule, since transgenic expression of GITRL on B cells induces strong effector CD4 T cell formation without any signs of immunopathology as seen in the above described models (Van Olffen *et al.,* unpublished data). The reason for this protected phenotype is that increased availability of GITRL not only provides costimulation for conventional T cells, but also activates and expands regulatory CD4 T cells. These regulatory T cells are fully functional and are even able to counteract the expanded pool of effector CD4 T cells in an EAE model, thereby delaying disease induction (Van Olffen *et al.*, unpublished data).

In conclusion, these transgenic models demonstrate the necessity of tightly regulated expression of costimulatory receptors and their ligands: even though constitutive costimulation does enhance T cell responses, these cells can also induce severe immunopathology, unless they are kept in check by regulatory T cells. CD27-CD70 interactions can play a potent role in T cell activation and should be tightly controlled, but comparison within the family clearly indicates that close relatives can act in a similar fashion. Therefore, we would like to suggest that although lack of a single costimulatory TNF(-R) superfamily member only moderately inhibits a productive immune response, constitutive stimulation through these receptors is rather detrimental for the host, due to collateral damage induced by excessive immune activation. This implies that the relationship between the amount of costimulation given through these receptors and the productivity of the ensuing immune response is not sigmoid, as one might intuitively have expected, but rather bell-

shaped (see *Figure 3*). To what extent signal strength rather than signal duration determines the outcome of the response is difficult to assess in these *in vivo* systems and probably depends on the type of immune activation model that is used. We anticipate that the importance of this model is most relevant for chronic infectious diseases and tumours, since these are conditions in which expression of costimulatory ligands can be disproportionate and lasting. It will therefore be important to assess to what extent excessive costimulation contributes to the course of the disease.

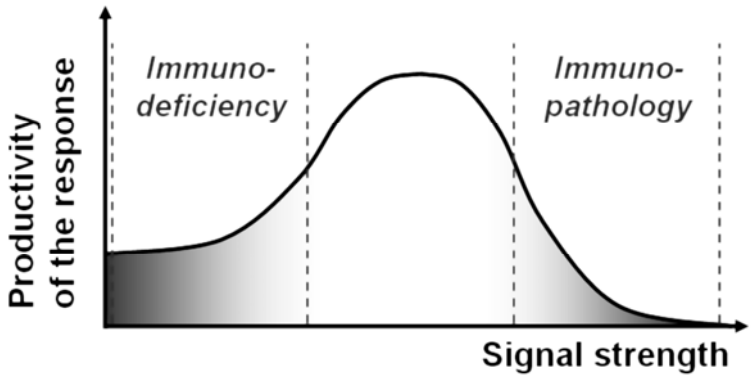

Figure 3: The strength of co-stimulatory signals delivered through members of the TNF-R superfamily determines the outcome of immune reactions.
Low level triggering (as in deficient mice or after treatment with blocking antibodies) diminishes, but hardly ever completely blocks immune responses (left side of the figure), whereas continuous and strong activation limits immune reactivity and leads via chronic immune activation to the collapse of the immune system (right side). Optimal responses depend on the proper dosing and timing of costimulatory signals (middle part of the graph).

Function of CD27 on non T cells

Although CD27 can play an important costimulatory role on T cells, this receptor is also expressed on a variety of other cell types. In humans, CD27 is induced on B cells through antigen receptor triggering and because its expression is maintained long-term after activation, it is also a typical marker for memory B cells (121-123). Triggering of CD27 on human B cells stimulates immunoglobulin production by promoting the differentiation to

plasma cells (124-126). Moreover, it was recently shown that interaction between activated CD8 T cells and freshly isolated B cells promotes survival and proliferation of the former, which is dependent on CD27 on the B cells and CD70 on the T cells (127). In mice, CD27 is not a marker for somatically hypermutated B cells as it is only expressed at the centroblast stage, after which expression is rapidly lost (128). On these cells, CD27 triggering promotes germinal center formation, most likely by supporting centroblast expansion. Still, CD27 is not absolutely required for adequate B cell responses, as CD27-deficiency does not affect isotype switch, somatic hypermutation or antibody production (128).

Apart from T and B cells, NK cells have also been reported to express CD27. In humans, CD27 expression was originally found on resting NK cells and activation with IL-2 increases its expression (129). Triggering of CD27 through CD70 in the presence of IL-2 or IL-12 directly enhances NK activity by increasing effector-target conjugate formation (130). Recently, we and others have found that CD27 expression can define phenotypically and functionally distinct human NK cell subsets, as the majority of human NK cells in peripheral blood is $CD27^{low}/CD56^{dim}$, while a minor population has a $CD27^{hi}/CD56^{bright}$ phenotype (131;132). The former population contains higher levels of perforin and granzyme B and is correspondingly more cytotoxic, whereas the latter population is better capable in production of the inflammatory cytokines IFN-γ and TNF-α. We found that CD27 expression on NK cells is controlled by IL-15 and that CD27 down-regulation is specifically induced by CD70 (132). In mice, CD27 is uniformely expressed on immature $CD11b^{low}$ NK cells, whereas the population of mature $CD11b^{hi}$ NK cells consists of two distinct subsets based on CD27 expression: $CD27^{hi}$ cells are more proliferative, produce more IFN-γ upon cytokine stimulation and are more cytotoxic than the $CD27^{low}$ population (133). Transplantation experiments suggest that the mature and functionally active $CD27^{hi}$ population can differentiate into the more resting $CD27^{low}$ subset, which indicates a lineage relationship

between these subsets, rather than the presence of two independent types of NK cells (133). In mice, CD27 triggering is sufficient to induce proliferation and IFN-γ production of freshly isolated NK cells, while prestimulation via CD27 also enhances the cytotoxic capacity of NK cells in an IFN-γ dependent manner (134). Little is known about NK cell function in either CD27-deficient or CD70-transgenic mice.

Expression of CD27 is also found on early hematopoietic stem cells in murine bone marrow (48;135), as well as more differentiated precursors, including the earliest lymphocyte precursors and common lymphoid progenitors (136;137). We found that CD27-deficient progenitor cells perform better in differentiation assays both *in vitro* and *in vivo*, while CD27-stimulation leads to a decrease in their differentiation capacity and an accumulation of early progenitor cells (48). The molecular mechanism of this effect is currently under investigation. CD27 has not been found on the earliest hematopoietic precursors in human bone marrow (135), but it is present on early B cell progenitors (138). Although the physiological role of CD27 expression on bone marrow precursors is not yet known, we have suggested that it is part of a cellular feedback mechanism, in which activated lymphocytes that express CD70 are able to affect hematopoiesis during immune activation (48).

CD27/CD70 interactions in pathophysiology

In a number of chronic clinical conditions that are associated with an enhanced activation of the immune system, an increase in CD70 expression has been documented. First, T cells from HIV-infected individuals express enhanced levels of CD70 upon activation, which contributes to their APC-like properties *in vitro* (52). Enhanced stimulatory potential of these non-professional APC may contribute to persistently high levels of immune activation in HIV infection and be related to disease progression which is supported by the demise of T cell system observed in CD70 trangenic mice. In patients with rheumatoid arthritis, CD70 is

significantly more expressed on CD4 T cells compared with age-matched controls (50). Interestingly, as CD70 on T cells lowers the activation threshold predominantly of low-avidity T cells, it suggests that high expression of CD70 might contribute to the breakdown of tolerance in this autoimmune disease. Likewise, T cells from SLE patients overexpress CD70 (51). This increased expression might contribute to B cell costimulation and subsequent immunoglobulin overproduction that may contribute to lupus. Although the role of CD27-CD70 interactions in the pathogenesis of human autoimmune disease needs clarification, blocking studies with anti-CD70 antibody show that this treatment can prevent EAE, the mouse model for multiple sclerosis (139). The preventive effect of anti-CD70 mAb was not due to the inhibition of T cell priming and antibody production from B cells, or immune deviation. However, TNF-alpha production was suppressed by treatment with anti-CD70 mAb, indicating that the ameliorating effect of anti-CD70 mAb appeared, at least in part, to be mediated by the inhibition of TNF-alpha production. These results indicate that the CD70-CD27 interaction plays a pivotal role in the development of cell-mediated autoimmune disease.

CD70 expression has been found on human malignancies from different origins including thymic carcinoma, renal cell carcinoma, gliobastoma, chronic lymphocytic leukemia, non-Hodgkin lymphoma and human T cell leukemia virus-1 induced T cell leukemia (reviewed in (140)). Since many of the tumors arising from the hematopoietic lineage also express CD27, a possible role for CD27-CD70 interactions in the regulation of tumor cell expansion and survival might be envisaged. Further, the high expression of CD70 on particular tumors implies that the molecule is an attractive candidate for active immunotherapy. Indeed, a humanized CD70 mAb has been engineered that possesses Fc-dependent antibody effector functions and mediates anti-tumor activity *in vivo* (141;142).

Finally, the ability of CD70 to induce a strong expansion of effector T cells *in vivo* (45), breakdown of tolerance (53) and induction responses to non-immunogenic tumors (46) makes CD70 an attractive adjuvant for active immunotherapy. In this respect it is promising that multimeric soluble CD70 is as able as membrane-bound costimulatory activity *in vivo* (54). Soluble CD70 has the advantage that dose and timing of costimulation can be tuned avoiding the side effects of excessive CD27-CD70 interactions.

Perspective

The large variety of costimulatory molecules that can be put into action during T cell activation generally enables an adequate and well-balanced T cell response upon encounter of most antigens and pathogens. As highlighted here, CD27 and its related family members assist activated T cells in their processes of survival, proliferation and acquisition of effector functions. They also seem to play a role in the formation of distinct T cell subsets that are generated during infection, such as effector T cells that target infected cells, effector memory T cells that provide immediate effector function upon re-challenge, and central memory T cells that have the potential to clonally expand upon secondary infection. However, the specific contribution of CD27 and other TNFR molecules in formation of these different T cell subsets is not yet known and it will not be an easy task to examine this, as at present contrasting and mutually exclusive views exist on T cell differentiation (143). Nevertheless, a better understanding on the role of CD27 and related molecules in development of short lived effectors versus long-lived memory T cells has high value, as this knowledge may guide the design of optimal vaccination strategies.

The role of CD27 has been well studied in acute infection models and CD27-driven costimulation seems to be beneficial for T cell responses, leading to enhanced pathogen clearance. Clearly, CD70 is constitutively expressed during chronic infection and in

autoimmune disease, but not much is known on the role that CD70 and CD27 play in chronic infection models. The only exception in this respect is infection with LCMV, but these studies rather indicate that CD70-driven responses are counter-effective on viral clearance (41;43). Transgenic mouse models have clearly revealed the powerful effector T cell differentiation capacity of CD27, but also indicate that CD70 can inititate inhibitory pathways mediated by inhibitory molecules PD-1 and IL-10 and activate apoptosis pathways driven through FasL and Fas. However, the importance of these regulatory events driven by CD27 in chronic diseases remains to be proven.

Although it is evident that transient signaling through CD27 and related costimulatory molecules is benificial for the immune response, sustained signaling rather results in immune pathology. At present we do not understand the underlying molecular mechanism, but new insight might come from studies on CD40 and TNFR-I, as it has been suggested that downstream signaling pathways through NF-κB and MAPKs are different between sustained and transient triggering of these receptors (144;145). Whether the same concept holds true for CD27 and its relatives and to what extent this could explain the induction of immune pathology is food for future research.

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English Summary

T cell activation requires the recognition of a cognate antigen bound to major histocompatibility complex (MHC) molecules via their T cell receptor (TCR). Hereafter, the clonal expansion, differentiation and polarization of T cells is dependent on costimulatory molecules and the cytokine environment. In **chapter 1**, T cell activation is described in more detail with a specific focus on the effects of costimulatory molecules, specifically members of the TNFR superfamily, on adaptive immune responses. This thesis focuses on the function of two members of the TNFR superfamily, namely CD27 and GITR, in T cell homeostasis and immune activation.

The glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is expressed on activated T cells and regulatory $CD4^+$ T cells. To gain insight in the function of GITR on these functionally opposing cell types, we developed a mouse model in which GITRL is constitutively expressed on B cells, and assessed the effects of chronic GITR signaling on T cell activation, differentiation and proliferation *in vivo* (**chapter 2**). We found that overexpression of GITRL induced an accumulation of both effector and regulatory $CD4^+$ T cells, but it did not affect $CD8^+$ T cells, nor naïve $CD4^+$ T cells..This increase was the consequence of increased proliferation of effector and regulatory $CD4^+$ T cells and not due to enhanced differentiation of naïve T cells. In addition, GITR ligation enhanced the activation state of regulatory T cells, but did not affect their suppressive capacity. Furthermore, the impact of GITRL overexpression on the $CD4^+$ T cell pool was functionally protective in an autoimmune model, i.e. experimental autoimmune encephalomyelitis (EAE), as it significantly delayed the onset of disease. Thus, we conclude that costimulation through GITR provides a functional balance between $CD4^+$ effector and regulatory T cells by enhancing the proliferation of both cell types.

Following recognition of an antigen by the B cell receptor, B cells generally require additional signals from helper T cells to further differentiate to plasma or memory B cell. We therefore postulated that the increase in regulatory and effector $CD4^+$ T cells in GITRL TG mice could affect the humoral immune system (**chapter 3**). Whereas B cell development and terminal differentiation of B cells in the spleen was not affected in GITRL TG mice, these mice showed increased serum IgA titers and decreased IgG3 levels, suggesting modulated B cell responses. When stimulating splenic B cells *in vitro*, GITRL TG B cells displayed normal immunoglobulin production, which indicates that there is no intrinsic B cell defect in these mice. Interestingly, compared to WT mice the numbers of mucosal B cell subsets were affected in GITRL TG mice, as peritoneal B1 B cells numbers were decreased and numbers of B2 B cells were increased, correlating with increased serum IgA immunoglobulin levels. The increased levels of IgA in GITRL TG mice did not protect these mice against an influenza infection compared to WT mice. In conclusion, the GITR-GITRL axis of costimulation modulates the numbers of mucosal related B cell subsets, abrogates IgG3 responses and promotes IgA responses *in vivo*.

Similar to effects observed in GITRL TG mice, transgenic overexpression of CD70, the ligand for CD27, results in expansion of IFNγ-producing $CD4^+$ T cells *in vivo*. However in contrast to GITRL TG mice, CD70 TG mice eventually deplete their naïve $CD4^+$ T cell compartment and show early mortality due to opportunistic pulmonary infections. As macrophages are key players in defense against pulmonary pathogens and IFNγ is an important factor in macrophage activation, we assessed the effects of chronic immune activation via CD70-driven costimulation on the myeloid compartment (**chapter 4**). CD70 TG mice showed an IFNγ-dependent increase in numbers of activated monocytes, which expressed high levels of MHC class II, compared to WT mice. These activated monocytes showed normal phagocytosis and migration characteristics *in vitro*, but displayed an enhanced

IFNγ-dependent susceptibility to apoptosis. As a consequence, monocytes from CD70 TG mice failed to accumulate at inflammatory sites *in vivo* and these mice were highly protected against atherosclerosis. These findings reveal that CD70-driven immune activation modulates the myeloid compartment, which on one hand increases the risk of opportunistic infections, but on the other hand protects from atherosclerosis.

Normally, costimulation of T cells via CD27 signaling is dependent on the transient expression of CD70 on cells of the immune system (T, B and DC's) upon activation. However, during chronic immune activation CD70 is constitutively and highly expressed on activated T cells. Chronic immune activation also results in enhanced formation and exhaustion of effector $CD8^+$ T cells and a failure to develop a memory $CD8^+$ T compartment. To assess the functional consequences of enhanced CD27 triggering on T cell homeostasis during chronic immune activation, we generated mice in which CD70 is constitutively expressed on T cells (CD2-CD70TG). These CD2-CD70TG mice showed increased numbers of $CD8^+$ effector T cells, phenotypically similar to exhausted cells found during chronic inflammation (**chapter 5**). Interestingly, the formation of $CD4^+$ effector T cells was not affected. CD70-driven costimulation enhanced the primary $CD8^+$ T cells response to influenza A infection, but showed impaired maintenance of memory $CD8^+$ T cells. Thus, we conclude that CD70 driven costimulation deregulates $CD8^+$ T cell homeostasis, reminiscent to events found in chronic inflammation.

Generally, the formation of $CD4^+$ helper T (T_H) cells is dependent on costimulatory molecules and polarizing cytokines. **In chapter 6**, we investigated whether CD27 signaling influences helper T cell formation by providing instructive, supportive or inhibitive signals for T_H cell differentiation. CD70-driven costimulation enhanced formation of IFNγ-producing T_H1 polarized cells in T_H1 prone C57Bl/6J mice, but did not influence helper T cell formation in T_H2 prone Balb/c mice. In addition, CD27 signaling did not affect the formation or activation

status of regulatory T cells *in vivo*. By stimulating $CD4^+$ T cells *in vitro* in the presence or absence of CD70-mediated costimulation, we found that CD27 signaling supports the formation of IFNγ, but also IL13 producing $CD4^+$ T cells depending on the cytokine environment and the genetic background, whereas it inhibited T_H17 polarization. In CD70 TG Balb/c mice the induction of allergic airway inflammation, a typical T_H2 polarizing pathology, resulted in increased formation of IFNγ producing $CD4^+$ T cells, without impairing T_H2 responses. Furthermore, in line with a supportive rather than an instructive role, CD27 signaling did not modulate transcription factor expression levels. In conclusion, these data indicate that CD27 signaling supports T_H1 differentiation, permits T_H2 formation, and inhibits T_H17 formation.

Chapter 7 reviews the current knowledge of costimulation via TNFR superfamily members in general and CD27 in particular and how adaptive immune responses are modulated depending on the timing, context and intensity of these costimulatory signals. In line with the observations presented in this thesis, we propose that excessive costimulation can affect the immunoregulatory balance of the immune system, which can lead to immune pathology.

Nederlandse Samenvatting

T cel activatie vindt plaats na herkenning van een verwant antigeen, welke gebonden is aan de major histocompatibility complex (MHC) molecuul, via hun T cel receptor (TCR). Hierna is de klonale expansie, differentiatie en polarisatie van T cellen afhankelijk van costimulatoire moleculen en de cytokine milieu. In **hoofdstuk 1**, wordt T cel activatie in meer detail beschreven met als focus de effecten van costimulatoire moleculen. Hierbij wordt er specifiek gekeken naar de effecten van de leden van de TNFR superfamilie op adaptieve immuun responsen. Dit proefschrift focust op de functie van twee leden van de TNFR superfamilie, namelijk CD27 en GITR, in T cel homeostase en immuun activatie.

De glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) komt tot expressie op geactiveerde T cellen en regulatoire $CD4^+$ T cellen. Om meer inzicht te verkrijgen in de functie van GITR op deze functioneel tegenovergestelde cel types, hebben wij een muis model ontwikkeld waarbij GITRL constitutief op B cellen tot expressie komt, en hebben wij de effecten beoordeeld van chronische GITR signalering op T cel activatie, differentiatie en proliferatie *in vivo* (**hoofdstuk 2**). Middels deze muizen hebben we gevonden dat overexpressie van GITRL een accumulatie van zowel effector als regulatoire T cellen induceert, maar geen effect heeft op $CD8^+$ T cellen noch op naïeve $CD4^+$ T cellen. De betreffende toename was de consequentie van toegenomen proliferatie van effector en regulatoire $CD4^+$ T cellen en niet door verhoogd differentiatie van naïeve T cellen. Tevens zorgde GITR ligatie voor een verhoogde activatie status van regulatoire T cellen, maar beïnvloede hun suppressieve capaciteit niet. Bovendien resulteerde de overexpressie van GITRL in een functioneel beschermend effect in een auto-immuun model, namelijk experimental autoimmune encephalomyelitis (EAE), omdat er een significante vertraging in de pathogenese van het model optrad. Hierbij concluderen we dat costimulatie middels GITR

een functioneel balans teweeg brengt tussen $CD4^+$ effector en regulatoire T cellen middels het versterken van de proliferatie van beide cel types.

Na herkenning van een antigen door de B cel receptor zijn er additionele signalen nodig van helper T cellen om verder te differentiëren tot plasma of memory B cel. Wij postuleerden dat de humoraal immuun systeem beïnvloed zou kunnen worden door de toename van regulatoire en effector $CD4^+$ T cellen (**hoofdstuk 3**). Alhoewel de B cel ontwikkeling en terminale differentiatie van B cellen in de milt niet beïnvloed was in GITRL TG muizen, hadden deze muizen verhoogd IgA en verlaagd IgG3 titers, welke suggestief is voor gemoduleerd B cel responsen. Na *in vitro* stimulatie van B cellen afkomstig van de milt, was een normale immunoglobuline productie te zien van GITRL TG B cellen, welke indicatief is dat er geen intrinsieke B defect is in deze muizen. Een interessante bevinding is dat de absolute aantallen van mucosale B cel subsets werd beïnvloed door transgene overexpressie van GITRL. GITRL TG muizen hadden een verlaging van de absolute aantallen van peritoneale B1 B cellen, terwijl de absolute aantallen van de B2 B cel subset was verhoogd; welke correleert met een verhoogd IgA immunoglobuline titer. De verhoogde IgA titers in GITRL TG muizen waren niet beschermend tegen een influenza infectie vergeleken met WT muizen. In conclusie, de GITR-GITRL axis van costimulatie moduleert de absolute aantallen van mucosaal gerelateerde B cel subsets, verlaagd IgG3 responsen en stimuleerd IgA responsen *in vivo*.

Vergelijkbaar met de effecten die geobserveerd werden in GITRL TG muizen, resulteert transgene overexpressie van CD70, de ligand voor CD27, in een expansie van IFNγ-producerende $CD4^+$ T cellen *in vivo*. In contrast met GITRL TG muizen, depleteren CD70 TG muizen uiteindelijk hun naïeve $CD4^+$ T cel compartiment, en ervaren een vroege mortaliteit door opportunistische pulmonaire infecties. Omdat macrofagen gewichtige spelers zijn in de verdediging tegen pulmonaire pathogenen en IFNγ een belangrijk factor in macrofaag activatie is, hebben wij de effecten bepaald van chronische immuun activatie via CD70-

gedreven costimulatie op de myeloïde compartiment (**hoofdstuk 4**). CD70 TG muizen hebben een IFNγ afhankelijke toename in absolute aantallen van geactiveerde monocyten, welke MHC klasse II hoog tot expressie hebben, vergeleken met WT muizen. Deze geactiveerde monocyten hadden normale fagocytose en migratie karakteristieken *in vitro*, maar hadden een verhoogd vatbaarheid voor IFNγ-afhankelijke apoptose. Hierdoor konden monocyten van CD70 TG muizen niet accumuleren bij inflammatoire locaties *in vivo* en waren deze muizen duidelijk beschermd tegen atherosclerose. Deze resultaten verduidelijken dat CD70-gedreven immuun activatie de myeloïde compartiment moduleert, welke aan de ene kant de risico op opportunistische infecties verhoogd en aan de andere kant beschermend werkt tegen atherosclerose.

Normaliter is costimulatie van T cellen via CD27 signalering afhankelijk van de transiënte expressie van CD70 op cellen van de immuun systeem (T, B en DC's) na activatie. Echter, gedurende chronische immuun activatie wordt CD70 constitutief en hoog tot expressie gebracht op geactiveerde T cellen. Chronische immuun activatie resulteert tevens in verhoogd formatie en uitputting van effector $CD8^+$ T cellen, en resulteert in het falen om een memory $CD8^+$ T cel compartiment te ontwikkelen. Om de functionele consequentie van verhoogd CD27 ligatie op T cel homeostase te bepalen gedurende chronische immuun activatie, hebben wij transgene muizen ontwikkeld welke CD70 constitutief tot expressie brengt op T cellen (CD2-CD70TG). De CD2-CD70TG muizen hadden verhoogd aantallen van $CD8^+$ effector T cellen, welke fenotypisch vergelijkbaar waren met uitgeputte cellen die gevonden worden tijdens chronische inflammatie (**hoofdstuk 5**). Een interessant punt is dat de formatie van $CD4^+$ effector T cellen niet was beïnvloed in CD2-CD70TG muizen. CD70 gedreven costimulatie verhoogd de primaire $CD8^+$ T cel response tegen influenza A infectie, maar liet een verzwakt handhaving van memory $CD8^+$ T cellen. Hierdoor concluderen wij dat CD70

gedreven costimulatie de $CD8^+$ T cel homeostase dereguleert, vergelijkbaar met gebeurtenissen die gevonden worden bij chronische inflammatie.

Normaal gesproken is de formatie van $CD4^+$helper T (T_H) cellen afhankelijk van costimulatoire moleculen en polariserende cytokines. **In hoofdstuk 6** hebben wij onderzocht of CD27 signalering de formatie van helper T cel formatie beïnvloed door het geven van instructieve, ondersteunende of inhibiterende signalen voor T_H differentiatie. CD70 gedreven costimulatie versterkt de formatie van IFNγ-producerende T_H1 gepolariseerde cellen in T_H1 vatbare C57Bl/6J muizen, maar beïnvloede niet de helper T cel formatie in T_H2 vatbare Balb/c muizen. Tevens beïnvloede CD27 signalering niet de formatie of activatie status van regulatoire T cellen *in vivo*. Door het stimuleren van $CD4^+$ T cellen *in vitro* in aanwezigheid of afwezigheid van CD70 gemedieerde costimulatie hebben wij gevonden dat CD27 signalering niet alleen de formatie van IFNγ producerende $CD4^+$ T cellen ondersteund maar ook van IL13 producerende $CD4^+$ T cellen, afhankelijk van de cytokine milieu en de genetische achtergrond, terwijl het T_H17 polarisatie blokkeert. In CD70 TG Balb/c muizen resulteert de inductie van allergische luchtweg inflammatie, een typische T_H2 polariserende pathologie, in versterkt formatie van IFNγ producerende $CD4^+$ T cellen, zonder T_H2 responsen te remmen. Tevens en in lijn met een ondersteunende (in plaats van instructieve) rol, moduleert CD27 signalering niet de expressie niveaus van transcriptie factoren. In conclusie zijn deze data indicatief dat CD27 signalering T_H1 differentiatie ondersteund, T_H2 formatie toestaat en T_H17 formatie blokkeert.

Hoofdstuk 7 laat een overzicht zien van de actuele kennis van costimulatie via leden van de TNFR superfamilie in het algemeen en CD27 in het bijzonder, en hoe adaptieve immuun responsen worden gemoduleerd afhankelijk van de timing, context en intensiteit van deze costimulatoire signalen. In lijn met de observaties gepresenteerd in dit proefschrift stellen wij

voor dat excessieve costimulatie de immunoregulatoire balans van de immuun system kan beïnvloeden en kan leiden tot immuun pathologie.

Publications

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