Modulation of B cell antibody production by antigen-specific IL-10 producing regulatory T cells

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ZUSAMMENFASSUNG

Die Fähigkeit von B Zellen Antigene zu präsentieren und Antikörper zu produzieren ist die notwendige Voraussetzung um die Adaptive Immunantwort auszulösen. Andere Schlüssel-Mediatoren des adaptiven Systems sind regulatorische T Zellen (Treg), die sowohl die T- als auch die B-Zell-Reaktionen modulieren. Derzeit gibt es drei verschiedene Treg Populationen. Zum einen gibt es natürliche regulatorische T Zellen (nTreg), die durch ihre Expression des IL-2 Rezeptors (CD25) und des Transkriptionsfaktors Foxp3 charakterisiert sind. Zum anderen gibt es Tr-1 und Th3 Zellen, die durch die Sekretion von IL-10 und TGF-β charakterisiert sind. Es ist festgestellt worden, daß IL-10 produzierende T Zellen das adaptive Immunsystem regulieren, indem sie B Zellen zur Sekretion von IgG4 anregen. Diese Induktion von IgG4 ist Zell Kontakt abhängig. Der Vorteil einer solchen nicht-entzündlichen B Zell-Antwort zeigt sich am hypo-responsiven Zustand von Patienten mit Allergien oder Helminth Infektionen wie Onchozerkose. Das Ziel der vorliegenden Dissertation war die Analyse der molekularen Mechanismen, die IL-10 produzierende T Zellen dazu befähigen die Produktion von IgG4 durch B Zellen zu induzieren. Für diese Untersuchungen wurden regulatorische T Zell Klone (Tr-TCC) aus Human PBMC generiert.

Der erste Ergebnis-Abschnitt dieser Arbeit gibt einen Überblick über die verschiedenen Aspekte von Antigen-spezifischer Tr-TCC-Generierung. Die Antigen-Spezifität wurde durch wiederholte Stimulation mit dem Tetanus-Toxoid (oder *Onchocerca Volvulus* Extrakt) allein, oder in Kombination mit Dexamethason und Vitamin D3 (DD3) induziert. Die Ergebnisse zeigen, daß die so generierten T Zellen die Fähigkeit haben die Proliferation von Tetanus-spezifischen nicht regulatorischen T Zellen zu unterdrücken. Diese Unterdrückung war Zell-Kontakt unabhängig und wird durch Zytokine wie IL-10 vermittelt. Die so erzeugten Tr-TCC wurden dann auf die Expression von regulatorischen T Zell Markern, wie CD25 und Foxp3, hin untersucht und mit nTregs verglichen. Interessanterweise wiesen die Phänotypen der Tr-TCC und nTreg Zellen einige Ähnlichkeiten zueinander auf.

Mit B:T-Zell-Co-Kultur-Assays konnte dargestellt werden, daß die generierten Tr-TCCs, abhängig vom Zell-Kontakt, B Zellen zur Sekretion von IgG4 induzieren. Weitere Experimente haben verdeutlicht, daß sowohl memory und als auch naive B Zellen erforderlich sind, um eine signifikante Menge an IgG4 zu produzieren. Es konnte gezeigt werden, daß Moleküle wie GITR, GITR-L, TGF-β, IL-10 und Foxp3 eine wichtige Rolle bei der Induktion von diesem

Immunglobulin spielen. Weiterhin wurde nachgewiesen, daß die Blockierung von GITR Molekülen selektiv die IgG4 Produktion verhindert, wie es auch bei neutralisierenden Antikörpern gegen GITR-L, IL-10 und TGF-β der Fall ist. Interessanterweise konnte die vom anti-GITR Antikörper induzierte IgG4 Blockade durch einen Überschuß an rekombinanten IL-10, nicht aber durch rTGF-β rückgängig gemacht werden.

Der Bedarf an Foxp3 bei diesem Prozeß war sehr überraschend, da Tr-1 Zellen Foxp3 nicht konstitutiv exprimieren und über eine aktive funktionelle Rolle dieses Transkriptionsfaktors bislang nichts bekannt ist. Um dies zu untersuchen wurde die Menge an Foxp3 während des Tr-TCC-Generierungs Prozesses gemessen. Hierbei wurde festgestellt, daß die Menge an konstitutiven Foxp3 nach jeder Stimulationsrunde zunimmt und mit der Fähigkeit von T Zellen korreliert IgG4 zu induzieren. Der funktionelle Bedarf dieses Transkriptionsfaktors konnte außerdem durch Ausschalten mit spezifische siRNAs nachgewiesen werden. Diese Tr-TCC Zellen induzierten IgG2 anstelle des IgG4.

Weiterhin konnte mit Hilfe von isolierten nTreg von unbehandelten gesunden Spendern IgG4 induziert werde, jedoch in geringerer Mengen als durch Tr-TCC. In weiterer Übereinstimmung war die IgG4 Induktion durch nTreg ($CD4^+CD25^+$) ebenfalls GITR und IL-10 abhängig. Isolierte $CD4^+CD25^-$ Effektor T Zellen induzierten die Produktion von IgG2, ein Ergebnis das gut mit dem „silencing“ von Foxp3 in Tr-TCC korreliert. In den letzten Experimenten konnte gezeigt werden, daß die B Zell-Aktivierung durch Toll-like-Rezeptoren die Fähigkeit von Tr-TCC beeinträchtigt IgG4 zu induzieren. Diese Ergebnisse bieten eine Hypothese, warum die Onchozerca Infektionen unterschiedlich verlaufen können: Hypo-Responsiv (hohes IgG4 und niedrige Pathologie) gegenüber dem Hyper-Responsiv (niedriges IgG4, hohes IgE und schwere Erkrankung).

SUMMARY

The ability of B cells to present antigen and produce antibodies has established their absolute requirement in mediating adaptive immune responses. Other key mediators of the adaptive system are regulatory T-cells (Treg) which modulate both T and B cell responses. There are currently three different Treg populations: naturally-occurring regulatory populations (nTreg) designated by the expression of the IL-2 receptor (CD25) and the transcription factor Foxp3. Alternatively there are Tr-1 and Th3 cells which are characterised by the secretion of IL-10 and TGF-β respectively. IL-10 producing T cells have been shown to regulate the adaptive immune system by inducing B cells to secrete IgG4 in a cell-contact dependent manner. The benefit of such non-inflammatory B cell responses is apparent in the hypo-responsive state of patients with allergy or helminth infections such as Onchocerciasis. The present thesis was aimed at analyzing the molecular mechanisms underlying the ability of IL-10 producing T cells to induce the production of the IgG4 by B cells. For these investigations, regulatory T cell clones (Tr-TCC) were generated from human PBMC.

The initial results section provides an overview regarding the different aspects of antigen-specific Tr-TCC generation. Antigen-specificity was induced via repeated rounds of stimulation with tetanus toxoid (or *Onchocerca Volvulus* extract) alone or on combination with dexamethasone and vitamin D3 (DD3). The results show the ability of Tr-TCC to suppress tetanus-specific reactive T cells in a cell-contact independent but IL-10-dependent manner. Generated Tr-TCC were then characterised for their expression of regulatory T cell markers such as CD25 and Foxp3 and compared with isolated nTreg: interestingly the cells had overlapping phenotypes. Using B:T cell co-culture assays, generated Tr-TCC were then shown to preferentially induce B cells to secrete IgG4 and required cell-contact. Experiments also demonstrated that both memory and naïve B cells were required to produce significant levels of IgG4. Mechanistically, molecules like GITR, GITR-L, TGF-β, IL-10 and Foxp3 were shown to play an important role in the induction of this immunoglobulin. It could be shown that blocking GITR molecules selectively prevented IgG4 production as did neutralizing Abs to GITR-L, IL-10 and TGF-β. Furthermore, the prevention of IgG4 induction by anti-GITR Abs was reversed by excess rIL-10 but not rTGF-β indicating a complex relationship. The requirement of Foxp3 in this process was surprising since Tr-1 cells do not constitutively express Foxp3 and an active functional role was not previously reported. To investigate this point further, the levels of Foxp3 were measured during the generation process; here it could be shown that levels of constitutive Foxp3 increased after

each round of stimulation and correlated with the ability of T cell lines to induce IgG4 in B cells. The functional requirement of this transcription factor was further shown after silencing Foxp3 in Tr-TCC using specific siRNA since Tr-TCC induced IgG2 instead of IgG4. IgG4 production was also shown using isolated nTreg from healthy untreated donors albeit weaker than Tr-TCC. In further correlation, IgG4 induction by nTreg was also GITR and IL-10 dependant. More interestingly, under the same conditions, isolated $CD4^{+}CD25^{-}$ effector T cells induced the production of IgG2, a result correlating with Foxp3 silenced Tr-TCC. In the final experiments, B cell activation using Toll-like receptor stimuli affected the ability of Tr-TCC to induce IgG4 in B cells. These preliminary findings provide a hypothesis for the mechanism into how the different outcomes of Onchocerca infection occur: hypo-responsive (high IgG4 and low pathology) versus hyper-responsiveness (low IgG4, high IgE and debilitating pathology).

THIS THESIS IS BASED ON THE FOLLOWING ORIGINAL PUBLICATIONS

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Poster presentation, "Antigen specific regulatory T cells and TLRs differentially control immunoglobulin G secretions by B cells" at the World Immune Regulation Meeting II special focus on "Regulatory & Th17 T cells. March 2008, Davos Switzerland.

LIST OF ABBREVIATIONS

Ab	antibody
ADCC	antibody dependent cellular cytotoxicity
Ag	antigen
APC	allophycocyanine
APCs	antigen presenting cells
αCD3	anti CD3
αCD28	anti CD28
BCP	boro-chloro-phenol
BCR	b cell receptor
bp	base pair
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFSE	carboxy fluoroscein succinimidyl ester
CTLA	cytotoxic T lymphocyte antigen
DC	dendritic cell
DD3	vitamin D3 and dexamethasone
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleic triphosphate
EDTA	ethylenediamine-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
Fab	fragment antigen binding
FACS	fluorescence activated cell sorting
$FADH_2$	flavin adenine dinucleotide reduced form
Fc	fragment crystallizable
FCS	foetal calf serum
FITC	fluoresceine isothiocyanate
$FMNH_2$	flavin mononucleotide reduced form
Foxp3	fork head box protein3
GATA3	GATA binding protein 3
GITR	glucocorticoid-induced tumor necrosis factor receptor
ICOS	inducible co-stimulator
IL	interleukin
IFN	interferon
Ig	immunoglobulin
LPS	lipopolysaccharide
µg	microgram
MACS	magnetic activated cell sorting
mg	milligram
MgCl2	magnesium chloride
mM	millimolar
mAb	monoclonal antibody
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid

NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
ng	nanogram
NK	natural killer cell
NKT	natural killer T cell
nTreg	natural occurring regulatory T cells
OD	optical density
O.v	*Onchocerca volvulus*
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PFA	paraformaldehyde
pg	picogram
PHA	phytohemagglutinin
PMA	phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonylfluorid
rpm	rotation per minute
RAG	recombination activating gene
RORγt	retinoid-related orphan receptor gamma-t
RT	room temperature
SB	specific buffy-coat
si RNA	small interfering RNA
SOC medium	super optimal culture medium
TBE	Tris-borate-EDTA
T-bet	T-box transcription factor
Tc	cytotoxic T cell
TCC	T cell clone
TCR	T cell receptor
Th	T helper
TGF	transforming growth factor
TLR	toll like receptor
TNF	tumor necrosis factor
Treg	regulatory T cells
Tr-TCC	T- regulatory T cell clone
Tris	2-amino-2-hydroxymethyl-propan-1,3-diol
TT	tetanus toxoid

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1 INTRODUCTION

1.1 AN OVERVIEW OF THE IMMUNE SYSTEM

The immune system is an association of organs, tissues and cells that coordinate their actions to prevent, clear or control infections elicited by pathogens such as bacteria, viruses, parasites, or other potential threats[1]. However, our environment contains many other harmless organisms which can actually activate the immune system in a positive protective manner. In addition, the immune system must be able to differentiate between "self" and "non-self" to avoid autoreactivity. Therefore, a balance between tolerance to harmless and "self" antigens and immunity against pathogens must be maintained [2-4]. There are two main components, of the immune system. The first, the innate system is composed of mucous membranes, phagocytes and killer cells which provide non-specific first line defence mechanisms against pathogens[5]. Cells of innate immunity are activated through receptors such as toll like receptors (TLR) that recognize genetically encoded pathogen-associated molecular patterns (PAMPs). These PAMPs are specific conserved structures, such as lipopolysaccharide from Gram-positive bacteria, which are released by infected cells, or components of pathogens that do not normally exist in the host. Signalling through TLR for example induces so-called "danger signals", which ultimately leads to rapid clearance of the infection[6]. Pathogens that overcome this initial immune response are confronted by a second more specific line of defence termed the adaptive immune system[7]. In contrast to the innate immune system, the adaptive immune response is highly antigen-specific, and requires more time to provide a protective response. The adaptive immune system is composed of different cell types including: B cells, $CD8^+$ T cells, $CD4^+$ T helper (Th) cells, naturally-occurring and adaptive regulatory T cells (Tregs), NKT cells[6, 7]... One of the main weapons of the adaptive immune system is its ability to produce antibodies that can specifically target epitopes located on pathogens. B cells are the immune components that produce antibodies

and this antibody production is highly modulated by the ongoing cytokine milieu and other factors produced by activated T cells[8, 9].

1.2 B CELLS

B cells are lymphocytes that develop from lymphoid progenitor cells in the bone marrow[10]. Lymphoid progenitor cells receive signals from bone marrow stromal cells to begin B cell development[11]. During the early stages of their development B cells express one important receptor molecule called the pre-B-cell receptor (pre-BCR) which regulates B cell development. The pre-BCR is a heterodimer composed of an immunoglobulin (Ig) heavy chain molecule (H chain) covalently associated with an immunoglobulin light chain-like molecule usually called the surrogate light chain[12-14]. When B cells develop from their precursors they initiate a complex series of differentiation and selection program which leads to rearrangements of the H chain gene segments[15]. A successful rearrangement of the heavy chain is a prerequisite for the rearrangement of the light chain. After rearrangement of both light and heavy chains, if the two chains form a viable immunoglobulin, then this complex directs the cell to stop rearranging to ensure that only a single specificity is produced. Correspondingly, the developing B cells which fail to make a productive rearrangement undergo apoptosis [16, 17]. There are also mechanisms for ensuring the destruction of any new B cell whose antibody reacts too strongly with self-proteins on the surface of host cells[17]. Immature B cells emerge from the bone marrow to the periphery and migrate into the spleen for their final maturation step[18]. After this final maturation step, B cells become responsive to antigens and are able to produce antibodies.

1.3 B CELL ACTIVATION AND ANTIBODY PRODUCTION

B cells can be activated in either T cell dependant or independent manners. In order to initiate antibody activation in the former, protein antigens must be simultaneously presented and recognized by the B cell and T cell receptors, whereas in the latter, non-protein antigens can activate B cells directly without the additional T cell receptor recognition.

1.3.1 T-DEPENDANT B CELL ACTIVATION

B cells become activated by T cells by acting as antigen presenting cells. Helper T cells recognize peptide fragments associated with MHC class II molecules on the surface of B cells. T cells then stimulate B cells by releasing lymphokines which act as growth and differentiation factors for the B cells, and also by providing cell contact dependant additional signals such as CD40-CD40L and CD28-B7 ligation[19]. The initial interaction occurs in the T cell area of secondary lymphoid tissues, where antigen-specific helper T cells and B cells are trapped as a consequence of antigen-specific binding. Further interactions between T cells and B cells occur after migration into the B-cell zone or follicle of secondary lymph organs and form a germinal center [6]. T cells help promote B cell clonal expansion and can direct the differentiation of the clonally expanded naive B cells into either, antibody-secreting plasma cells or memory B cells[6, 20, 21]. During B cell differentiation and according to the cytokine milieu generated by the helper T cell-B cell interactions, the antibody isotype can be re-directed. Furthermore, the properties of antibodies produced by B cells can be modified by somatic hypermutation of the V-region gene (variable region gene). Somatic hypermutation and selection for high-affinity binding antibodies occurs in the germinal centers. Helper T cells control these processes by selectively activating cells that have retained their specificity for the antigen and by inducing proliferation and differentiation into plasma cells and memory B cells[6, 21].

1.3.2 T-INDEPENDENT B CELL ACTIVATION

Non-protein antigens such as polysaccharides can stimulate B cells in the absence of helper T cells. This T independent B cell activation involves the antigen-mediated cross-linking of surface Ig (sIg) receptors on the B cell themselves[22]. T independent activation does not lead to the production of memory B cells and induces only limited isotype switching. However, responses to these types of antigens, for example lipopolysaccahride (LPS) play a critical role in host defence against pathogens whose surface antigens cannot elicit peptide-specific T-cell responses[6].

1.4 ANTIBODIES

The main function of B cells is to present antigens to circulating T cells and produce antibodies which in turn bind antigens. Once produced, antibodies are exported by exocytose and reside

within the plasma membrane whereas others are secreted[23]. Antibodies are found in the plasma and also bound to specific receptors on the invariant (Fc) region of immunoglobulins. They are also found in secretory fluids such as mucus, milk and sweat[23-25]. All antibodies have a similar overall structure with two light and two heavy chains which are linked by both covalent (disulphide bridges) and non-covalent forces. They are made up of a series of domains of related amino acid sequence which possess common secondary and tertiary structures. These conserved structures are frequently found in proteins involved in cell-cell interactions and are especially important when studying immunology[26, 27].

1.4.1 ANTIBODY CLASSES

There are five main antibody classes in mice[28, 29] and humans[30, 31]. The different types of known antibody are: IgM, IgG, IgA, IgD, and IgE[30]. Certain classes are further divided into subclasses. For example, in humans IgG has four subtypes; G1, G2, G3, G4 whereas IgA has two: A1 and A2. In mice there are four IgG subclasses G1, G2a, G2b and G3. In contrast to humans there is no mouse IgG4. Members of each subclass have the same isotype and depend on the structure of the adopted heavy chain. The heavy chain is described by Greek letters (mu, gamma, alpha, delta, and epsilon). Antibodies of each isotype have different properties in terms of complement fixation and their ability to bind to immunoglobulin (Ig) receptors. There are two light chain isotypes kappa and lambda; and each B cell expresses only one light chain isotype. Table 1 depicts the different types of antibody, their serum levels, their ability elicit opsonization and to activate complement, NK and mast cells.

	IgM	IgG1	IgG2	IgG3	IgG4	IgA1/2	IgE	IgD
Form	Pentamer	Monomer	Monomer	Monomer	Monomer	Dimer	Monomer	Monomer
Serum level (mg/ml)	1.5	9.0	3.0	1.0	0.5	2.5	$3x10^{-5}$	0.04
Complement activation	Strong	Strong	Low	Strong	No	No	No	No
NK cell sensitization	No	Yes	No	Yes	No	No	No	No
Mast cell sensitization	No	Low	No	Low	No	No	Strong	No
Opsonization	Low	Strong	-	Middle	Low	Middle	No	No
Neutralization	Low	Middle	Middle	Middle	Middle	Middle	No	No

Table 1.1: The different types of human immunoglobulin and their functions[6].

1.4.2 IMMUNOGLOBULIN G SUBCLASSES

IgGs represent one of the most important immunoglobulin classes; they are secreted late on in the ensuing immune response once B cells have gone through affinity maturation. Affinity maturation is the consistent improvement or specificity of the antibody to the antigen; this allows a greater affinity of antibodies during persistent antigen exposure. IgGs are also secreted by memory B cells during secondary immune responses. IgGs have two antigen binding sites which have very high affinity for the same antigen. Earlier studies using specific polyclonal rabbit anti-serum against human IgG myeloma proteins revealed the existence of the four human IgG subclasses: IgG1, IgG2, IgG3 and IgG4[32-34]. The relative serum concentrations of human IgG subclasses in clinically healthy subjects are as follows: IgG1 > IgG2 > IgG3 = IgG4[35, 36]. Variations in IgG subclasses are an indication of clinical perturbations. A reduction in just one IgG subclass indicates that there is a defect in humoral immunity, whereas increases show a potential ongoing infection.

The four subclasses show more than 95% homology in the amino acid sequence of the constant domains of the γ-heavy chains. The four IgG subclasses show the most differences in their amino acid composition and structure of the 'hinge region', which is the part of the molecule that contains disulfide bonds between the heavy chains. This region, between the Fab (Fragment antigen binding) arms and the two carboxy-terminal domains C_H2 and C_H3) of both heavy chains, determines the flexibility of the molecule[37]. However, the most important difference between IgG subclasses is their function: activation of complement (IgG1>IgG2>IgG3) or induction of ADCC (IgG1 and IgG3). IgG1 and 2 are the most potent pro-inflammatory immunoglobulins and have a high affinity to complement. In contrast, IgG3 has a lower affinity for complement and IgG4 is unique since it is unable to fix complement (Table 1).

1.4.3 ANTIBODY FUNCTIONS

Antibodies play diverse roles in controlling an ongoing infection. This section briefly describes some of these functions.

1.4.3.1 Agglutination

Antibodies are able to induce agglutination of pathogen: bacteria and viruses. IgM, because of its particular tetramer structure and its ability to change its tri-dimensional conformation is particularly suitable for this process.

1.4.3.2 Opsonization

Antibodies (especially IgG) and complement elements like C3b are able to "tag" microorganisms for destruction, this process is termed opsonization. IgG antibodies bind to the antigens with their Fab end so that phagocytes with their Fc gamma receptors can bind to the coated particles and internalize them[38].

1.4.3.3 Neutralization

Antibodies can also neutralize targets directly by binding to a vital part of the pathogen or to it released toxins. For example, the human monoclonal antibody called 53-2-3 binds to the tetanus toxin with a high affinity and reduces its toxicity with an efficiency of more than 99% [39].

1.4.3.4 Antibody dependent cellular cyto-toxicity (ADCC)

Neutrophils, eosinophils, phagocytes and NK cells all mediate ADCC and activation of the lytic machinery only occurs after ligation of the low affinity Fc gamma RIII molecule, CD16[40, 41]. ADCC is a vital mechanism against viral and bacterial infections.

1.4.3.5 Immediate (type I) hypersensitivity

Immediate (type I) hypersensitivity is mediated through IgE antibodies. Ligation of the Fc epsilonR1 receptor on mast cells and basophiles leads to the production of inflammatory and vasoactive mediators (histamine), lipid derived mediators (leukotrienes, prostaglandins, platelet activating factor) and cytokines. This phenomenon manifests as allergy but is clearly of benefit in clearance of extracellular parasites.

1.5 T-LYMPHOCYTES

T cells are lymphocytes that originate from the bone marrow like B cells but mature in the thymus[8] and play a central role in adaptive immune responses[42]. They serve as crucial effector cells through antigen-specific cyto-toxic activity and production of soluble mediators called

lymphokines[43]. T lymphocytes have several classes and the two most distinguishable groups are the cyto-toxic or killer T cells[44] that express the surface marker CD8 and the helper T cell (Th) recognized by surface marker CD4. Both T cell groups originate from the same progenitor that develops into a class of double positive thymocytes $CD4^+CD8^+$ T cells that can further differentiate into "single" positive $CD4^+$ helper T cells or $CD8^+$ cyto-toxic T cells[45]. Despite their common origin these two groups of T cells play completely different roles in the course of an immune reaction[46].

1.5.1 CYTOTOXIC T CELLS

Cytotoxic T cells or $CD8^+$ T cells are activated by antigens (virus peptides for example) within MHC class I molecules and are thus licensed to kill infected cells. Tc cells play an important role in the destruction of cells that have been infected or altered. They are key players in cell mediated immune responses[47].

1.5.2 HELPER T CELLS

$CD4^+$ helper T cells are activated by antigens presented within MHC class II molecules[47] and upon activation secrete B cell activation factors and cytokines that support B cells activation and in turn antibody production. In addition, T cells also recruit other T cells into the site of infection and begin to proliferate themselves.

Th1 and Th2 are the main helper T cell subsets. The two Th subsets are morphologically identical expressing the TCR and displaying the same T cells markers on their surface[48, 49]. However, upon activation they produce completely different cytokines and play different roles in the course of an immune reaction. In brief, Th1 cells produce high amounts of IL-2 and IFN-γ and are particularly important in cell-mediated immune responses. In contrast, the Th2 subset is very important in activating and supporting B cell responses through cytokines like IL-4, IL-5 and IL-6[48, 50-52].

1.5.2.1 Th cell activation

T cell activation is similar for both Th and Tc cells[53]. After being released from the thymus, Th cells go into the blood stream and lymphatic circulation. When Th cells infiltrate a lymph node, they get the opportunity to interact with antigen presenting cells (APCs) such as B cells,

macrophages, and DCs. T cells interact through their TCR with antigen presented in association with MHC molecules on APCs as described above. The alpha and beta chains of the TCR are associated with a group of five proteins called CD3. The CD3 chains sense the ligation of peptide antigen to the alpha-beta chains and activate a group of ten immunoreceptor tyrosine activation motifs (ITAMs), residing within the CD3 chains, which activate the T cell and begin the immune response[54]. TCR only recognize antigens presented on the surface of APCs in association with MHC molecules[55]. The recognition of MHC restricted antigens by the TCR-CD3 complex does not require any other molecules, however, accessory signals provided by co-stimulatory molecules on APCs such as CD80 and CD86, which bind to CD28, and the inducible co-stimulator (ICOS), which binds to ICOS ligand (ICOS-L) are necessary to obtain full T cell activation[56, 57]. As a result of activation, Th cells produce cytokines like IL-2, up-regulate surface proteins (such as CD25, CTLA-4 and amongst others CD69) and begin to proliferate[58]. According to the cytokine milieu, Th cells can differentiate into different subclasses: Th1, Th2, Th17 or regulatory T cells.

1.5.2.2 Helper T cell differentiation:

There are four major differentiation pathways for $CD4^+$ T cells, specifically: Th1, Th2, Th17, and Treg. The decision to undergo one differentiation pathway is tightly associated with the type of antigen being presented, the co stimulatory factors and the cytokine milieu generated by the APCs and the innate immune system. Th cells activated in the presence of IL-12 or interferon gamma (IFN-γ) for example differentiate into Th1 cells and express the transcription factor T-bet[59-61]. T-bet induces expression of IFN-γ, and up-regulates the IL-12 receptor β (IL-12Rβ). Generally, Th1 cells secrete IFN-γ, promote cyto-toxic T cell responses, and induce protective immune responses against intracellular pathogens. Interestingly, the Th1 cytokines IL-12 and IFN-γ inhibit other Th cell subsets by essentially blocking the expression of the critical Th2 transcription factor, GATA binding protein 3 (GATA3)[62] and by promoting IFN-γ production, which is known to inhibit the differentiation of Th17 cells[63-65]. Th17 cells are a relatively new Th subclass and have been shown to promote inflammation. Recent data have also suggested a role for these cells in eliciting autoimmune pathology. In contrast to Th2 cells which produce IL-4, IL-5 and Th1 cells characterized by their secretion of IL-12 and IFN-γ, Th17 cells secrete IL-17 and TNF-α. They are further characterized by the expression of the retinoic acid-related orphan receptor γt (RORγt)[66].

As mentioned above, upon T cell activation through TCR ligation, in the presence of co-stimulatory molecules, $CD4^+$ T cells proliferate and differentiate according to the cytokine microenvironment. When $CD4^+$ T cells are activated in the presence of high concentrations of IL-12 they usually differentiate into Th1 cells and produce IL-2, IFN-γ and TNF. In contrast, naïve $CD4^+$ T cells activated in a microenvironment with low IL-12 but simultaneous high levels of IL-4, differentiate into Th2 T cells and produce IL-4, IL-5, IL-10 and IL-13. The recently discovered Th17 lineage develops from naïve T cells after activation in the presence of TGF-β and IL-6[67-70]. Tregs can be induced by stimulating $CD4^+$ T cells with TGF-β during activation[67-70]. Tregs produce IL-10 and TGF-β and can suppress in either cell-contact dependent or independent manners. Transcription factor T-bet is essential for Th1 cells whereas GATA3 is a critical Th2 transcription factor. RORγt has been shown to be an important Th17 marker[66] and Foxp3 has become the main marker for Tregs.

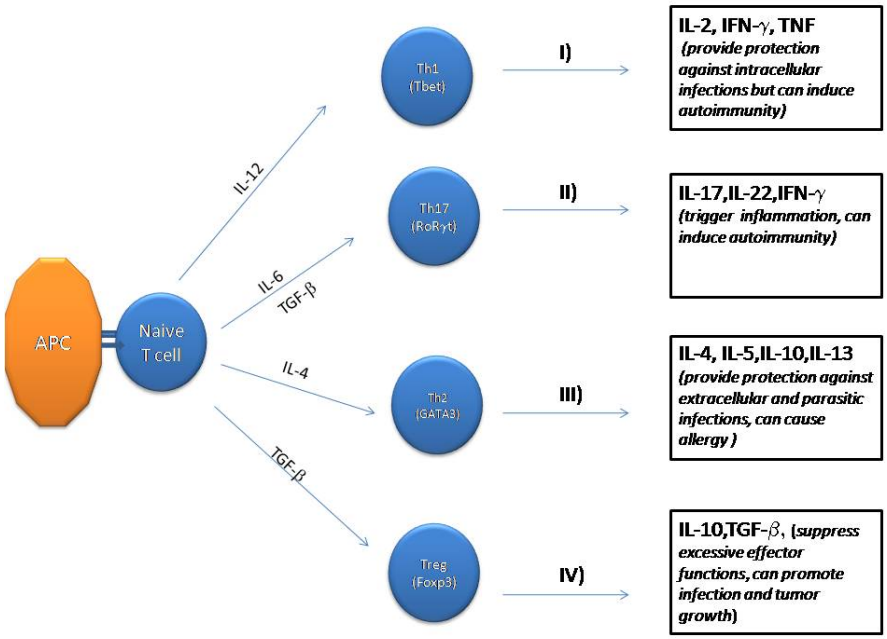


Figure 1.1: Different pathways for Th cell differentiation(adapted from[71]).
There are four different pathways for the differentiation of naïve T cells. I)Under influence of proinflammatory cytokines like IL-12, naïve T cells differentiate into Th1 cells that express the transcription factor T-bet and produce cytokines like IL-2, IFN-γ, and TNF. Th1 responses provide protection against intracellular infections but can also induce autoimmunity. II) The second pathway is governed by the combination of IL-6 and TGF-β and lead to the development of Th17 cells. Th17 cells are characterized by the expression of the retinoic acid orphan receptor RoRγt; they trigger inflammation by producing cytokines like IL-17, IL-22 and IFN-γ and are known to participate in the development of autoimmunity. III) The third pathway conduces to the development of Th2 cells. Th2 cells secrete IL-4, IL-5, IL-10, IL-13, express the transcription factor GATA3 and are known to provide protection against extracellular and parasitic infections. However, Th2 responses through the induction of antibodies like IgE can also cause allergy*)*.IV) The last pathway is mainly governed by the presence of TGF-β (or other regulatory signals). Here naïve T cells are committed into cells with regulatory properties that are characterized by the expression of Foxp3, the production of cytokines like IL-10 and TGF-β and which function is to suppress excessive effector responses. Regulatory T cells can however promote infection and tumor growth.

1.5.3 REGULATORY T CELLS

In contrast to Th1, Th2, and Th17 cells that all induce pro-inflammatory effects and elicit the activation of other T cell subpopulations, the regulatory T cells dampen responses by inducing anti-inflammatory signals. In 1971, the concept of regulatory or "suppressor" T cells was suggested by the work of Gershon and Kondo who showed in adoptive transfer experiments the possibility to transfer antigen-specific tolerance to naive animals[72]. However this concept quickly lost credibility[73]. The reasons for this were the inability to identify specific surface markers associated with these suppressor T cells. In addition, due to advances in molecular technology and thus elucidation of T-cell receptor (TCR) genes, it appeared that suppressor T cells did not have functional gene rearrangements. Finally, suppressor T cells were thought to be governed in their activity by the murine I-J locus, which was supposed to code for soluble suppressor factors. The use of hybrid DNA technology to analyze this locus in the early 1980s, gave a lethal challenge to the suppressor T cell theory as it showed that the putative I-J region just did not exist. Although the whole concept of suppressive T cells was discredited and described as an artefact, many experimental observations remained difficult to interpret without postulating some form of active down-regulation of the immune response[73, 74]. More than 20 years later, in 1995, after the global shame on the suppressor T cell theory, a phenotypic characterization of one class of regulatory T cell was finally described by the group of Shimon Sakaguchi[75]. Since then, the existence of $CD4^+CD25^+$ regulatory T cells (Treg) cells, which arise naturally from the thymus is widely approved and tremendously investigated in both rodent models[76-80] and man[81-85]. These studies have demonstrated that the cells are essential for controlling autoimmune responses[76, 86, 87].

1.5.3.1 Subclasses of regulatory T cells (Treg)

Two main groups of T cells with regulatory activity have been described in mouse and man. Naturally occurring $CD4^+$ Tregs (nTregs) represent the most described subset. nTregs are generated in the thymus and constitutively express CD25 and Foxp3[87] and are mainly restricted to auto-antigen recognition and control responses to "self". However, their depletion also leads to stronger effector responses against foreign antigens[88, 89] highlighting their role as general regulators of the immune system. The molecular mechanisms of immunosuppression by nTreg have been investigated and the most important requirements appear to be direct cell-cell contact with the target cells as well as TGF-β and IL-10[82, 90]. The second group of Treg named

“induced" Treg can be specific for both “self” and “non-self „antigens. Their immunosuppressive activities are often associated with the production of soluble factors like IL-10 or TGF-β[91-95]. Different subgroups of induced Treg have been described using diverse experimental setups. One important group, Tr-1, is characterized by their ability to produce large amounts of IL-10. These cells are interesting for therapeutic purposes since they can be induced *in vitro*. Groux *et al*., described in 1997 the generation of IL-10 producing T cells called Tr-1 cells in both human and rodent models using chronic activation of $CD4^+$ T cells in the presence of interleukin IL-10. The obtained $CD4^+$ T cells displayed high levels of IL-10 but low proliferation, low levels of IL-2 and no IL-4. In addition, these cells were antigen-specific and could suppress the proliferation and immune responses of antigen-specific effector $CD4^+$ T cells in response to antigen both *in vitro* and *in vivo*. Moreover, these cells could actively down regulate ongoing pathology[96].

In 2002, Barrat *et al*., proposed a protocol using the combination of the immunosuppressive drugs vitamin D3 and dexamethasone to induce both human and mouse naive $CD4^+$ T cells to differentiate *in vitro* into IL-10 producing regulatory T cells. In contrast to the previously described *in vitro* derived $CD4^+$ T cells, these cells produced IL-10 but no IL-5 and IFN-γ and furthermore, retained strong proliferative capacity[97, 98]. Different methods have been described to expand $CD25^+Foxp3^+$ regulatory T cells *in vitro* using a combination of anti-CD3/anti-CD28 and IL-2[99]. Recent publications indicate that $CD25^+$ Tregs not only develop in the thymus, but can also differentiate from naive T cells in the periphery[100]. A protocol using TGF-β was recently proposed to generate *in vitro* TGF-β-induced Tregs (Ti-Tregs). The resulting *in vitro*-induced regulatory T cells express markers of conventional Treg such as CD25 and the genetic program committing transcription factor Foxp3[100]. Functionally, these Ti-Tregs suppress T-cell activation and proliferation and are able to control inflammation in different animal models[100].

1.5.3.2 Suppressive mechanisms

Although it is now well established that regulatory T cells suppress immune responses, the mechanisms used for this suppression are not completely elucidated. Two main groups of mechanisms have been described including cell contact dependant and cytokine mediated suppression. Cell contact mechanisms are mediated mostly by surface TGF-β and CTLA-4[101-104], whereas secreted TGF-β and IL-10 mediate cell contact independent mechanisms[105]. Natural Tregs expressing CD25 and Foxp3 are believed to use contact dependent mechanisms in contrast to antigen-induced IL-10 positive regulatory T cells suppression is mediated through soluble factors like IL-10 and TGF-β[105].

1.5.3.3 Regulatory T cells in infection

Whereas Treg are desired for the inhibition of auto-immunity, they have been shown to have a negative role during certain infections. Several pathogens that lead to chronic infection have been found to induce Treg both *in vitro* and *in vivo*. This phenomenon has been well examined using *Bordetella pertussis* infection. Survival of the bacteria has been shown to be enhanced by the induction of specific Treg to the bacteria's filament-associated haemagglutinine (FHA). Similar situations using adaptive Treg which benefit both host and invading parasite or virus have been identified during infections with Candida[106, 107],Hepatitis C virus[108, 109], Leishmania[110], Filaria[111, 112] and malaria[44, 113, 114]. In the latter parasitic infection it was recently shown that the increase in $CD25^+$ Treg in infected patients corresponded with malaria parasite growth and such evidence indicates that the parasites, bacteria or viruses manipulate the suppressive T cell responses for their own advantage[44]. In filarial helminth infections, it has been reported that in situations of immune hypo-responsiveness, functional antigen-specific Treg have been identified which constitutively express CTLA-4 and produce high levels of IL-10 or IL-10 and TGF-β[115, 116]. This phenomenon is described in more detail in the following section.

1.5.3.4 Regulation of the humoral immune responses: IgG4 production in infectious and allergic diseases

As mentioned above the Tregs are known to prevent the development of autoimmune diseases and are also involved in prevention of sensitization to allergens[77, 87, 117, 118]. A possible use of Tregs to cure or prevent allergic diseases is a currently discussed issue[77]. Th2 responses to allergens are known to be suppressed by $CD4^+CD25^+$ Tregs or IL-10 producing Tr-1 cells [119, 120]. In animal models, Tregs could be induced using high doses of antigen and the induction of such Tregs prevented subsequent development of allergen sensitization and airway inflammation in inhaled challenge models[121-124]. For many years, allergen-injection immunotherapy has been used for the treatment of allergic disease, and this therapy is thought to induce protective IL-10 Tr-1 cells which suppress Th2 responses and induce a switch from IgE to IgG4 antibody production[125, 126]. Figure 1.2 illustrates the course of such a deviation of the immune response due to the induction of IL-10 producing Tr-1 cells. Furthermore, it could be demonstrated that peripheral blood mononuclear cells (PBMC) of allergic patients, that were successfully hyposensitized, produced more IL-10 in response to these allergens and in addition higher production of IgG4[124, 127-130].

Thus in allergy therapy, IgG4 antibody production induces protection against a given antigen whereas IgE antibodies are linked to ongoing allergic responses[131].

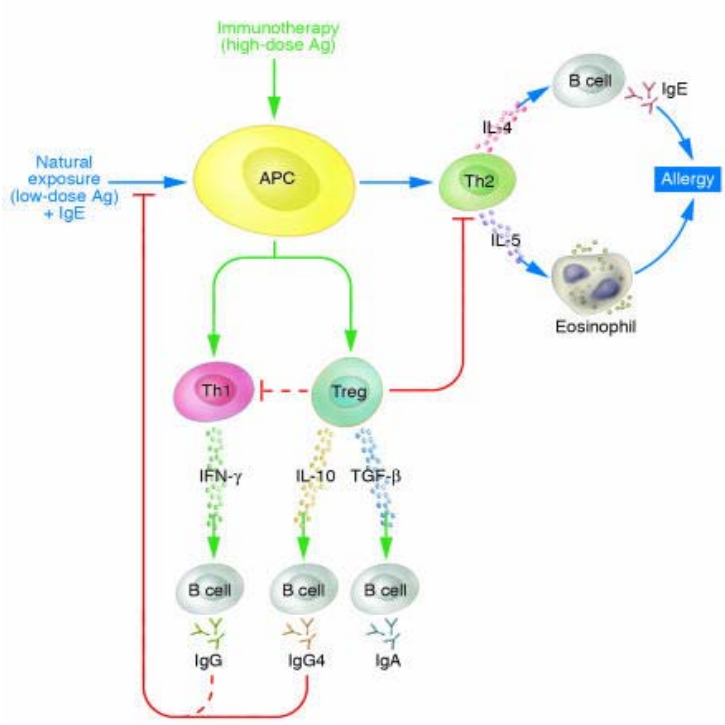


Figure1.2: Potential mechanisms of conventional allergen immunotherapy.

Natural environmental exposure to allergens induces the activation or maintenance of atopic Th2 T cell responses leading to allergy (blue arrows). High-dose allergen exposure during immunotherapy leads to immune deviation of Th2 responses in favor of a Th1 response and the generation of IL-10 and TGF-β–producing Tregs. The cytokines produced by Tregs induce preferential switching of B cell responses in favor of IgG4 antibodies. IgGs and especially IgG4 competes with IgE for the circulating allergen which subsequently down regulates IgE-dependent Th2 responses. Furthermore, Treg may directly inhibit Th2 and Th1 T cell proliferation (red blocked lines). Blue arrows represent immune response pathway to natural exposure (low-doses antigen and IgE); dotted lines represent possible means of action not yet fully understood[130]

Interestingly aspects of nematode filarial infections also correlate with the observations found during allergic responses. In Onchocerciasis for example, there are two possible outcomes in the development of infection: the generalized onchocerciasis, where infected individuals with high parasitemia and microfilaria present few clinical symptoms and the hyper responsive onchocerciasis where individuals demonstrate low parasitemia and few microfilaria but intense dermal pathology (also termed sowda form). In the latter form, dermal and ocular complications, including blindness are caused by the reaction of the host's immune system to the parasite, particularly the microfilaria[132, 133]. Interestingly, persons with the generalized form exhibit significantly higher ratios of IgG4 to IgG1 and IgG4 to IgE[134]. Prominent IgG4 levels in hypo-responsive patients positively correlate with worm load[135, 136].

In addition, a high parasite load correlates with IL-10 which stems from $CD4^+$ T cells and possibly macrophages. These IL-10 producing T cells have been shown to elicit the production of the non complement fixing immunoglobulin IgG4 by B cells. This IgG4 production is one of the suspected mechanisms involved in the development of generalized onchocerciasis. In contrast, worm specific IgE antibody levels correlate positively with microfilaria clearance but also to immunopathology[137, 138] and such, low parasite loads were found in hyper responsive patients with high levels of IL-4 and IL-5[139, 140].

Furthermore, it has been reported that adaptive regulatory T cells induced by filarial antigens can down-regulate both Th1 and Th2 effector cells responses in addition to inducing IgG4 production by B cells. These regulatory mechanisms encouraged the development of the generalized form of onchocerciasis, characterized by low pro-inflammatory reaction[141] by inhibiting proinflammatory signals that may lead to hypersensitivity. Figure 1.3 illustrates this key role of regulatory T cells in avoiding the development of the hyper reactive form of onchocerciasis.

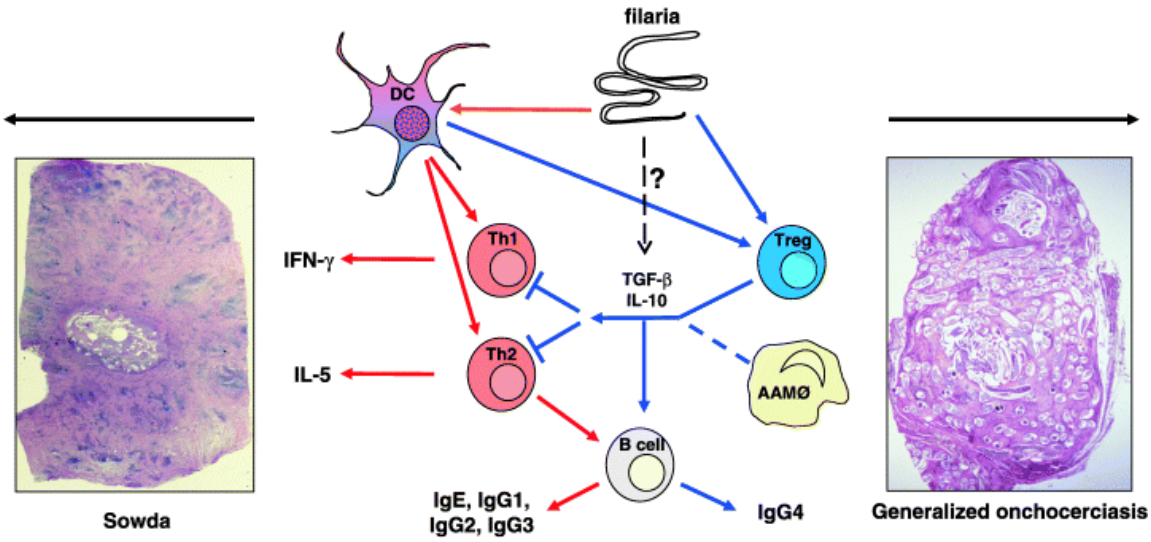


Figure 1.3: Network of interaction in the immunomodulation during filarial infection.

The polar forms of host reactivity in human onchocerciasis: generalized onchocerciasis vs. Sowda (hyper-reactive onchocerciasis). Red lines represent pathways conducing to parasite clearance and hypersensitivity (Sowda form) including Th1 and Th2 effector cytokines and blue lines represent tolerogenic pathways including regulatory T cells immunosuppressive cytokine, IL-10 and TGF-β, as well as the production of IgG4 by B cells, conducing to parasite tolerance (Generalized onchocerciasis)[141].

1.6 AIM OF THIS THESIS

Previous studies have identified that regulatory cytokines like IL-10 and TGF-β were responsible for the antigen-specific cellular hypo-responsiveness in chronic human helminth infections[142]. Furthermore, T cell clones (TCC) with regulatory properties, characterized by IL-10^{high}IL-

4^{low}IFN-γ^{low} and generated using a combination of tetanus toxoid antigen and IL-10 were demonstrated in direct T-B co-culture to be able to induce IgG4 production from B cells. This IgG4 induction was cell-contact dependant and IL-10 was an absolute requirement for this response[143]. More recently in patients with autoimmune pancreatitis, $CD4^{+}CD25^{high}$ Tregs, were associated with high levels of serum IgG4 and to be involved in shifting B cells into IgG4-producing plasma cells[144].

The present work was aimed at analyzing the components required by regulatory T cells to induce IgG4 secretion from B cells. The first task was to generate T cell lines (TCL) using tetanus toxoid or O.v extract and DD3 (vitamin D3 and dexamethasone) which enhances IL-10 producing cells *in vitro*. After multiple rounds of antigen-specific stimulation, IL-10 producing TCL were then cloned and characterized to decipher whether they possessed a Th1 (non Tr-TCC), Th2 or regulatory T cell (Tr-TCC) profile. Upon generation of IL-10^{high}IL-4^{low}IFN-γ^{low} Tr-TCC, the ability of these cells to suppress both antigen-specific and non-specific Th1 cells was tested using a variety of specific assays. Suppressive Tr-TCC were then analyzed for their ability to induce different IgG subclasses *in vitro* using a T:B cell co-culture assay. Tr-TCC with the ability to preferentially drive IgG4 were then used to decipher the mechanisms and components thereof, that were involve in this IgG class switch. Investigations that were performed in order to elucidate these mechanisms included 1) neutralization experiments with antibodies to GITR, GITRL, CTLA-4, ICOS, IL-10 and TGF-β, 2) silencing Foxp3 with specific siRNA using nucleofection techniques and 3) PCR of T cell transcription factors. In addition, the phenotypic characteristics and functional activity of Tr-TCC was compared with nTreg ($CD4^{+}CD25^{+}$) that were directly isolated from healthy PBMC. These experiments were performed to determine whether the mechanism involved in IgG4 induction was restricted to IL-10 producing Tr-TCC or were perhaps a common feature for all Treg. Finally, it was investigated whether simultaneous TLR activation on the B cells or the Tr-TCC themselves could influence the outcome of IgG responses.

2 MATERIALS AND METHODS

To describe the materials and methods used throughout this work, this chapter is divided into five sections. In section 2.1, there is a general summary of the necessary materials. This section is followed by detailed accounts regarding cell culture 2.2, cell immunology assays 2.3, and molecular biology procedures 2.4. Chapter subsections which are of particular interest to this work are the protocols used to generate the regulatory T cell clones (2.3.3), the techniques used to identify the suppressive capacity of these clones (2.3.2) and the co-culture assays performed to detect the ability of those clones to induce B cells to produce IgG4 (2.3.7) The protocols for the various buffers and solutions referred to in this chapter are described in appendices A-E.

2.1 MATERIALS

This section covers the variety of materials that were used throughout the study, beginning with clarification about the different blood samples obtained from the University blood bank and continuing with accounts of the plastic ware and the required antibodies. Finally, there is a description about how the different antigens were prepared.

2.1.1 BLOOD SAMPLES, PBMCS, APCS / FEEDER CELLS

Blood samples used in this study were collected from healthy European donors and were kindly provided by the Institute for Experimental Haematology and Transfusion Medicine, University Clinic Bonn, Germany. The only selection criteria were the willingness of the donors to participate in the study. The study was approved by the University Clinic Bonn ethic committee ("Ethikkommission der Medizinischen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn"). PBMCs were separated from citrate venous blood by gradient centrifugation on Ficoll-

Plaque (density 1.077; PAA, Germany). The isolated PBMC were either immediately used or cryo-preserved (see section 2.2.3). The HLA type of the donors was characterized by PCR (kindly performed by the Department of Clinical Medicine Bonn) to ensure that no allogenic responses could occur in cell cultures in which no autologous APCs or B cells were available. Twenty donors were consistently used throughout this study. Table 2.1 below shows the patients numbers and the corresponding information about their haplotypes.

PATIENT	HLA-A	HLA-B	DRB	PATIENT	HLA-A	HLA-B	DRB
SB1	02 03	07 15	11/15 (3*5*)	SB11	01 68	07 08	03/07 (3*4*)
SB2	01 02	07 08	03/15 (3*5*)	SB12	01 24	08 15	03/14 (-*3*)
SB3	02 26	07 56	03/07 (3*4*)	SB13	01 02	08 44	03/04 (3*4*)
SB4	03 26	35 38	07/15 (4*5*)	SB14	01 24	08 40	03/11 (3*/-*)
SB5	03 29	18 45	03/04 /3*4*)	SB15	02 /--	38 44	11/- (3*/-*)
SB6	02 /--	13 39	04/13 (4*3*)	SB16	03 31	07 18	03/15 (3*5*)
SB7	26 66	14 27	01/04 (4*5*)	SB17	24 26	07 /--	04/15 (4*5*)
SB8	01 24	35 37	13/15 (4*5*)	SB18	02 24	07 39	08/11 (-*3*)
SB9	02 /--	27 40	03/15 (3*5*)	SB19	01 68	07 14	03/15 (3*5*)
SB10	02 /--	27 44	01/14 (-*5*)	SB20	11 24	40 51	11/15 (3*5*)

Table 2.1 Donor patient number and information about their haplotypes.

2.1.2 PLASTIC AND GLASSWARE

All plastic and glassware equipment was supplied by one of the following firms: Eppendorf, Hamburg, Germany, Becton Dickinson, Heidelberg, Germany, or Greiner, Friekenhausen Germany.

2.1.3 ANTIBODIES AND MICROBEADS

Anti-GITR-APC, blocking anti-GITR, anti-IL-10, anti-TGF-β antibodies and their corresponding isotype controls were obtained from R&D systems, (Wiesbaden, Germany). PE-conjugated anti-Foxp3, anti-ICOS and blocking anti-ICOS antibody were from eBioscience (San Diego USA). All other antibodies used for cell culture, flow cytometry or ELISA were purchased from BD

Biosciences (Heidelberg, Germany). Anti CD3/CD28 T cell expansion beads as well as magnetic cell sorting beads were purchased from Dynal/Invitrogen GmbH (Karlsruhe, Germany).

2.1.4 ANTIGENS AND RECOMBINANT PROTEINS

2.1.4.1 Onchocerca volvulus extract

Aqueous soluble *Onchocerca volvulus* extracts were prepared from adult female worms that were isolated from Onchocerca nodules from infected patients. Worms were isolated after collagenase digestion of the nodules and then shock frozen in liquid nitrogen[145]. For antigen preparation, mortar and pestle were first placed on ice and pre-cooled by filling the mortar with liquid nitrogen. The eluting buffer which contained E64 (10 μM, PMSF, 500 μM, Benzamidin 6.4 mM, in PBS pH7.4) was freshly prepared and chilled on ice. The worms were then pulverized in liquid nitrogen and the powder was collected in a chilled falcon tube. Then 1 ml per worm of the eluting buffer was added and the solution was mixed. The lysate was incubated overnight at 4°C under gentle rotation and then centrifuged at 4°C for 1hour 30 minutes at 150,000xg. The supernatant containing the soluble proteins was double filtered through a 4.5 μm filter in order to remove large debris and then through a 0.2 μm sterile filter. The protein concentration was determined by Bradford assay and Coomassie solutions and the antigens were stored at -80°C until use.

2.1.4.2 Tetanus toxoid

Tetanus toxoid was kindly provided by Dr. C. Hungerer (Chiron Behring, Marburg, Germany).

2.1.4.3 Recombinant proteins

Several recombinant proteins were throughout this study; as ELISA standard (rIL-2, rIL-4, rIL-10, rTGF-β and rIFN-γ) or as cell culture supplement or source of exogenous active proteins (rIL-2, rIL-10, rTGF-β rGITR and rGITRL). All recombinant proteins used in this study were purchased from BD Biosciences (Heidelberg, Germany).

2.2 CELL CULTURE PROCEDURES

2.2.1 ISOLATION OF PBMCS

Peripheral blood mononuclear cells (PBMCs) or PBLs for peripheral blood lymphocytes were isolated from heparanized whole blood using a ficoll based density gradient separation method[146, 147]. The entire isolation procedure was carried out on ice. The heparanized blood was diluted 1:2 in RPMI medium supplemented with 2 mM L-glutamine and 50 µg/ml gentamycin and penicillin (RPMI+++). The diluted blood sample was then gently pipetted onto 15ml of Ficoll that was already within in a 50 ml falcon tube. The suspension was then centrifuged for 20 minutes at 4°C, 800g without brake. The white layer containing the leukocytes was then carefully collected into a new tube using a 2 ml-pipette and washed twice with RPMI+++ to remove the remaining ficoll. For washing, 20 ml of RPMI+++ was added to the cell pellet, gently mixed and centrifuged for 8 minutes at 4°C and 400g. The supernatant was discarded and the washing step was repeated again.

2.2.2 CELL VIABILITY AND COUNTING

The number of the living cells was determined using a trypan blue based exclusion method and was expressed in number of cells per ml. The cell suspension was diluted 1:2 or 1:5 with a solution of 0.4% trypan blue. 10µl of the mixed solution was then loaded onto a cell counting chamber or haematocytometer, (Neubauer, Assistent, Germany), and the living cells (non-coloured) were counted. PBMCs were not used when more than 5% of the cells were dead (blue coloured).

2.2.3 FREEZING OF ISOLATED CELLS

Cell suspensions were adjusted to a concentration of 2-3 x 10^7 cells/ml, depending on whether PBLs, feeder cells, lymphocytes lines or T-cell clones were to be frozen. The freezing medium, pre-cooled on ice, was slowly added drop by drop to the same volume of suspended cells, with intermittent mixing, and quickly frozen in a Nunc cryotube (2 ml/tube) at –80°C for at least 24 hours and then in liquid nitrogen.

2.2.4 FREEZING OF CULTURED CELLS

At the end of the stimulation cycle, cultured cells were cryo-conserved by freezing up to 2-5 x 10^6cells/tube. For this purpose, 100-150µl of supernatant per well was removed from the cultures wells and pooled in a 15 ml falcon tube. After centrifugation at 1300 rpm for 8 min at 4°C, the pellet was re-suspended in 1ml RPMI 1640 medium containing 10% FCS. The cell concentration was adjusted to 2-5 x 10^6 cells/ml and the cells were frozen as described above.

2.2.5 THAWING OF FROZEN CELLS

As with freezing, all thawing procedures were fulfilled on ice. The cryotubes were removed from liquid nitrogen or from -80°C, and quickly thawed in 2-3 minutes between the palms of both hands. The content of the cryotube was poured into a 15ml falcon tube and 10ml of pre-cooled culture medium was slowly added over a 5-10 minute period with frequent mixing. After centrifugation at 1300 rpm for 8 minutes at 4°C, to wash away the freezing medium which is toxic for the cells, cells were washed once again with 10ml culture medium, re-suspended in 1 ml, adjusted to the appropriate concentration and used in stimulation assays.

2.3 CELL IMMUNOLOGY PROCEDURES

2.3.1 IRRADIATION OF PBMCS FOR USE AS FEEDER CELLS OR APCS

After isolation of the living cells, PBMCs destined for use as APCs or feeder cells were γ-irradiated for 15 minute at 4000rads using a Gamma cell irradiator (Atomic Energy of Canada). This rendered them unable to proliferate but did not affect their ability to present antigen and thus suitable as APCs and or feeder cells since there could be no interference with target cell proliferation. Irradiated cells were adjusted to 1x10^7cells/ml and frozen until used.

2.3.2 ASSESSMENT OF CELL ACTIVATION AND PROLIFERATION

During the characterisation phase of the generated clones several different techniques were used to assess cell activation and proliferation.

2.3.2.1 Tritium incorporation

Cells undergoing proliferation increase their rate of protein and DNA synthesis. The increase in DNA synthesis can be measured by adding ^{3}[H] thymidine, a radioisotope-labelled DNA precursor, to the cell culture medium. The amount of tritium taken up by the dividing cells is proportional to the level of cell proliferation. In short, cells were cultured in the presence or absence of antigen presenting cells and specific antigens, inhibiting cells or controls for 72 hours. For the last 18 hours, 25 µl/well of 0.2 µCi ^{3}[H]-thymidine was added to the wells. The cells were then lysed by freezing at -20°C. Thereafter, the supernatant was filtrated with a 5% polyethyleneimine-treated Whatman GF/B filter paper under vacuum. The filters were then dried at 37°C. 10ml/filter of Fisher Scintisafe 30% Liquid Scintillation Cocktail was then added and the filters are sealed and the trapped radioactivity was analyzed using a Beckman Coulter LS 6500 liquid scintillation counter. Proliferation was expressed as counts per minute (cpm).

2.3.2.2 CFSE based assays

Another method for the determination of cell proliferation was the use of Carboxy Fluoroscein Succinimidyl Ester (CFSE). CFSE is a dye which in moderate concentrations is not harmful to cells. Upon entering cells, it undergoes esterase cleavage and diffuses throughout the cytoplasm. As cells divide, the CFSE is split equally between the daughter cells resulting in diminished CFSE signal detection[148, 149] which can be observed by flow cytometry. Thus the amount of cell division made by the labelled cells can be determined. In this work we used this property of *in vitro* labelling of CFSE to T cells to quantify their proliferation after antigen or mitogenic stimulation or their ability to inhibit other cell types. In brief, $1x10^{5}$ conventional $CD4^{+}CD25^{-}$ effector T cells or antigen-specific effector T cell lines or clones with a Th1 phenotype were used as reporter cells. These cells were incubated with generated Tr-TCC ($1x10^{5}$) that were previously labelled with 2.5 µM of CFSE. The reporter cells were then stimulated with either anti CD3/CD28 expansion beads (0.125µl beads/200µl) or with APCs loaded with antigen. Following stimulation, the reduction of CFSE was measured on days 3 and 7 using the FACS Canto™ flow cytometry machine and data were analyzed using the FACS Canto Diva™ software.

2.3.2.3 Alamar-Blue based assay

Alamar-Blue is an indicator dye that can quantitatively measure the proliferation of a variety of human or animal cells. The dye is a sensitive oxidation-reduction (REDOX) indicator that

undergoes colorimetric changes (from blue to pink) as well as changes in fluorescent signal in response to metabolic activities (reduction).

In some experiments the reduction of Alamar-Blue due to cellular activity was used to assess the proliferation of T cells. 10% v/v of Alamar-Blue was added to $1x10^5$ activated T cells and controls and the colour change was assessed after 24-72 incubation at 37°C. Alamar-Blue is reduced by $FMNH_2$, $FADH_2$, NADH, NADPH, all components of cellular metabolism that are upregulated during proliferation. Upon reduction the dye originally blue, changes it colour and fluorescence and becomes pink. These changes can be measured using the spectrophotometer at 565 and 595 nm

2.3.2.4 Activation assay: CD69 up-regulation

The CD69 (Leu-23) is a phosphorylated 28 to 32-kDa disulfide-linked homodimer protein that is rapidly induced after lymphocyte activation. CD69 is not present on the surface of resting T cells but appears on their surface after activation[150]. In this work the levels of CD69 before and after stimulation were assessed 24 hours following TCR ligation with anti-CD3 or antigen stimulation by FACS analysis and its up-regulation was used as an indication for T cell activation. $1x10^5$ T cells were incubated with antigen and $1x10^5$ APCs or anti-CD3/CD28 for 24 hours. Cells were then harvested and stained with fluorescently-labelled antibodies to detect CD4 or CD3 T cell populations and their levels of CD69. Controls of non-stimulated T cells as well as T cells stimulated with non-specific antigens were also examined.

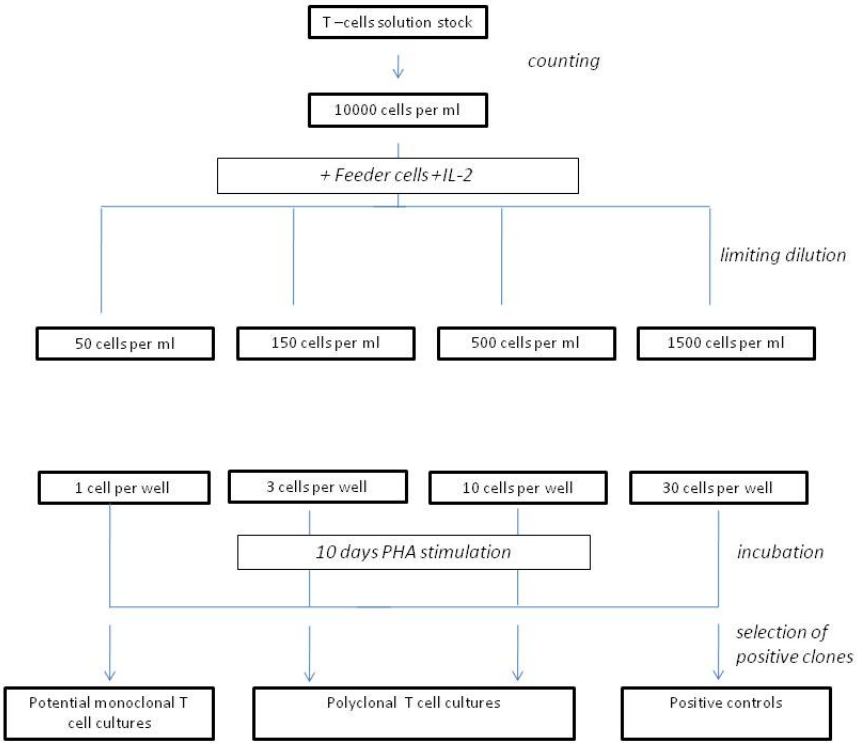
2.3.3 Generation and characterization of regulatory T cell lines (TCL) and T cell clones (TCC)

2.3.3.1 Successive stimulation of PBMC in the presence of antigen and dexamethasone plus vitamin D3 (DD3)

For the generation of TCL, isolated PBMC were stimulated in a 96-well round bottom well plate at a concentration of $1x10^5$ cells with 10 µg/ml tetanus toxoid or 10µg/ml O.v antigen. Some cells were also stimulated with a combination of antigen and vitamin D3 (15ng/ml) and dexamethasone (12ng/ml). The culture medium was composed of RPMI+++supplemented with 10 % FCS. After 10 days, cells underwent another round of stimulation with the same antigens with or without DD3 and in the presence of irradiated autologous APCs ($1x10^5$cells/well). This step was repeated again 10 days thereafter. After the last stimulation TCL were directly used, frozen or pre-stimulated with PHA (1µg/ml) for T cell cloning.

2.3.3.2 T cell cloning

Cells were pre-stimulated with PHA (1µg/ml) for 72 h in the presence of recombinant human IL-2 (100 U/ml) and autologous feeder cells. The pre-stimulated cells were then harvested, counted and diluted to obtain a stock solution of 10,000 cells per ml. This cell suspension was then used to create serial dilutions so that different concentrations of cells were plated per well (20µl) (Scheme 2.1): 30 cells/well, 10 cells/well, 3 cells/well and 1/well in Terasaki plates (Greiner). Cells were plated with irradiated PBMCs ($1x10^4$/well) used as feeder cells with 1µg/ml PHA and 200U/ml IL-2. Plates were then incubated for 10 days at 37°C in 5% CO_2 in boxes containing distilled water to ensure the small volume of medium in the wells did not dry out.



Scheme 2.1: Generation of antigen-specific T cell clones.

TCL were pre-stimulated with PHA (1µg/ml) for 72 h in the presence of recombinant IL-2 (100 U/ml) and autologous feeder cells. Pre-stimulated cells were then harvested and stock solution of $1x10^4$ cells/well was prepared. This stock was then used to prepare different concentrations of cells. Cells were plated to obtain 30, 10, 3 and 1 cell/well on Terasaki plates (Greiner). Cells were stimulated with 1µg/ml PHA and 100U/ml IL-2 in presence of 10^6 Cells/ml irradiated PBMCs used as feeder cells. Plates were then incubated for 10 days at 37°C in 5% CO_2. After 10 days, proliferated were selected. T cell clones were preferentially harvested from plates with 1 cell per well.

At day 9 after cloning, the wells were screened using a Leica inversed microscope and positive clones which had visibly proliferated were selected and picked the following day. T cell clones

were preferentially harvested from plates with less than 10% positive clones. Picked cells were then transferred onto 96-wells plates in a total volume of 200µl with 1µg/ml PHA and 100 U/ml IL-2 in RPMI/10%FCS and cultured at 37°C for 24 hours. The medium was then changed to dilute the PHA concentration and cells were incubated for further 9 days.

2.3.3.3 Expansion of T-cell clones

After the first 10 days of stimulation with PHA, the picked T cell clones were further re-stimulated with anti-CD3/CD28 (0.125µl/well) T cell expansion Dynal beads in the presence of 100U/ml IL-2. Expander beads allowed the clones to grow without contaminating them with feeder cells and antigen so that they could then be used "pure" in further assays.

2.3.3.4 Antigen specificity and cytokine analysis of the T cell clones

To find out whether the generated T cell clones were specific to tetanus toxoid or O.v antigens they were subjected to a proliferation test with the appropriate antigen. Matched feeder cells were used as antigen presenting cells (APCs) as follows: $1x10^5$APCs/well in culture medium supplemented with 10% FCS were incubated either in medium alone or with O.v antigen (25µg/ml) or tetanus toxoid antigen (10µg/ml). The T cell clones were washed in culture medium and then added at a concentration of $5\text{-}10x10^4$/well. After 72 hours, 100µl of supernatant was collected for cytokine analysis using ELISA. The proliferation rate was then determined after a further 18 h incubation in presence of ^{3}[H] thymidine (see section 2.3.2.1).

2.3.4 CYTOKINE ELISA

After T cell culture, supernatants were collected after 24 h for IL-2 and IL-4 and 72 hours for IL-10, IFN-γ and TGF-β measurement. Cytokines were quantified using cytokine-specific sandwich ELISA. ELISA-plates were coated with 50 µl /well of the appropriate anti-cytokine mAb (2 µg/ml; 8D4-8, JES3- 9D7, and NIB42 Becton Dickinson; for IL-4, IL-10, TGF-β and IFN-γ, respectively) diluted in 0.1 M $NaHCO_3$-Na_2HCO_3 buffer. Plates were incubated overnight at 4°C (or alternatively at 37°C for 2 hours). After incubation, plates were washed four times with PBS containing 0.05 % Tween-20 and blocked for 1 h at 37°C with 200 µl of 1 % BSA/ PBS per well. Supernatants or recombinant cytokine standards (all from Becton Dickinson) were then diluted 1:2 and added at a volume of 50µl/well. Plates were incubated overnight at 4°C (or 2 hours at 37°C), then washed, and the corresponding biotinylated secondary antibody (1µg/ml) was added at 100 µl / well. Plates were then incubated for 2 h at room temperature and washed four times

with 1x PBS containing 0.05% Tween-20. Streptavidin-peroxidase complex (1:10,000) was then added to each well at 100µl/well. After 1 h of incubation at room temperature, plates were developed by adding 100 µl/ well of substrate solution containing 0.3 mg/ml of TMB and 10^{-3} ml/ml of H_2O_2 (Roth, Karlsruhe, Germany) in substrate buffer (0.2M Na_2HPO_4). After 15 minutes of incubation at room temperature, the reaction was stopped by adding 25 µl of 4N H_2SO_4 /well and plates were measured at 450 nm using an ELISA plate reader (Molecular Devices, Sunnyvale USA).

2.3.5 FLOW CYTOMETRY ANALYSIS

2.3.5.1 Surface markers

For surface markers (CD4, CD19, GITR, ICOS, CD69), $5x10^4$ cells were re-suspended in 100 µl of PBS and blocked with 2µl of human Fc-block (Sigma Aldrich). 8µl/$5x10^4$ cells of the indicated antibodies or appropriate isotype controls were added, gently mixed by vortexing and incubated at 4°C for 30 min. Cells were washed twice with 1x PBS, fixed in 4% PFA and analysed using the FACSCanto® flow cytometer (BD Biosciences). Lymphocyte populations were gated based on forward and side scatter and at least 30,000 events were acquired for each experiment. Data were then analyzed with FACS Diva® software (Becton Dickinson).

2.3.5.2 Intracellular Foxp3 staining

Intracellular Foxp3 staining was performed according to the manufacturers' protocol (eBioscience). In brief, pre-surface stained cells were permeabilized using the fix/perm buffer provided by the supplier. The cells were then washed and blocked for 15 minutes with 2µL of rat serum in a total volume of 100 µL. Immediately after, Foxp3-PE (PCH 101) was added for a further 30 minutes. After two additional washing steps, the cells were acquired using the FACSCanto® flow cytometer. T lymphocyte populations were gated based on forward and side scatter. At least 30,000 events were acquired for the analysis of $Foxp3^+$ cells. Data was then analyzed with FACS Diva® software.

2.3.5.3 Intracellular cytokines staining

$1x10^5$ cells were stimulated with PMA-ionomycin (50ng/ml PMA and 1µM ionomycin) for either 3h-4h for IFN-γ, 5h for IL-10 and 8-10h for TGF-β. Protein secretion was blocked for the last 3 hours of the incubation using 100ng/ml of monesin (Golgi stop) solution (BD Biosciences)

according to the manufacturer's protocol. Cells were then stained for surface proteins such as CD4, CD3 CD19, and GITR and then permeabilized using the eBioscience permeabilization reagent for 30 minutes. After a washing step, cells were stained using 5µl of the corresponding antibody towards IFN-γ, IL-10, TGF-β, IL-4 or their isotype controls after 30 minutes. Cells were then washed twice and acquired using the FACSCanto® flow cytometer. T lymphocyte populations were gated based on forward and side scatter. At least 30,000 events were acquired for the analysis of cells. Data were then analyzed with FACS Diva® software.

2.3.6 MAGNETIC CELLS SORTING

2.3.6.1 Isolation of CD19+ B cells

Dynabeads® CD19 (111.03), CD19 detachabeads® (125.06), and Dynabeads CD4 (113.03) were all purchased from Dynal/Invitrogen (Karlsruhe, Germany) and were routinely used to isolate $CD19^+$ B cells and $CD4^+$ T cells according to the manufacturer's protocols. Briefly, 200µl of specific dynabeads were added to a 15 ml tube and washed twice with 5 ml of 2 % FCS/PBS using the Dynal MPC® (Dynal magnetic particle concentrator). Thereafter, beads were re-suspended in RPMI 1640 medium containing 10% FCS. Approximately, $1x10^5$ PBMC were added to the $CD19^+$ Dynabeads and incubated for 20 min at 4°C under gentle vortexing. The rosetted cells were then washed four times with 2% FCS/PBS and re-suspended in 500µl of RPMI 1640 medium with 10 % FCS. CD19 detachabeads (100µl) were then added and incubated for 45 min at room temperature under gentle rotation. The suspension containing the detached beads was collected and the detached beads were washed twice. To analyse the purity of the isolated cells, small fractions were double stained before and after isolation with APC-labelled anti-CD20 and PE-labelled anti-CD45 antibody. Fluorescence was measured using a FACSCanto® flow cytometer and analysis was performed using the FACS Diva software. Analysis was performed in accordance with classical lymphocyte gates (Fig 2.1).

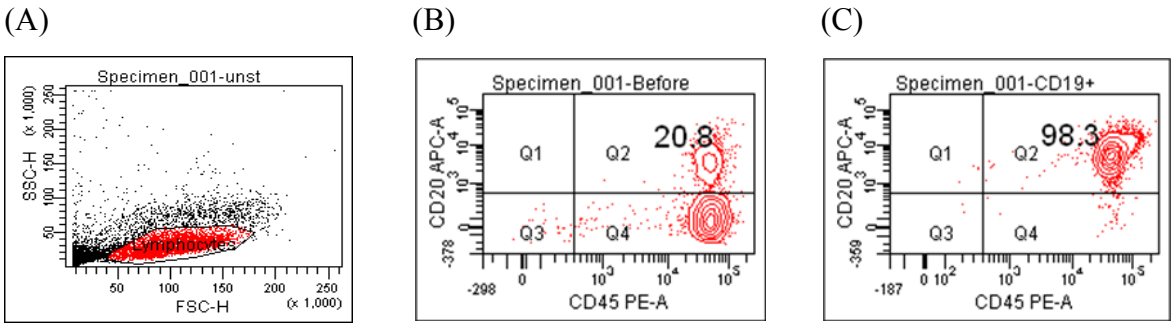


Figure 2.1: FACS controls for B cells isolation.

A classic lymphocyte gate was use for analysis (A). Total PBMC contains approximately 20% $CD20^+$B cells (B). The isolated B cells had a purity of approximately 98% (C). The purity of the isolations of B cells using Dynabeads and detachabeads was routinely > 98% in each experiment.

2.3.6.2 Isolation of $CD4^+$ T cells

$CD4^+$ T cells were isolated using the positive $CD4^+$ isolation kit from Dynal/Invitrogen in accordance with manufacturer's instructions. Briefly, 200 µl of $CD4^+$ dynabeads were added to a 15 ml tube and washed twice with 5 ml of 2 % FCS/PBS using the Dynal MPC®. Beads were then re-suspended in 10% FCS containing RPMI 1640 medium. Approximately, $1x10^5$ PBMC were added to the $CD4^+$ dynal beads and incubated for 20 min at 4°C under gentle vortexing. The rosetted cells were then washed four times with 2% FCS/PBS and re-suspended in 500 µl of medium. 100µl of detach-beads was then added; the suspension was mixed by inversion and incubated for 45 min at room temperature. The tube containing the suspension of the detached beads and the free $CD4^+$ cells was then decanted on the magnet and the solution containing the $CD4^+$ cells was collected in a new tube.

To analyse the purity of the isolated cells, small fractions were double stained before and after isolation with APC-labelled anti-CD4 and PE-labelled anti-CD45 antibody. Fluorescence was measured using a FACSCanto® flow cytometer and analysis was performed using the FACS Diva software. The purity of the isolation was routinely > 99%.

2.3.6.3 Isolation of $CD4^+CD25^+$ regulatory T cells

$CD4^+CD25^+$ double positive regulatory T cells were isolated using the $CD4^+CD25^+$ regulatory T cell isolation kit from Miltenyi Biotech according to the manufacturer's protocol. Briefly, CD4 T cells were first isolated by negative selection and then incubated with CD25 microbeads. Positive cells were then enriched by positive selection. $CD4^+$ $CD25^+$ T cells were run over a second magnetic column to increase the purity. The purity in all assays was routinely > 90%.

The expression of Foxp3 in the isolated cells was controlled per FACS analysis and was always around 90%.

2.3.6.4 Isolation of naive and memory CD19$^+$ B cells

Naive and memory B cells were isolated using the CD27$^+$ B cell isolation kit from Miltenyi Biotech according to the manufacturer's instructions. Briefly, CD19$^+$ B cells were isolated as described in section 2.3.7.1 and subsequently incubated with CD27 microbeads and separated by positive selection from the CD27$^-$ B cells. The CD27$^-$ fraction was used as naïve B cells.

2.3.7 *IN VITRO* IMMUNOGLOBULIN PRODUCTION ASSAY

T cells affect many aspects of B cell development and function[151]. T cell derived lymphokines and growth factors (B cell growth Factors (BCGF) as well as isotype differentiation factors initiate and control B cell maturation and immunoglobulin production[152-161]. Furthermore, membrane bound ligands on T cells play a critical role in B cell differentiation and antibody production. Signals received through CD27[162], OX40[163] and TNF-R1[164], CD40 or BAFF-R (B cell Activator Factor of TNF Family Receptor)[165] all having their ligand on T cell, promote B cell survival, proliferation, and immunonoglobulin secretion. In this work, an assay was designed to assess T-B cell reactions during co-culture. T cells were activated with anti-CD3/CD28 (10µg/ml/2.5µg/ml), to produce soluble factors and activate membrane associated ligands which could induce B cells to secrete immunoglobulins. The culture medium was composed of supplemented RPMI 1640 and 10% FCS. B cells were isolated as described in section 2.3.7. Neutralizing antibodies in pre-titrated concentrations or corresponding isotype controls was added to specifically block the function of proteins of interest in the co-culture systems. B and T cells were then co-cultivated in a ratio of 1:2 ($5x10^4$ B cells: $1x10^5$ T cells) for 14 days at 37°C to allow full B cell differentiation and immunoglobulin secretion. IgG1, 2, 3 and 4 levels were then measured in supernatants using IgG subclass kits (PeliClass kit, Sanquin, Amsterdam, Netherlands) according to the manufacturer's instructions. Detection ranges were 6-89 ng/ml for IgG1, 25-400 ng/ml for IgG2, 3-48 ng/ml for IgG3 and 4-64 ng/ml for IgG4, respectively.

2.3.8 TRANSWELL ASSAYS

To test the necessity of cell-contact in immunoglobulin productions, transwell assays were used in which different T and B cells were co-cultivated as described in 2.3.7, but within 24-well cell

culture plates and separated with a tissue culture insert (8 µm; Nunc). Generated Tr-TCC (2.5 x 10^5/well) were co-cultured in the upper chamber with 1.25 x 10^5 $CD19^+$ B cells/well in the lower chamber. Tr-TCC cells were then stimulated with anti-CD3/CD28 (10µg/ml/2.5µg/ml) and cultured for 14 days). B cells were left either alone in the lower chamber or were incubated with an autologous non-regulatory T cell clones (non-Tr-TCC). Supernatant in the lower wells was then measured for IgG subclass production using the PeliClass kit.

The same type of transwell experiment was performed to test the necessity of cell-contact in the inhibition of effector T cell proliferation. Here, 2.5 x 10^5/well of Tr-TCC were co-cultured in the upper chamber with 2.5 x 10^5/well CFSE labelled (or alamar Blue stained) non Tr-TCC in the lower compartment. Both T cell types were stimulated with anti-CD3/CD28 (10µg/ml/2.5µg/ml) and cultured for 2 to 3 days. And the dilution of CFSE or the reduction of Alamar-Blue was accessed either by FACS analysis or spectrophotometric measurements using respectively FACSCanto® flow cytometer or a Molecular Devices ELISA plate reader.

2.4 MOLECULAR BIOLOGY PROCEDURES

2.4.1 RNA ISOLATION

RNA isolation was performed using the Trizol reagent from Invitrogen and a four step protocol which was modified from the version provided by the manufacturer:

2.4.1.1 Homogenization

Cells were collected from culture plates and pelleted by centrifugation. The supernatant was discarded and the cells lysed in TRIzol-Reagent by repetitive pipetting. 1 ml of the reagent was used per 10×10^6cells. The homogenate was then centrifuged at 12,000g for 10 minutes at 4°C. The supernatant containing RNA was then collected.

2.4.1.2 Separation

200µl of BCP (boro-chloro-phenol) per 1 ml of TRIzol Reagent was added to the collected supernatant. Samples were mixed vigorously for 15 seconds and incubated at 20°C for 3 minutes. Samples were then centrifuged at 12,000g for 15 minutes at 4°C.

2.4.1.3 Precipitation

The aqueous phase was then transferred to a fresh tube and RNA was precipitated from this phase with 500µl of isopropyl alcohol per 1 ml of TRIzol. Samples were incubated for 10 minutes at room temperature and re-centrifuged at 12,000g for 10 minutes at 4°C.

2.4.1.4 RNA Wash

The supernatant was discarded and the RNA pellet washed with 75% ethanol using 1 ml of 75% ethanol per 1 ml of TRIzol Reagent. Samples were mixed by vortexing and centrifuged at 7,500g for 5 minutes at 4°C. The RNA pellet was then air-dried for 5 minutes and the RNA dissolved in RNAse free water and conserved at -80°C or directly transcribed into cDNA. The purity of the isolated mRNA was assessed using a spectrophotometer at 260/280 nm, the ratio was routinely between 1.6-1.9. For reverse transcription and real time PCR an additional genomic DNA digestion was performed by incubating RNA samples in genomic deoxyribonucleic acid (DNA) Wipeout Buffer (Qiagen) at 42°C for 2 minutes.

2.4.2 REVERSE TRANSCRIPTION AND PCR

2.4.2.1 Reverse transcription

1 µg of total RNA was reverse transcribed with the Omniscript RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with oligo-d(T) primer (Roche, Manheim, Germany). The latter binds to the poly-A tail of mRNA and leads to selective transcription. In brief, reagents listed below (Table 2.2) were combined and vortexed for 5 sec. The RNA template was then added to the pre-prepared mix and vortexed for a further 5 sec. This was followed by a 60 min incubation at 37°C to reverse transcribe the template RNA into cDNA. The cDNA was then stored at –20°C or directly used for PCR.

Component	Volume	Final concentration
10 x RT-buffer	2.0 µl	1x
dNTP mix	2.0 µl	0.5 mM (each dNTP)
Oligo-dT primer (10 µM)	2.0 µl	1 µM
Rnase inhibitor (10 units/µl)	1.0 µl	10 units
Omniscript Reverse Transcriptase	1.0 µl	4 units
Rnase-free water	Variable	
Template RNA	Variable	
Total reaction volume	***20 µl***	

Table 2.2: Reaction mix for reverse transcription.

2.4.2.2 Real Time PCR

1µL of cDNA were used in the PCR reactions. The "master mix" was prepared according to the recipe depicted in Table 2.3. According to the number of samples to be tested, a master mix without cDNA was first prepared and this was then aliquoted into 100µL rotor gene adapted real time PCR STRIP tubes (Corbett research, Sydney, Australia). The corresponding cDNA were then added to the PCR master mix. Real Time PCR was performed in a total volume of 20µl.

Component	Volume	Final concentration
10 x buffer	2.0 µl	1x
$MgCl_2$	2.4 µl	3.0 mM
dNTP	0.1 µl	
Primer 1 (forward)	1.2 µl	300mM
Primer 2 (reverse)	1.2 µl	300nM
SybrGreen	0.2µl	
Hot start Taq Polymerase (250 U/µl)	0.1µl	
DNA	Variable	50ng
Rnase free water	Variable	
Total reaction volume	***20 µl***	

Table 2.3: Reaction mix for real-time-PCR reaction.

Reaction conditions performed in a Rotorgene (Corbett research) were 15 min at 95°C, followed by 45 cycles of 15s at 94°C, 20s at 58°C and 20s at 72°C. Temperature change rates were 20°C/s. Copy numbers were determined using a plasmid standard and normalized to expression of the housekeeping gene β-actin that is known to be expressed constitutively. Primers used are given in Table 2.4. Primers were designed using the Primer 3 online program.

PCR Primers List	
β-actin FW	GAT GAG ATT GGC ATG GCT TTA
β-actin RV	AAC CGA CTG CTG TCA CCT TC
Tbet-FW	CAC CTG TTG TGG TCC AAG TTT
Tbet-RV	AAC ATC CTG TAG TGG CTG GTG
GATA3-FW	CCC GGT CCA GCA CAG AAG
GATA3-RV	CGG TCC AGC ACA GGC AG

Table 2.4: Sequences of primers used in this study of primers.

2.4.3 AGAROSE GEL AND ELECTROPHORESIS

1g of agarose powder was mixed with 50 ml of TBE 1x buffer and then heated in a microwave oven until completely dissolved. 1µl of ethidium bromide (Biomol) was then added to the gel (final concentration 0.5 µg/ml) to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60°C, the gel was poured into a casting tray containing a sample comb and allowed to solidify at room temperature under a chemical vertical flow fume hood. After the gel had solidified, the comb was removed; the gel was then inserted horizontally into the electrophoresis chamber and covered with buffer. Samples containing PCR products mixed with 2µl 6x loading buffer were then pipetted into the wells. The samples were then allowed to migrate to the cathode end using 100V (250mA) for 1h. The bands were revealed using a UV transilluminator and a picture of the gel was taken for analysis.

2.4.4 PREPARATION OF PLASMIDS FOR REAL TIME PCR NORMALIZATION.

To design plasmids for normalization of mRNA, PCR products were cloned into TOPO Cloning vectors (Invitrogene, Groningen, Netherlands) according to the manufacturer's instructions. 4 µl of the PCR product, 1 µl salt solution and 1 µl TOPO vector was mixed gently and incubated for 5 min at RT. The mix was then placed on ice and 2 µl of this reaction mix was added to competent *Escherichia coli* bacteria. The suspension was then mixed gently and incubated for 5 min on ice then heated for 0.5 min at 42°C and immediately transferred on ice. Finally, 250 µl of SOC medium (super optimal culture medium) was added. 50µl of this solution was then spread on a pre-warmed selection plate and incubated overnight at 37°C. Transformed colonies, which could be distinguished from non-transformed colonies because they were white and not blue were then picked and cultured overnight in LB medium. Alkaline lysis was performed to isolate the plasmids from bacteria suspension using the NucleoSpin Plasmid kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer´s instructions.

2.4.5 SMALL-INTERFERRING RNA (SIRNA) NUCLEOFECTION

To study the function and necessity of Foxp3 in the generated regulatory T cell clones, nucleofection assays were performed using Foxp3 specific small interfering RNA (siRNA). Specific siRNA, siRNA controls (Table 2.5) and transfection reagents used in this study were provided by Qiagen and were used according to the manufacturer indications.

DESIGNATION	TARGET SEQUENCES
Hs_Foxp3_1	CCAGCAGGTGTTCCAACCCTA
Hs_Foxp3_2	CCACAACATGGACTAACTTCAA
Hs_Foxp3_3	CACGCATGTTTGCCTTCTTCA
Hs_Foxp3_4	CAGGCCACATTTCATGCACCA
Control MAPK-1	AATGCTGACTCCAAAGCTCTG
Negative control	None

Table 2.5 siRNA used in this study and their target sequences.

In short, $2x10^5$ cells were seeded onto a 24 well plate in RPMI/10%FCS medium and returned to 37°C until transfection. 375ng of designated siRNA in 100µl medium was diluted in 6µl HiPerFect solution and incubated for 10 mins at 37°C (Qiagen, Germany). This step allowed the formation of transfection complexes. The siRNA transfection mix was added drop wise to the cells, mixed gently and incubated for 6 hours at 37°C. An additional 400µl medium was then added to the cells and incubated for a further 72 hours. Transfection efficiency was monitored using flow cytometric analysis. Tr-TCC and B cells co-cultures were then performed as described above in section 2.3.8.

3 RESULTS

This chapter is divided in five main parts. The first sections present different aspects of the generation of antigen induced regulatory T cell clones (Tr-TCC) using the combination of vitamin D3 and dexamethasone (DD3). These aspects include the induction of IL-10 in DD3 treated T cell lines (TCL) (3.1.1), the antigen specificity and cell-contact independent suppressive properties of the generated Tr-TCC (3.1.6 and 3.1.7). The next series of sections (3.2) describes the results of different experiments designed to decipher the mechanisms used by the generated Tr-TCC to induce B cells to preferentially secrete the immune regulatory antibody IgG4. Data presented in this section demonstrate the necessity of contact between Tr-TCC and B cells (3.2.2) and the key roles play by molecules like GITR, GITRL (3.2.3), TGF-β, IL-10 (3.2.4) and Foxp3 (3.2.8) in this Tr-TCC dependant IgG4 induction. Data presented in the third sections show that IgG4 induction is not exclusive for IL-10 producing regulatory T cells (Tr-TCC) by demonstrating that also isolated $CD4^+CD25^+Foxp3^+$ regulatory T cells from healthy untreated patients can direct B cells to produce IgG4 albeit weaker than Tr-TCC (3.3). Further sections also demonstrate that both memory and naïve B cells are required within the co-culture system to produce significant levels of IgG4 (3.4). In the final section (3.5) the research focuses on the role of TLR and demonstrates that whereas TLR on Tr-TCC themselves are redundant for the induction of IgG4; activation of TLR on the B cell population elicits pro inflammatory immunoglobulin production rather than IgG4.

3.1 GENERATION AND CHARACTERIZATION OF REGULATORY T CELL LINES (TCL) AND CLONES (TCC)

Within this results section, several aspects regarding the generation of regulatory T cell clones (Tr-TCC) are described. The first part deals with the initial generation and characterization of these regulatory T cells. In brief, it is demonstrated how the addition of dexamethasone and

vitamin D3 can drive T cells into an IL-10 producing phenotype whereas without dexamethasone they remain as strong Th1-like T cells. In addition the generated Tr-TCC possess characteristics of Tr-1-like cells but in addition display Foxp3 aswell.

To generate the T cell clones, Peripheral Blood Mononuclear Cells (PBMCs) were isolated from buffy coats obtained from known haplotype healthy donors (see section 2.1.1). Total PBMCs were then stimulated with tetanus toxoid (TT) in presence or absence of vitamin D3 and dexamethasone (DD3) for 10 days. Cells were then re-stimulated twice for 10 days with the same antigen (TT) alone or together with DD3 in the presence of irradiated PBL acting as antigen presenting cells (APCs). T cell lines produce high levels of IL-10 (Fig 3.1 and 3.2) and display characteristic regulatory T cell markers such as GITR, TGF-β, Foxp3 and are anergic in the absence of IL-2 and present significant suppressive properties (data not shown). In order to characterize the generated regulatory T cells, the T cell lines generated with O.v or TT were cloned by limiting dilution (see section 2.3.4) and single T cell clones were used for the rest of the work. The clones were analyzed for their specificity to antigen by a proliferation assay (Fig 3.9). They were also analyzed for cytokine production after stimulation through the T cell receptor (TCR) using anti-CD3 and anti-CD28 antibodies (Fig 3.3).

3.1.1 VITAMIN D3 AND DEXAMETHASONE (DD3) ELICIT IL-10 PRODUCING CELLS.

The predisposition of DD3 to drive IL-10 producing cells is well-established[97]. In the studies described here, DD3 was used to bias the generation of tetanus toxoid specific IL-10 producing T cells. After incubation with antigen and DD3 for 10 days, cell cultures were examined for their Th propensity by intracellular staining. In brief, cell cultures were re-stimulated with PMA and ionomycin for 3 hours for IFN-γ and 5 hours for IL-10 in presence of Golgi stop solution as described in section 2.3.5.3. Thereafter cells were surface stained with anti-CD4 Ab and intracellularly with fluorescently labelled antibodies to IL-10 and IFN-γ.
The dot-plot diagrams depicted in figure 3.1, display a representative picture of induced T cell lines from a specific donor (designated SB1, see section 2.1.1). As can be clearly seen, the presence DD3 in the cell cultures elicits strong IL-10 producing cells even in the presence of prominent Th1-inducing cytokines such as tetanus toxoid.

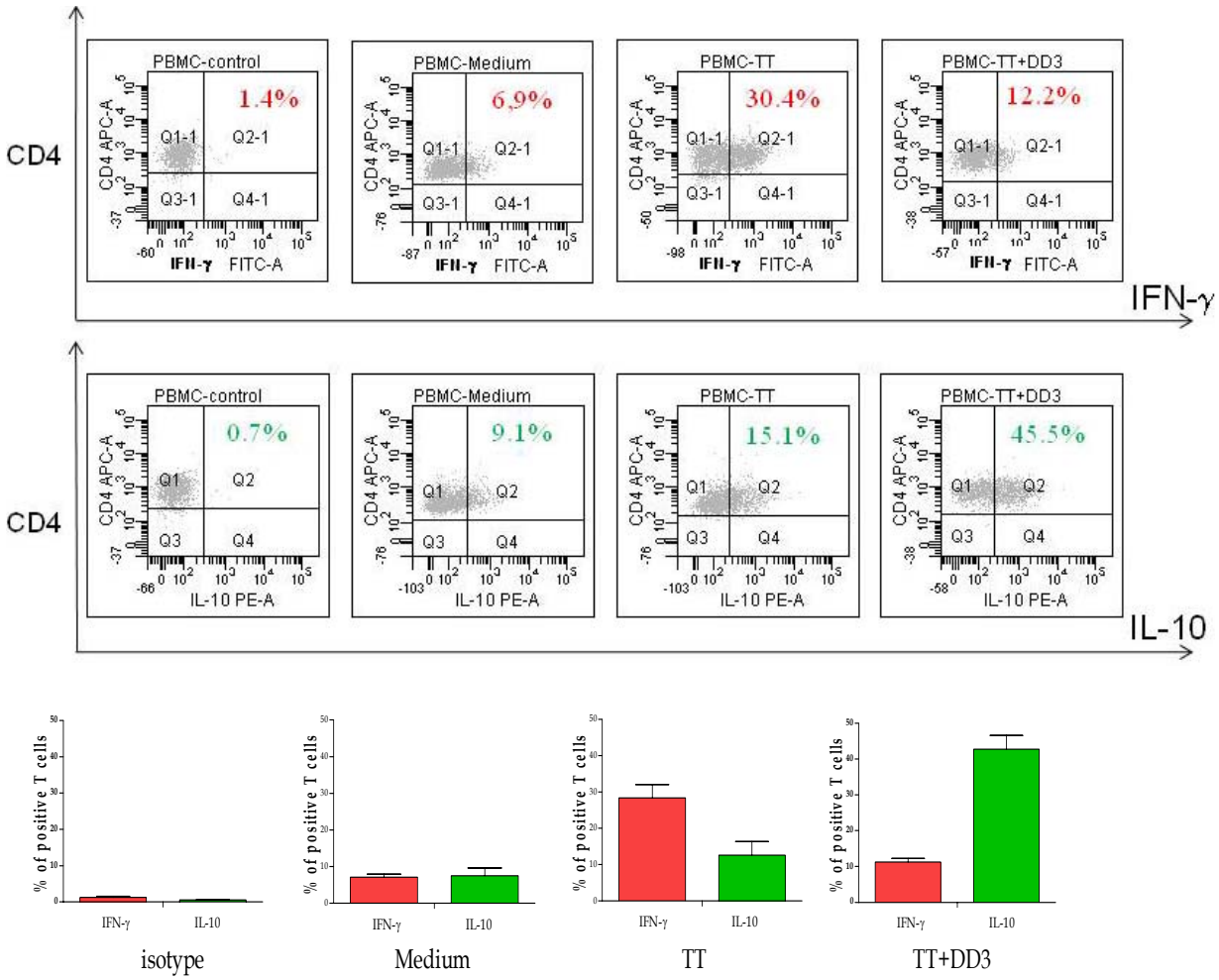


Figure 3.1: DD3 induce IL-10 producing T cells.

Freshly isolated PBMCs ($1x10^5$/ml) were plated in RPMI medium containing 10% FCS. Cells were then left unstimulated (Med), stimulated with either tetanus toxoid antigen alone (TT) or with TT and DD3 (TT+DD3). After 10 days of culture, cells were analyzed with flow cytometric techniques for their intracellular levels of IL-10 and/or IFN-γ after re-stimulation with PMA/ionomycin. The displayed dot plots show the levels of IFN-γ (upper panel) and IL-10 (lower panel) within the $CD4^+$ T cell population. Graphs represent the percentage of IFN-γ (red bars) and IL-10 (green bars) positive $CD4^+$ T cells in PBMC stained with isotype controls (control) or PBMC stained with anti-IL-10-PE or anti IFN-γ-FITC after treatment with medium (med) TT antigen alone (TT) or TT and vitamin D3 + Dexamethasone (TT+DD3).

3.1.2 CYTOKINE PROFILE DURING THE GENERATION OF TCL

As shown above, exposure of cells to the combination of vitamin D3 and dexamethasone generates high levels of IL-10 producing cells. Previous studies have generated IL-10 producing regulatory cells from patients inflicted with the parasite helminth *Onchocerca volvulus*[111]. In order to mimic an ongoing infection *in vitro*, PBMCs isolated from healthy donors were subjected to multiple rounds of antigen-specific stimulation. Following each round of stimulation, the culture supernatants were tested for the presence of different cytokines.

To find out the role of Th1, Th2 and Tr-1 polarising cytokines respectively IFN-γ, IL-4 and IL-10 in the DD3 induced IL-10$^+$ cell development, neutralizing antibodies against these cytokines was added to the cultures.

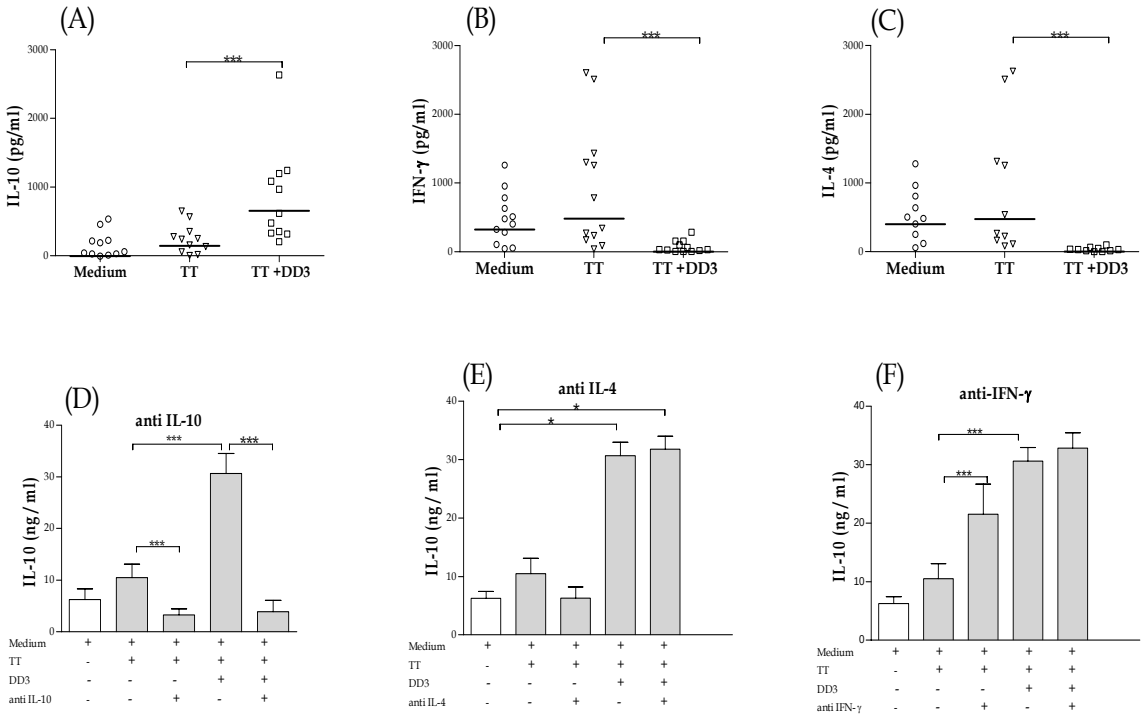


Figure 3.2: PBMC cultured with antigen and DD3 produce higher amounts of IL-10.

Following each round of stimulation, the cell culture supernatant was tested for the presence of IL-10, IFN-γ and IL-4 by ELISA (A-C respectively). Each symbol depicts the levels of cytokine found in the supernatant 3 days after the first re-stimulation in which cultures received fresh APCs (irradiated) and renewed antigen source in the presence or absence of DD3. Graphs A-C show the accumulated data from cell cultures derived from 12 different donors. Graphs D, E and F represent the IL-10 production of $2x10^5$ cells/well of PBMCs from three different patients stimulated in the presence of medium alone, TT or TT + DD3 in the presence (+) or absence (-) of 10µg/ml of neutralizing antibodies against IL-10, IL-4 or IFN-γ respectively. Bars represent means ± SD of three independent experiments. Asterisks indicate significant differences between the groups indicated by brackets, *$p<$ 0.05, ***$p<$ 0.001.

As shown in figure 3.2, PBMC cultures re-stimulated with antigen and DD3 show a higher propensity to produce IL-10$^+$ cells than those cultured in the presence of antigen alone. In correlation, those cells that were stimulated with antigen alone retained their Th1-type phenotype (Fig 3.2 A-C). Furthermore, neutralizing antibodies against IL-10 as expected completely inhibited the development of IL-10 producing cells (Fig 3.2 D) whereas blocking IL-4 or IFN-γ did not significantly affect the levels of IL-10 secreted by the TCL (Fig 3.2 E, F)

3.1.3 CLONING STRATEGY TO OBTAIN TR-TCC

To obtain homogenous T cell populations that could be used for further experiments, we performed a cloning per serial dilution. Using the data obtained in section 3.1.2, prominent IL-10 but low IL-4 and IFN-γ producing TCL were first pre-stimulated with PHA for three days to allow generalized T cell expansion through proliferation. Cells were then cloned per serial dilution in Terasaki plates as described in section 2.3.3. Ten days after cloning, culture wells that contained stably growing clones were transferred to 96-well culture plates and allowed to expand further in the presence of IL-2 and anti-CD3/anti-CD28 expander beads.

	TT	TT/DD3	O.v	O.v/DD3	αCD3	αCD3/ DD3
Generated TCL	112	145	55	120	82	120
Nr. generated TCC	220	760	52	14	15	95
Nr. Ag-specific TCC	63	22	8	3	-	-
Nr. IL-10$^+$ TCC	1	6	1	2	2	2
Nr. of TCC capable of inducing IgG4	0	6	1	3	0	2

Table 3.1. Total number of generated TCL and the derived numbers of TCC and Tr-TCC.

Table 3.1 shows the total numbers of TCL that were generated using either TT or O.v antigen or αCD3 stimulation. From this overview it is also clear that in comparison to O.v antigen, TT and DD3 stimulation allows for a more positive generation of TCC. However, it is also interesting to note that within these 760 TCC, only ≈3% were Ag specific and ultimately only 11 clones produced abundant IL-10 upon antigen specific stimulation.

This cytokine propensity was only tested after a sufficient amount of clone was obtained after the cloning step. The TCC profile was judged upon their cytokine expulsion after both antigen-specific and non-antigen-specific stimulation. In brief, TCC were co-incubated with either TT or αCD3/αCD28 in the presence of irradiated autologous APCs for 4 days. Culture supernatant was removed after 48 and 96 hours for cytokine assessment using ELISA. Figure 3.3 shows the cytokine profile of the generated Tr-TCC after αCD3/αCD28 (Fig 3.3 A) and tetanus toxoid stimulation (Fig 3.3 B) respectively. In addition to their prominent IL-10 response to αCD3 stimulation, the generated Tr-TCC also responded in a similar manner to their specific antigen,

namely tetanus toxoid. As observed in the two figures, only a portion of the generated Tr-TCC produced IL-10 upon re-stimulation. The TCC which did not produce any IFN-γ and IL-4 were used in further experiments. Those which showed a non-regulatory phenotype, that is, produced specifically IFN-γ were designated as non-Tr-TCC and used as control cells in the experiments described in the following sections.

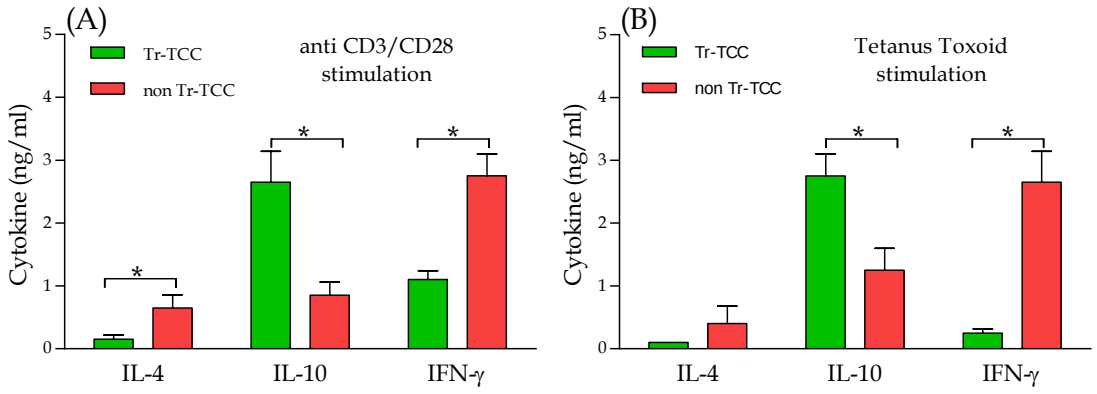


Figure 3.3: Generated Tr-TCC secrete IL-10 upon stimulation antigen-specific or αCD3/αCD28 stimulation.
(A) $1x10^5$ Tr-TCC were cultured with irradiated syngenic feeder cells ($1x10^5$) and stimulated with αCD3/αCD28 (10/2.5 µg/ml) for 3 days. Bars represent the mean and SD of 6 tested TCC. Thereafter, culture supernatant was removed and tested for levels of IL-10, IFN-γ and IL-4 by ELISA. (B) $2x10^5$ Tr-TCC were co-cultured with $1x10^5$ irradiated APCs and TT antigen (10µg/ml) for 96 hours. Symbols represent the cytokine secretions produced by 10 tested Tr-TCC and 7 non Tr-TCC. Cell culture supernatant was removed and tested for IL-4 (48 hours) and IL-10, TGF-β and IFN-γ (96 hours). Cytokine levels were quantified using Molecular Devices ELISA plate reader and softmax pro software. Statistical analysis was performed using Student's t test (* $p<0.05$).

3.1.4 EXPRESSION OF REGULATORY T CELL MARKERS

Naturally occurring regulatory T cells are characterized through their expression of distinct surface markers including CD25, GITR and CTLA-4. These thymus derived Tregs also express the transcription factor Foxp3 which has become the pre-requisite marker for their identification. As mentioned in the introduction, there are other distinct populations of Treg cells such as Tr-1 and Th3 cells. The phenotypic characterization of these populations is not as clear cut as natural Tregs. Tr-1 cells are actually defined by their ability to produce large quantities of IL-10 and to suppress proliferation of effector T ells in a cell contact independent manner in opposition to natural Tregs that apparently need cell contact. Since the Tr-TCC described here were selected on the basis of their ability to secrete IL-10 upon stimulation, we were interested to determine whether these Tr-TCC expressed Treg-specific markers. Thus, using flow cytometric techniques,

Tr-TCC (and non Tr-TCC as controls) were stained for distinct Treg cell surface markers and intracellularly for levels of Foxp3. As a further comparison we also included nTreg in this assessment. These cells were obtained from healthy donors and were isolated using $CD25^+$ Treg isolation kit from Miltenyi Biotech (see section 2.3.6.3). Figure 3.4 shows the mean fluorescence intensity, minus the intensity measured on cells stained with the corresponding isotype controls, of the different Treg markers (CD25, CD127, Foxp3, GITR, CTLA-4, and ICOS) on resting cell populations.

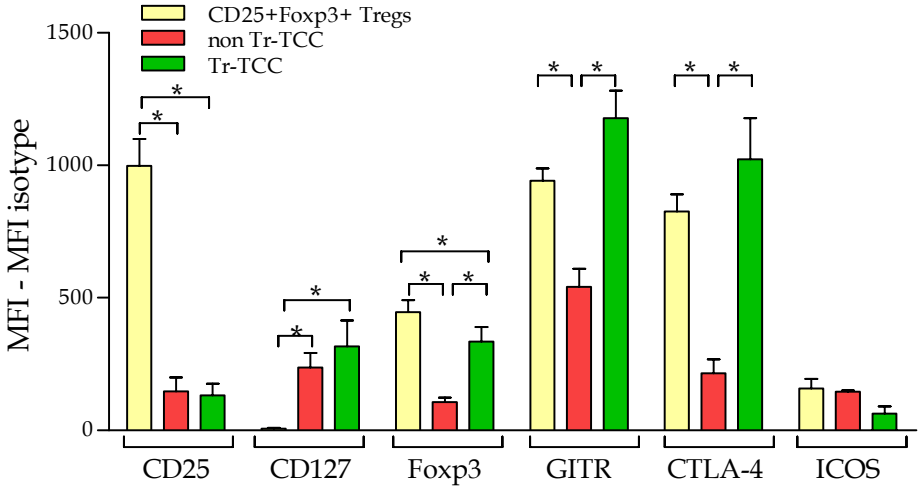


Figure 3.4: Resting Tr-TCC express Foxp3, GITR and other Treg markers.
$1x10^5$ resting cells were stained with APC-labelled anti-CD4 antibody and either: 1) PE-labelled anti-CD25 antibody 2) PE-labelled anti-CD127 antibody 3) PE-labelled anti-GITR antibody, 4) PE-labelled anti-CTLA-4 antibody and 5) PE-labelled anti-ICOS antibody. After staining with anti-CD4, some cells underwent intracellular staining with PE-labelled anti-Foxp3 antibody. Following acquisition with the FACS Canto™ the mean fluorescence intensity (MFI) of the cell populations was determined using FACS Diva software. Bars represent the mean ± SD of MFI on nTreg (yellow bars), non-Tr-TCC (red bars) and Tr-TCC (green bars) after deduction of MFI on cells after isotype control staining. Asterisks indicate significant differences between the groups indicated by brackets,*$p< 0.05$.

One can clearly see from figure 3.4 that resting Tr-TCC express approximately the same levels of CTLA-4, GITR and Foxp3 as isolated nTreg cells. In contrast they show a higher expression of CD127 but lower levels of ICOS and CD25. Interestingly Tr-TCC express almost the same levels of CD25 and CD127 as non-Tr-TCC. MFI for ICOS expression levels were not exceptionally elevated in any of the analyzed cells types. These data clearly reflect that categorisation of Tr-TCC is not a simple matter. Although they possess characteristics of Tr-1-like cells they also have common features to nTreg such as elevated Foxp3 levels.

3.1.5 DD3 TREATMENT REDUCES EXPRESSION OF BOTH TBET AND GATA3.

The TCC which are generated upon activation with tetanus toxoid and DD3 produce significant levels of IL-10 but low levels of IFN-γ and IL-4. Since these cytokines are characteristic markers for Th1 and Th2 type cells and are governed by key transcription factors such as Tbet for Th1 cells and GATA-3 for Th2 cells, we decided to investigate whether the presence of DD3 influenced the expression of these transcription factors. In brief, bulk PBMC isolated from healthy blood spenders were cultured for 72 hours with TT in the presence or absence of DD3. Thereafter, total RNA was prepared and quantitative PCR was performed using primers specific for GATA3 and Tbet. The results show that the treatment with DD3 down regulates the expression of both Tbet and GATA3 (Fig 3.5) during the first round of TCL generation.

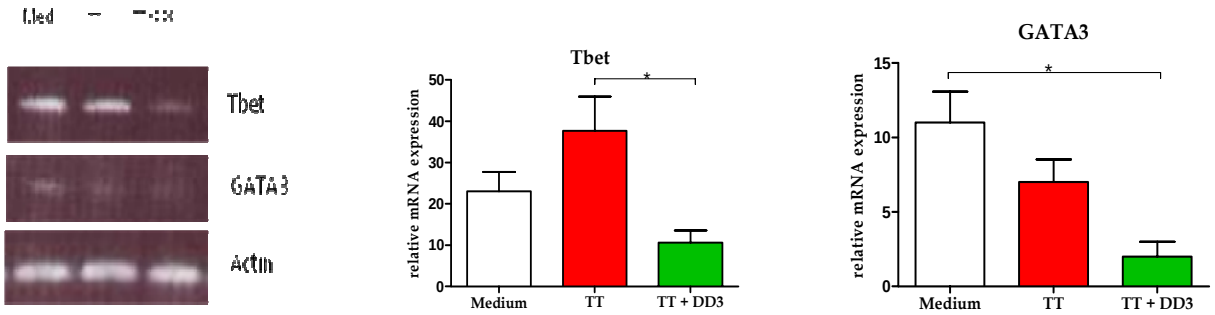


Figure 3.5: DD3 treatment reduced both Tbet and GATA3 expression in human PBMCs
Human PBMCs ($1x10^5$) were cultured in either medium alone (white bars), with tetanus toxoid antigen (red bars) or with tetanus toxoid in the presence of DD3 (green bars). The cells were harvested after 5 days of culture. Thereafter, 1ng of total mRNA was revers transcribed and analyzed by real-time PCR for Tbet and GATA3. Bars show the mean ± SD of three independent experiments. Asterisks indicate significant differences between the groups indicated by brackets,*$p< 0.05$.

However, after T cell cloning, the Tr-TCC expressed significantly higher levels of Tbet compared to GATA3 upon stimulation with αCD3/αCD28 expander beads (Fig 3.6). This justifies the expression of some IFN-γ but no IL-4 in the generated Tr-TCC (Figs 3.3 and 3.4).

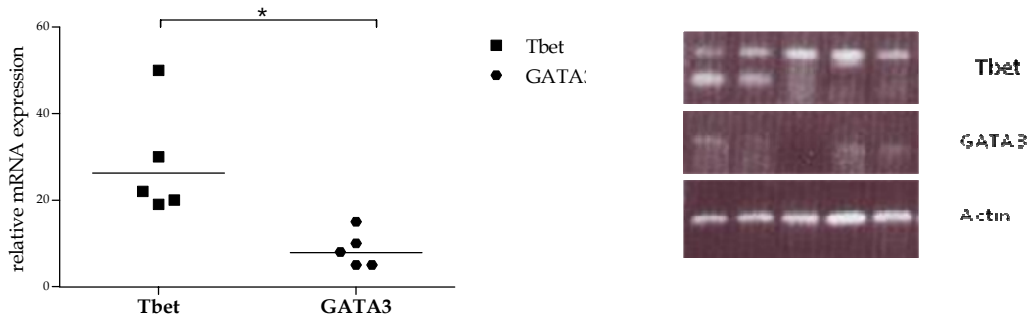


Figure 3.6: Tr-TCCs express Tbet rather than GATA3

1x10^5 Tr-TCC were activated with CD3/CD28 beads *in vitro*. The T cells were harvested after 5 days of stimulation and RNA was prepared. mRNA levels of Tbet and GATA3 were quantified by real-time PCR. Data represent the results of 3 independent experiments using 5 different T cell clones. Asterisks indicate significant differences between the groups indicated by brackets,*$p< 0.05$.

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3.1.6 ANTIGEN SPECIFICITY

Upon establishing that the generated Tr-TCC produced high quantities of IL-10 and reflected a regulatory phenotype in their surface marker expression and Foxp3, the following section demonstrates the ability of these cells to respond in an immunologically positive manner. Tetanus-toxoid derived TCC for example were tested for their response to tetanus using several parameters. First, they showed an up-regulation of CD69 when exposed to tetanus antigen but not O.v antigen (Fig. 3.7A). They also showed a weak increase in proliferation upon antigen re-stimulation (Fig. 3.7B). Similar results was obtained using O.v generated Tr-TCC.

Most interestingly, Tr-TCC respond to their specific antigen by secreting high levels of IL-10 (Fig 3.7C). An increase in IFN-γ was also detectable upon antigen stimulation but was not significant when compared to the non-stimulated values. Increased levels of IL-4 were not detectable and although there a small increase in TGF-β production upon antigen recognition it is obviously not the dominant cytokine when one compares it to the IL-10 response.

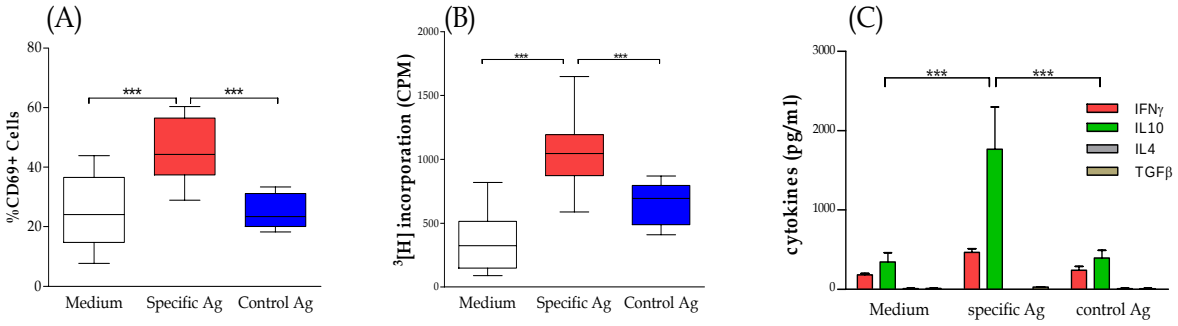

Figure 3.7: Tr-TCC specifically upregulate CD69, proliferate and produce IL-10 and IFN-γ upon antigen stimulation.

$2x10^5$ tetanus-toxoid specific TCC were stimulated with TT or O.v antigen (A). After 24 hours, the expression of CD69 was measured on the $CD4^+$ T cell population using the FACS Canto™. $2x10^5$ tetanus-toxoid specific TCC were co-cultured with $1x10^5$ irradiated APC and stimulated with TT for 96 hours (B). ^{3}[H] thymidine was added for the last 18 hours of culture. Bars represent means ±SD Tr-TCC activity in three independent experiments using 6 different TCC. Asterisks indicate significant differences between the groups indicated by brackets, ***$p< 0.001$.

3.1.7 SUPPRESSIVE PROPERTIES

Regulatory T cells are renowned for their ability to suppress effector T cell responses or B cell activation[166-168]. Since the generated regulatory T cell clones here were slightly different to both nTreg cells and Tr-1-like cells we assessed their suppressive capacity using four different methods. These methods included; 1) their capacity to suppress the proliferation of CFSE-labelled non regulatory T cell clones (or PBMCs) after αCD3/αCD28 stimulation; 2) their ability to inhibit tritium incorporation in co-culture with activated non regulatory TCC 3) their capacity to hinder cytokine production from activated Th1 cells as measured by ELISA and 4) T cell suppression measured using Alamar Blue. These methods allowed the measurement of Tr-TCC suppressive capacity in both antigen-specific and non-specific settings. For antigen-specific suppression; Tr-TCC were co-cultured in a 1:1 ratio with autologous CFSE-labelled TT specific non-Tr-TCC in the presence of TT antigen and irradiated APCs. After 72 hours, the proliferation of the CFSE labelled non-Tr-TCC was measured according to the dilution of CFSE staining. The same experimental settings were used in the presence of neutralizing antibodies against IL-10 and TGF-β to determine the role of these cytokines in the putative suppressive functions of Tr-TCC.

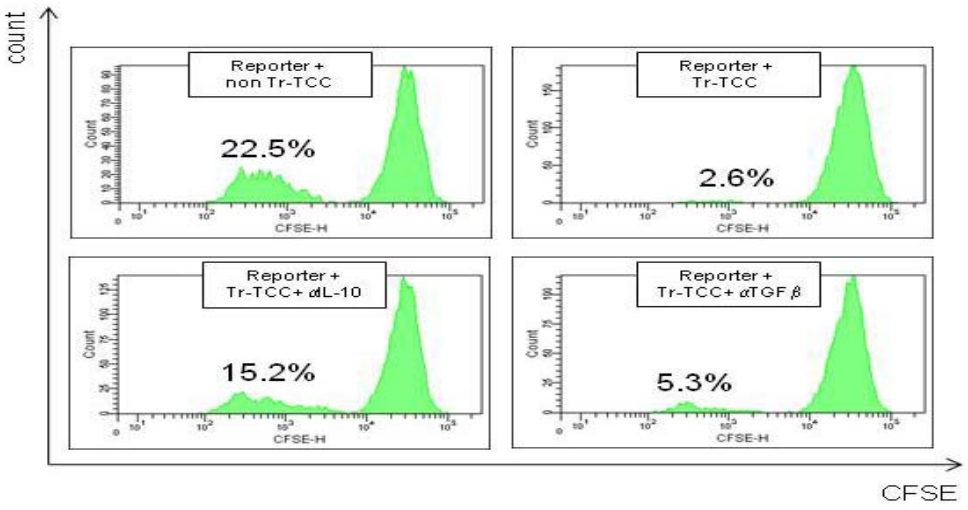

Figure 3.8: *In vitro* suppressive capacity of Tr-TCC.
$5x10^4$ Tr-TCC were co-cultured with $1x10^5$ CFSE labelled autologous non Tr-TCC (reporter) generated from the same donor using only tetanus toxoid antigen. Cells have been stimulated with 10µl/ml TT antigen as previously described. In some cultures 10µg/ml of either anti-IL-10 or anti-TGF-β was added to the cultures to determine the role of these cytokines. The dilution of CFSE was assessed via FACS analysis 3 days after stimulation. Numbers on the histograms represent the percentage of cells that have proliferated according to the control at day 0. Data are representative of three different experiments performed in triplicate.

As expected, CFSE labelled non-Tr-TCC (+ non labelled non Tr-TCC) proliferated strongly when stimulated with TT (upper panel left). Upon addition of Tr-TCC, this proliferation was drastically reduced (upper panel right) and this suppressive nature of the Tr-TCC was reversed with the addition of anti-IL-10 to the culture (lower panel left) and moderately by anti-TGF-β (lower panel right). In control experiments, in which non-Tr-TCC were added instead of Tr-TCC, blocking IL-10 Ab increased the amount of proliferation of CFSE-labelled non-Tr-TCC activated with TT antigen (not shown). The same tendencies were observed when the cells are stimulated with αCD3/αCD28 proliferation beads (data not shown). These results show that IL-10 is essential for the suppressive activities of Tr-TCC on other T cells.

Interestingly, the Tr-TCC were also able to dampen αCD3/αCD28-induced proliferation of a Th1 clone (S1 TCC) derived from a patient infected with the filarial nematode *Onchocerca volvulus* (Fig 3.9) demonstrating that the suppressive cells could also function in an Ag-non-specific setting. Within this experimental setup, the same inhibition levels could also be achieved by adding supernatant from activated Tr-TCC to the proliferating cultures (data not shown), indicating that it is the cytokine milieu, most likely IL-10 according to the data obtained in figure 3.8 that mediates suppression.

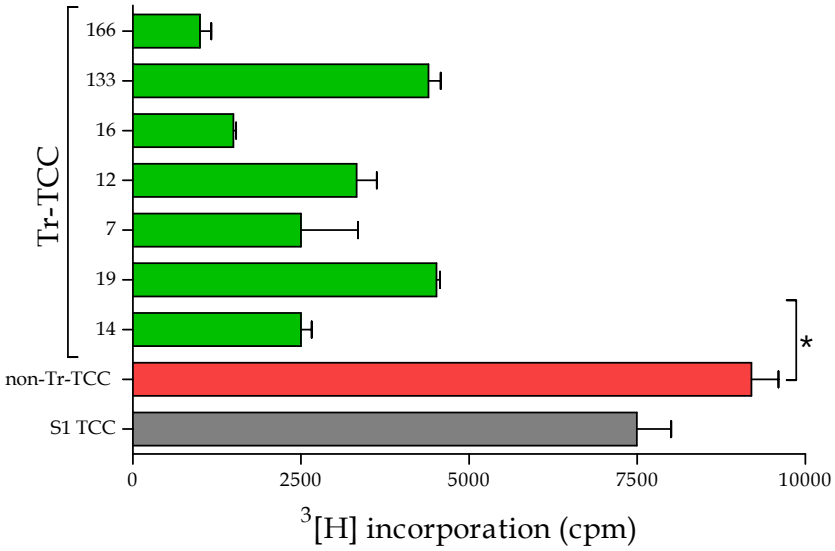

Figure 3.9: *In vitro* suppressive capacity of Tr-TCC using tritium incorporation (non-specific inhibition).
$5x10^4$ Tr-TCC or non-Tr-TCC ($5x10^4$) were co-cultured with a Th1-clone (S1-TCC) derived from an *Onchocerca volvulus*-infected patient and stimulated with αCD3 for 72 hours. During the last 18 hours ^{3}[H]-thymidine-incorporation was added and proliferation measured by liquid scintillation. Bars represent mean ± SD of three pooled experiments. Asterisks indicate significant differences between the groups indicated by brackets, *p<0.05.

In a T-T co-culture system, it was further examined whether Tr-TCC could also suppress cytokine expression from activated non Tr-TCC cells. In brief, $1x10^5$ non Tr-TCC were co-stimulated with irradiated APC ($1x10^5$) and stimulated with tetanus antigen for 72 hours. In some cultures, $1x10^5$ Tr-TCC were also applied. Figure 3.9 shows the results of three independent assays using 3 different Tr-TCC. As can be observed, the IFN-γ secreted by non-Tr-TCC (red bars) can be significantly suppressed with the addition of Tr-TCC (green bars).

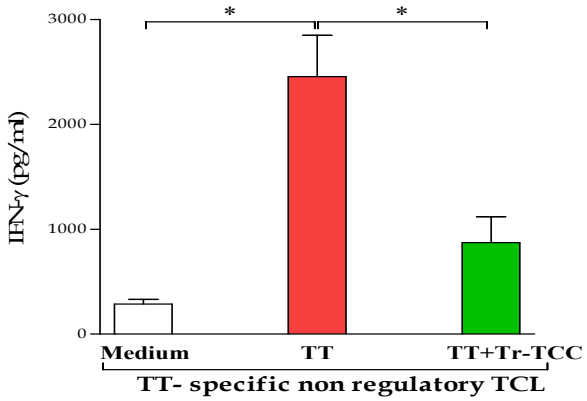

Figure 3.9: Tr-TCC are capable of suppressing IFN-γ producing T cell lines.
$1x10^5$ non-Tr-TCC were stimulated with tetanus (TT) antigen (10µg/ml) in the presence of irradiated APC ($3x10^5$) and in some cultures, $1x10^5$ Tr-TCC aswell. After 72 hours the culture supernatant was removed and tested for IFN-γ by ELISA. Bars represent the mean ± SD of three independent assays using 3 different Tr-TCC. Asterisks indicate significant differences between the groups indicated by brackets,*p< 0.05

In the final method, it was investigated whether there was a reduction in the percentage of Alamar Blue positive cells using a transwell co-culture system described in section 2.3.8. In the assay performed here, non-Tr-TCC were stimulated with anti CD3/CD28 in the presence or absence of Tr-TCC in the upper transwell. After 24 hours, the reduction of Alamar Blue was then determined. As shown in figure 3.10 activated non-Tr-TCC cells show a significantly greater amount of Alamar blue reaction showing that these cells have proliferated. In contrast, the reduction of Alamar blue is lower when co-cultured with Tr-TCC indicating the suppression of activated Th1 cells. In further experiments in which the Tr-TCC cells were placed in the upper chamber, it can be seen that Th1 cells are still suppressed demonstrating that the ability of Tr-TCC to suppress Th1 cells is not cell-contact dependent. Furthermore, addition of neutralizing antibody against IL-10 was able reverse the suppression of Th1 cells proliferation by Tr-TCC. The blockade of TGF-β did also but only partially reverse the suppressive capacities of Tr-TCC (Fig 3.10).

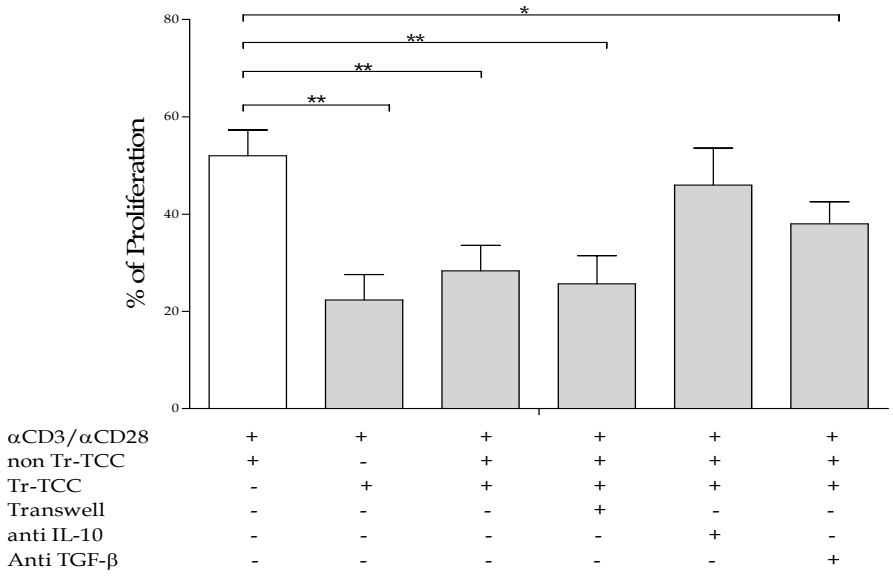

Figure 3.10*: In vitro* suppressive capacity of Tr-TCC on non Th1 cells.
Non Tr-TCC with Th1 profile (high IFN-γ) and IL-10 producing Tr-TCC were used in a transwell assay. Both T cell populations were previously activated with αCD3/αCD28, non-Tr-TCC were cultured in the lower chamber. Tr-TCC were placed in the upper chamber. 10% of AlamarBlue was added to the cultures and the reduction of Alamar blue was measured 72h after stimulation using a spectrophotometer. Bars represent the mean ± SD of the percentage of reduction of AlamarBlue in three independent experiments calculated according to a control without T cells. Asterisks indicate significant differences between the groups indicated by brackets, *$p< 0.05$, **$p<0.01$.

From the above transwell experiments it became clear that the ability of Tr-TCC to suppress Th1 cell proliferation was cell-contact independent and indicated that suppression is mediated through cytokines like IL-10 and TGF-β.

3.2 ANTIGEN-SPECIFIC REGULATORY T CELLS INDUCE AUTOLOGOUS B CELLS TO PRODUCE IGG4

As mentioned in the introduction, IgG4 behave differently to other IgG subclasses, in that it does not fix complement and cannot initiate ADCC. IgG4 is found in conditions where IL-10 is increased as, for example, in allergic diseases after immunotherapy[127-129] or chronic helminth infections[135]. IL-10 producing Tr-1 or Tr-1-like cells have been implicated in the induction of IgG4 from B cells[143]. However the mechanisms associated with IgG4 production are not well characterized. Within this work, a model was established to elicit IgG4 from B cell *in vitro*. In brief, activated IL-10 producing cell clones, with a regulatory phenotype (Tr-TCC) were co-cultured in the presence of autologous B cells for 14 days. Thereafter, the supernatant was measured for levels of immunoglobulins. To decipher molecules that were involved in this induction, different blocking and/or recombinant antibodies where added to the cultures.

3.2.1 PREFERENTIAL IGG4 INDUCTION BY TR-TCC

Upon activation, B cells can differentiate and produce antibodies. The type and amount of antibodies produced is dependent on the disease or infection and is tightly controlled by activated T cells. As mentioned above, to access the molecular components associated between IgG4-induction, B cells and regulatory T cells various assays using an *in vitro* model were performed. In brief, $CD19^+$ B cells were separated from an autologous donor and co-cultured in 1:3 ratio with Tr-TCC stimulated with αCD3/αCD28. After 14 days of culture the concentration of IgG1, 2, 3 and 4 was measured with a specific ELISA kit.

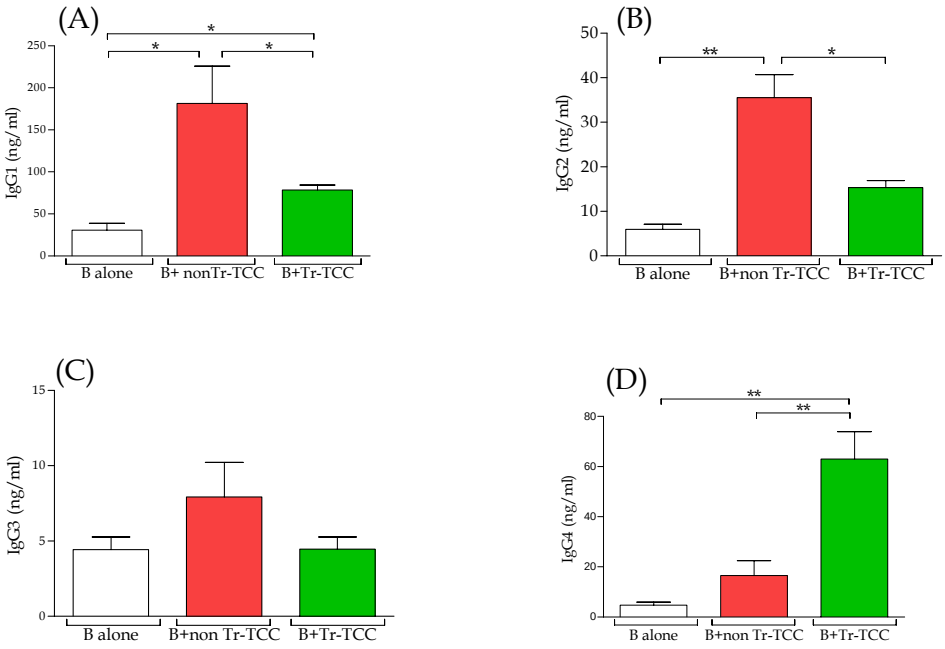

Figure 3.11: B cells preferentially secrete IgG4 when co-cultured with activated Tr-TCC.
$3x10^4$ $CD19^+$ B cells were co-cultivated with autologous Tr-TCC ($1x10^5$) or non Tr-TCC ($1x10^5$). Cultures were then stimulated with αCD3/αCD28 (10µg/ml/2.5µg/ml). After 14 days of culture the concentration of IgG1, 2, 3 and 4 was measured per ELISA. Bars shows means ± SD of IgG levels in three independent experiments. Asterisks indicate significant differences between the groups indicated by brackets, **P*<0.05, ***P<0.01*.

To identify the specific nature of the Tr-TCC, control cultures containing non-Tr-TCC were also included in the assay. The results in figure 3.11 show that B cells produce only moderate levels of immunoglobulin when cultured alone (white bars). With non Tr-TCC (red bars), B cells produced no IgG4, high levels of IgG1 and IgG2 and some IgG3. In contrast, activated Tr-TCC preferentially produced IgG4 and no IgG3 (green bars). B cells in these cultures also produced IgG1 and IgG2 albeit less than that induced by non-Tr-TCC.

3.2.2 IgG INDUCTION IN THE CO-CULTURE SYSTEM IS CELL CONTACT DEPENDANT

From section 3.1.7, it was observed that the suppressive capacity of Tr-TCC was cell-contact independent. Thus, in deciphering the mechanism through which Tr-TCC could induce B cells to produce IgG4, a transwell system was employed to investigate whether it was necessary to have cell contact or not. Surprisingly, and in contrast to what was observed with their suppressive properties on T cells, the separation of Tr-TCC and B cells led to the abrogation of IgG4 production (Fig 3.12D) suggesting the necessity of cell-cell contact in this process. However, this abrogation was not exclusive for IgG4 production since the separation of regulatory T cells and B

cells by the membrane also led to reductions in IgG1 and IgG2 aswell. In additional experimental cultures, non Tr-TCC were added to the lower part of the transwell assay, that is, in contact with B cells. In correlation to the results shown in the previous section (3.2.1), non-Tr-TCC were not able to elicit IgG4 production from B cells but could induce IgG1 and IgG2 (Fig 3.12 A and B respectively). The direct necessity of Tr-TCC was then further shown by the recovery of IgG4 production in cultures of non Tr-TCC and B cells and Tr-TCC in the upper chamber (Fig 3.12 *bar 5*). Thus, it appears that factors released from the Tr-TCC such as cytokines in association with cell contact mechanisms that can also be provided by non Tr-TCC were critical for the induction of IgG4. To substantiate this hypothesis section 3.1.4 shows the critical requirement of IL-10.

Most interestingly, the production of both IgG1 and 2 (Fig 3.12 A and B) induced by non Tr-TCC (in contact with the B cells) is slightly reduced by the addition of Tr-TCC in the upper compartment. Suggesting that these soluble factors secreted by Tr-TCC are also able to dampen IgG1 and 2 induction by non Tr-TCC. However this reduction was not statistically significant.

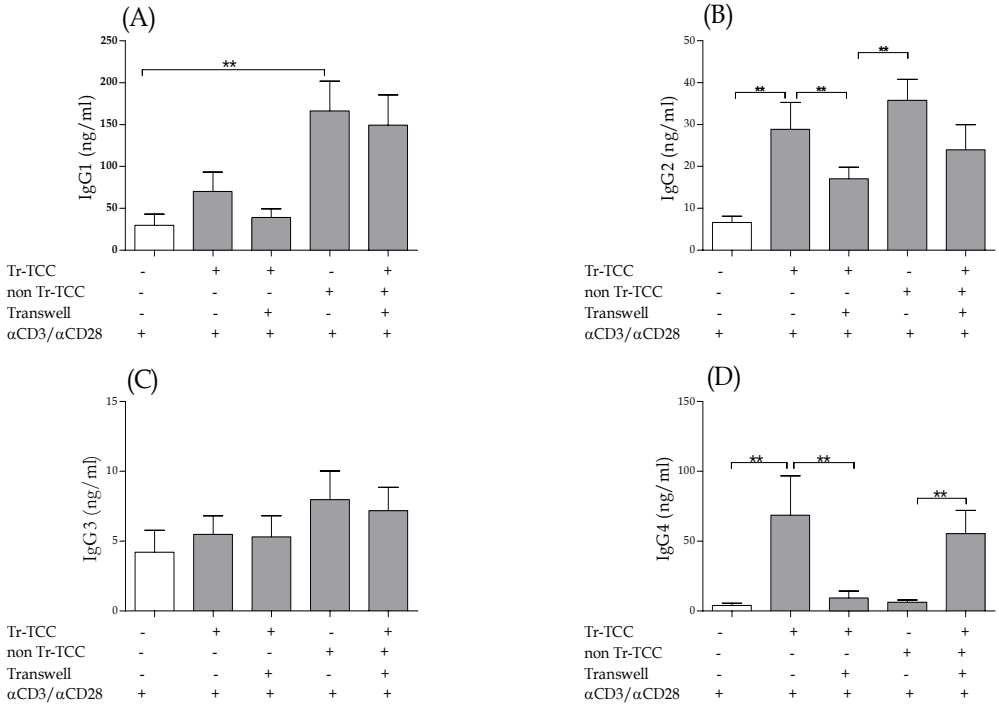

Figure 3.12: IgG4 induction by Tr-TCC is cell contact dependant.
$3x10^4$ $CD19^+$ B cells were co-cultivated with autologous Tr-TCC ($1x10^5$) or non Tr-TCC ($1x10^5$). Cultures were then stimulated with αCD3/αCD28 (10µg/ml/2.5µg/ml). Transwell systems were established using Nuncs tissue culture inserts containing 0.2µm nanopore membranes. B cells were cultivated alone or with non Tr-TCC in the lower compartment. Tr-TCC were added to the upper compartment. After 14 days of culture the concentration of IgG1-4 (A-D respectively) was measured per ELISA. Bars show means ± SD of IgGs in three independent experiments. Asterisks indicate significant differences between the groups indicated by brackets,$^{**}p < 0.01$.

3.2.3 IgG4 induction requires functional GITR-GITRL interaction.

From the above experiments it became apparent that surface molecules, in addition to soluble secretions released from activated Tr-TCC, played a key role in the production of IgG4. Since our generated T cell clones express high levels of GITR (Fig 3.4) and B cells are known to express the ligand of GITR (GITRL) on their surface[169], we hypothesized that this surface molecules may play a role during the induction of IgG4. To characterize this hypothetical role of GITR and GITRL, we used specific GITR or GITRL neutralizing antibodies in the T-B co-culture experiments.

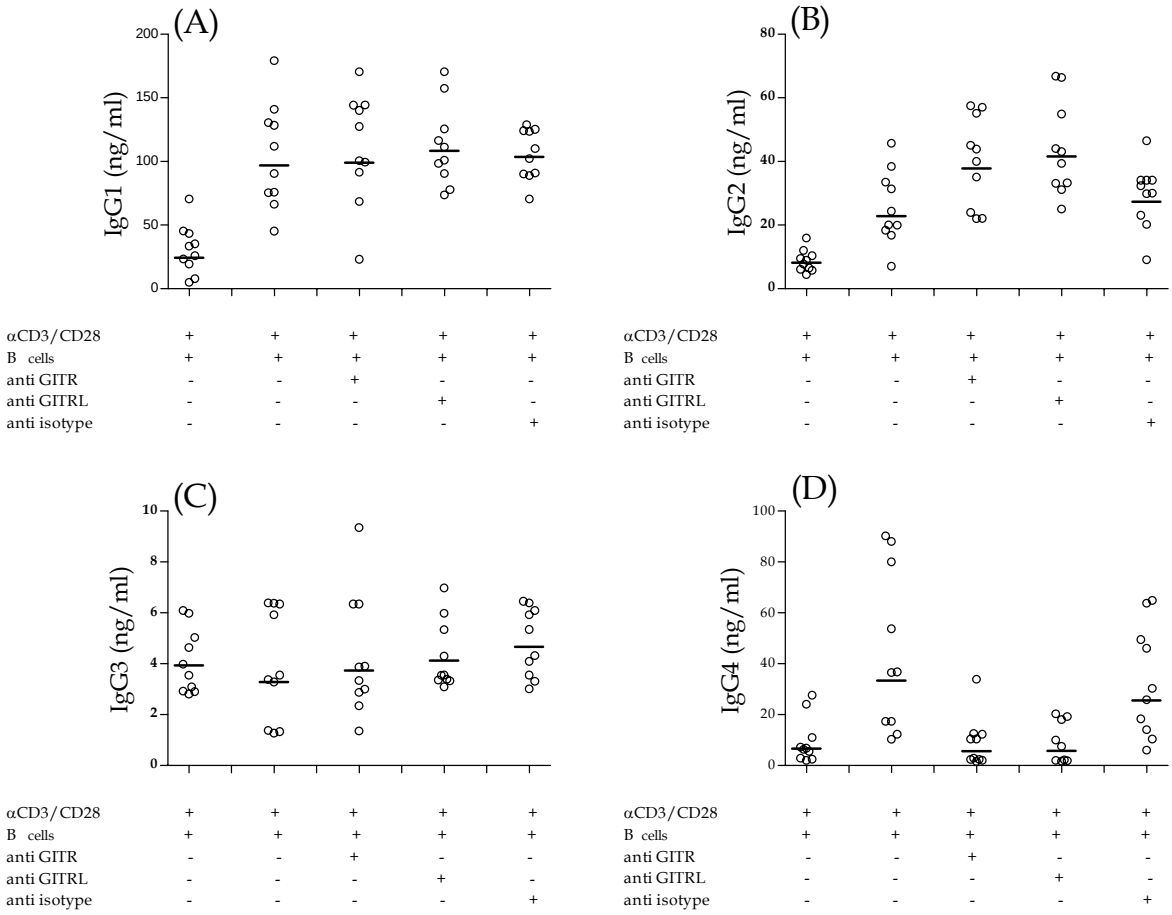

Figure 3.13: GITR-GITRL interactions control the ability of Tr cells to induce B cells to preferentially secrete IgG4.
$1x10^5$ Tr-TCC were incubated with $3x10^4$ $CD19^+$ autologous B cells and stimulated with αCD3/αCD28 in presence or absence of neutralizing antibodies against GITR, GITRL or isotype controls. After 14 days the culture supernatant was analysed for the production of IgG subtypes using ELISA. Scatter plots represent levels of IgG induced by 10 different O.v and TT specific Tr-TCC.

Once again co-cultures of T cells and B cells could not elicit production of IgG3 above baseline values (B cells alone) and these levels were not particularly affected by the addition of anti-GITR or anti-GITRL (Fig 3.13 C). Data from these experiments also revealed that blocking GITR or it ligand in the co-cultures significantly increased IgG2 production by B cells but did not affect IgG1 production (Fig 3.13 B and A respectively). Interestingly, using blocking antibodies to GITR or its ligand completely inhibited the IgG4 production by B cells (Fig 3.13 D), this effect was not seen with the isotype control.

3.2.4 THE RELEVANCE OF OTHER MOLECULES EXPRESSED ON TR-TCC

To investigate the role of other molecules expressed by Tr-TCC such as IL-10, TGF-β, CTLA-4 and ICOS, we performed additional *in vitro* co-culture assays. In brief, Tr-TCC and B cells were stimulated with αCD3/αCD28 in the presence or absence of neutralizing antibodies to the above mentioned molecules. IgG4 levels in the culture supernatant were once again measured after 14 days.

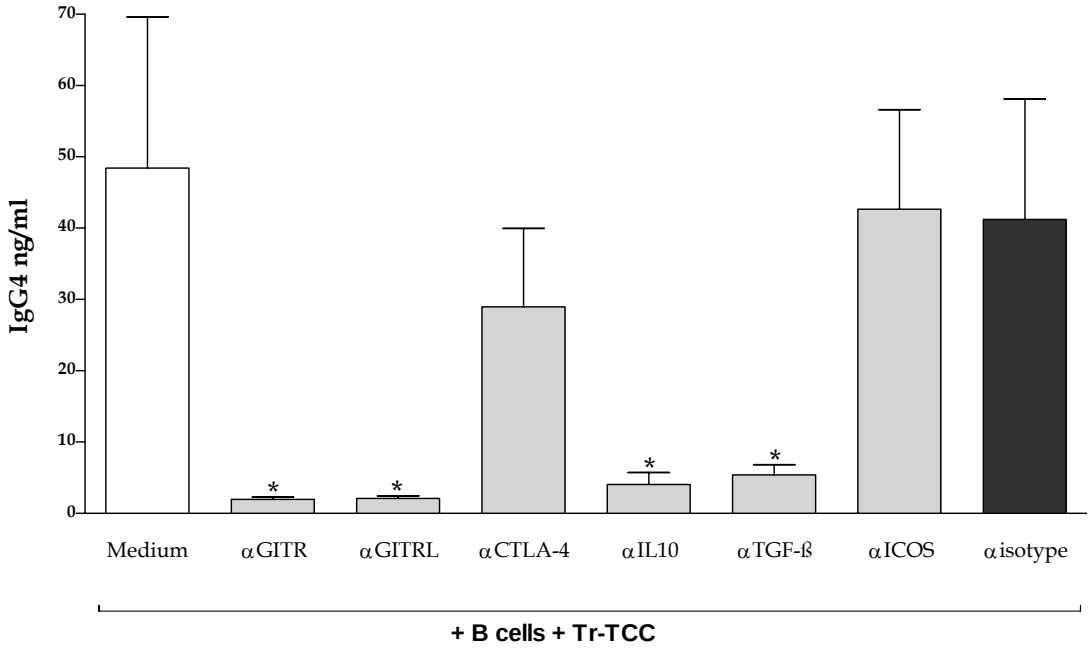

Figure 3.14: IL-10 and TGF-β are also vital components in the IgG4 inductive capacity of Tr-TCC.
$1x10^5$ Tr-TCC were incubated with $3x10^4$ $CD19^+$ autologous B cells and stimulated with αCD3/αCD28 (10µg/2.5µg/ml) in the presence or absence of neutralizing antibodies against GITR (2µg/ml), GITRL (15µg/ml) CTLA-4 (25µg/ml), IL-10 (10µg/ml). TGF-β (10µg/ml), ICOS (5µg/ml) or isotype controls. After 14 days the culture supernatant was analysed for the production of IgG4 using ELISA. Bars represent mean ± SD of three independent experiments using a total of 3 Tr-TCC. Asterisks indicate significant differences between the indicated bars and the isotype control, $*p < 0.05$.

Data presented in figure 3.14 clearly show that activated Tr-TCC can induce B cells to produce IgG4 and that this ability is completely abrogated by the addition of GITR or GITRL blocking antibodies (c.f. bars 1 to 2 and 3). Upon testing other regulatory T cell molecules the results also reveal a necessity for IL-10 and TGF-β aswell since blocking these cytokines also reduced IgG4 levels (c.f. bars 1 to 5 and 6). Interestingly, and despite its high constitutive expression of this molecule on Tr-TCC (Fig 3.4), the addition of blocking antibodies to CTLA-4 had only minor effects on the induction of IgG4 (bar 4). Antibodies against ICOS seem to not affect the ability of Tr-TCC to induce IgG4 at all. Since blocking CTLA-4 showed some influence on the ability of Tr-TCC to induce IgG4 production by B cells, we performed additional dose response assays using the neutralising antibodies to both ICOS and CTLA-4. Figure 3.15 demonstrates that high levels of antibody in the culture wells did not significantly affect the capacity or Tr-TCC to induce IgG4. In contrast, whereas only partial effects could be seen with 25µg/ml of anti-CTLA-4, complete abrogation of IgG4 production could be achieved with just 2µg/ml of anti-GITR antibody.

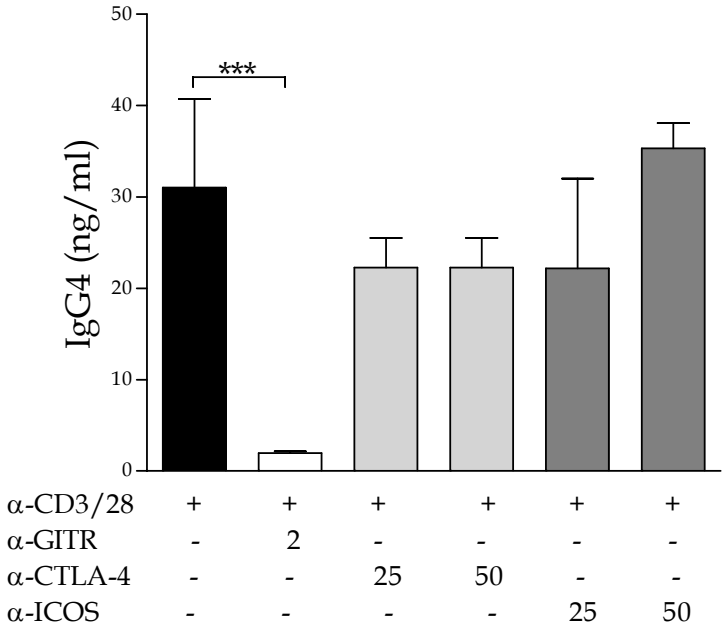

Figure 3.15: Increasing anti-ICOS and anti-CTLA-4 antibodies did not affect the ability of Tr-TCC to induce IgG4
$1x10^5$ Tr-TCC were incubated with $3x10^4$ $CD19^+$ autologous B cells and stimulated with αCD3/αCD28 (10/2.5µg/ml) in the presence or absence of anti-GITR (2µg/ml) or increasing concentrations of neutralizing antibodies against CTLA-4 or ICOS (25 and 50 µg/ml). After 14 days the culture supernatant was analysed for the production of IgG4 using ELISA. Bars represent mean ± SD of three independent experiments using a total of 3 Tr-TCC. Asterisks indicate significant differences between the groups indicated by brackets,***$p< 0.001$.

3.2.5 CONNECTIONS BETWEEN IL-10, TGF-β AND GITR IN THE INDUCTION OF IGG4

To further characterize the mechanisms that lead to the expression of IgG4 by B cells in the presence of Tr-TCC, we co-cultured Tr-TCC and autologous B cells with anti-GITR antibody in the presence or absence of recombinant IL-10 or TGF-β. As expected, anti-GITR monoclonal antibody was able to inhibit the production of IgG4 (Fig 3.16 c.f. bars 1 and 2). Interestingly this IgG4 production could be recovered by recombinant IL-10 (Fig 3.16 bars 3). Surprisingly, recombinant TGF-β could not release the inhibition of IgG4 by anti-GITR (bar 4) and rIL-10 failed to recover IgG4 in the presence of αTGF-β (bar 5). These results indicate that GITR/GITRL interaction modulates IgG4 induction using an IL-10-dependent pathway that leads to preferential production of IgG4 by Tr-TCC. Furthermore, since intracellular and membrane-bound TGF-β could be measured on Tr-TCC (data not shown) we also conclude that endogenous TGF-β expression is essential for the induction of IgG4 (Fig 3.16 *cf.* bars 5). This might explain why anti-TGF-β prohibited the induction of IgG4 despite the presence of rIL-10 but exogenous rTGF-β failed to increase IgG4 (Fig 3.16 *cf.* bars 5 and 6).

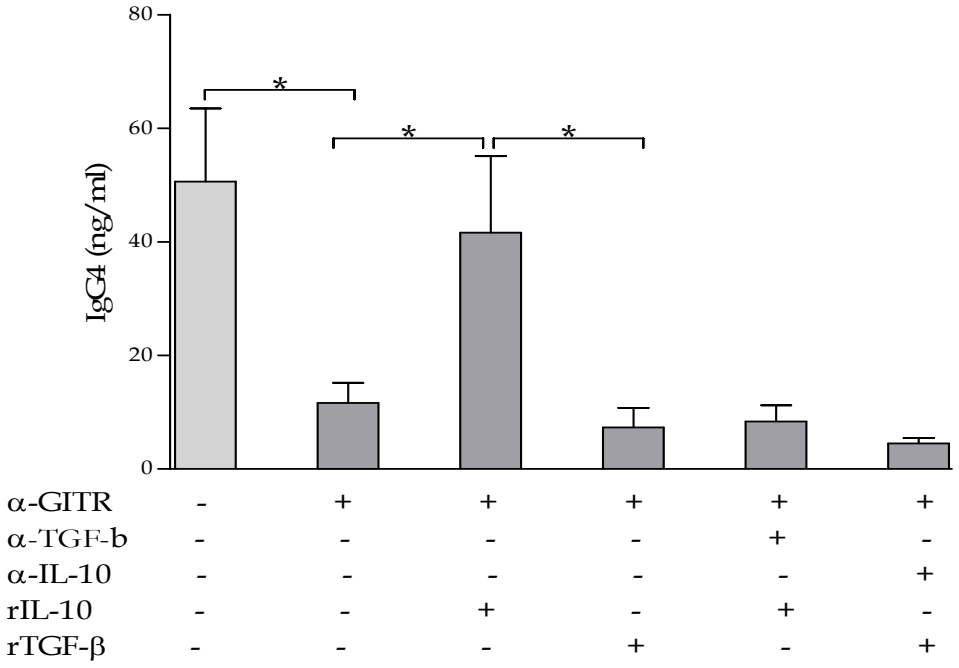

Figure 3.16: Recombinant IL-10 but not TGF-β recovers IgG4 production after GITR blockade.
$1x10^5$ Tr-TCC were incubated with $3x10^4$ $CD19^+$ autologous B cells and stimulated with αCD3/αCD28 (10/2.5µg/ml) in presence or absence of neutralizing antibodies against GITR, TGF-β or IL-10 and in combination with recombinant IL-10 or TGF-β. After 14 days the culture supernatant was analysed for the production of IgG4 using ELISA. Bars represent mean ± SD of three independent experiments using a total of 6 Tr-TCC. Asterisks indicate significant differences between the groups indicated by brackets,*$p< 0.05$.

3.2.6 GITR or GITRL interactions positively control the expression of IL-10 by Tr-TCC

The data obtained in section 3.2.5 strongly suggested that IgG4 production by B cells upon regulatory T cells activation was dependent on GITR/GITRL interactions which were in turn dependent on IL-10. Using flow cytometric analysis to further characterize the relationship between IL-10 and GITR/GITRL signalling, we measured the percentage of IL-10 producing Tr-TCC after co-culture with B cells in the presence or absence of anti-GITR antibody. In unstimulated controls, resting Tr-TCC produce only nominal amounts of IL-10 (Fig 3.17 A). Upon activation there is a strong release of this cytokine figure (3.17 B). The number of IL-10 producing Tr-TCC was significantly decreased in the presence of either anti-GITR or anti-GITRL antibodies (Fig 3.17 C and D respectively) suggesting that GITR/GITRL interactions induces IL-10 release by Tr-TCC. This phenomenon was only detected with the cytokine IL-10 since levels of TGF-β were not altered in the presence of blocking antibodies against GITR (data not shown).

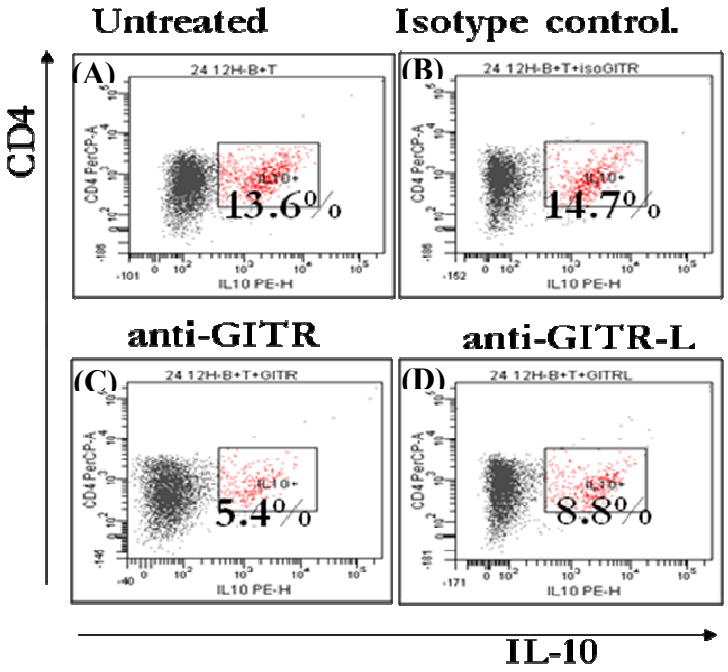

Figure 3.17: αGITR and αGITRL antibodies inhibit IL-10 production from Tr-TCC.
Tr-TCC were co-cultured with $CD19^+$ B cells ($1x10^5$) and stimulated with αCD3/αCD28 (10µg/2.5µg/ml) either alone or in the presence of α-GITR (1-2 µg/ml) or α-GITRL (15µg/ml) for 36 hours. Thereafter, cells were stained with PerCP-labelled CD4 antibody and intracellularly stained for IL-10 (A-D). Cytokine levels were measured using flow cytometry and depicted images represent data found with 4-6 clones.

Since non-Tr-TCC do not produce IL-10, similar experiments were performed as described above but instead of measuring IL-10, we measured IFN-γ instead. In these experiments, levels of this Th1 cytokine, in the presence of either blocking antibody were not altered showing that the GITR/GITRL interaction was only affecting the Tr-TCC (data not shown).

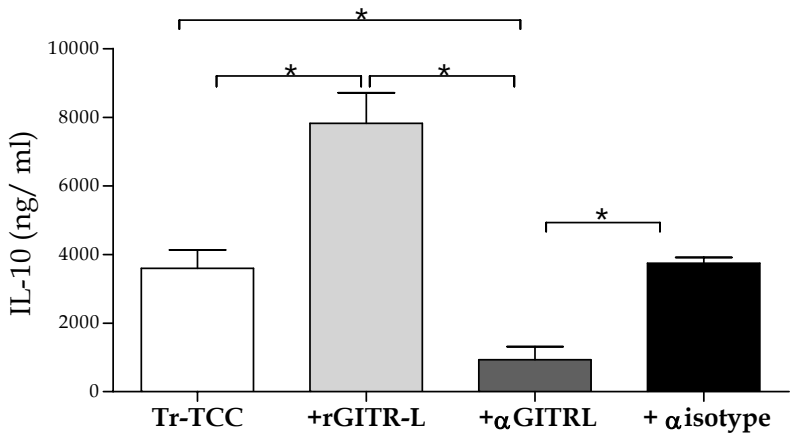

Figure 3.18: GITR ligation by its natural ligand (GITRL) increases IL-10 secretion by Tr-TCC.
Tr-TCC ($2x10^5$) were activated with 10μg/ml αCD3 and cultured in the presence of recombinant GITRL (20μg/ml) or neutralizing antibodies against GITR (αGITR) or control isotype. The production of IL-10 in culture supernatants was then measured after 72 hours incubation at 37°C using ELISA. Bars represent mean ± SD of three independent experiments using a total of 3 Tr-TCC. Asterisks indicate significant differences between the groups indicated by brackets,*$p< 0.05$.

To show a direct connection between GITR/GITRL interaction and IL-10 up-regulation in Tr-TCC further experiments were performed using Tr-TCC alone (Fig 3.18). Here, IL-10 levels were analysed in the culture supernatants of activated Tr-TCC in the presence of recombinant GITRL. As can be seen from the data, stimulation of GITR by its natural ligand GITRL instigates the production of IL-10 by these cells and in association with the other data presented in figures 3.14, 3.17 and 3.18, clearly indicates that the GITR signalling pathway and IL-10 are necessary components for the induction of IgG4 by B cells. In further assays, we then investigated the action of different concentrations of recombinant GITR or GITRL on the capacity of Tr-TCC to induce IgG4.

The data in figure 3.19 suggest a dose dependant increase of IgG4 production by recombinant GITR and/or GITRL with an optimum at 40μg/ml and 20 μg/ml respectively. The addition of either rGITR or rGITRL, led to an increase in IgG4 production with extra GITRL having the more drastic effect on IgG4 production. Interestingly, addition of recombinant proteins over

30ng/ml actually resulted in an inhibition of IgG4 production: the reason for this remains unclear from the present data. Nevertheless, these results do confirm the important role of GITR/GITRL in the specific regulation of IgG4.

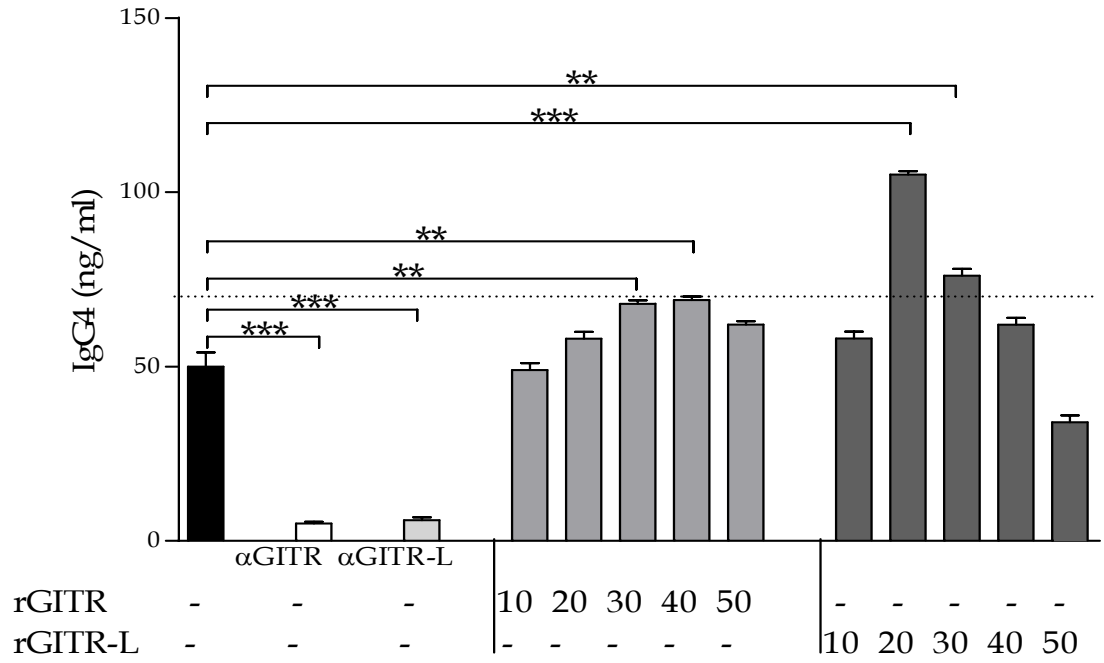

Figure 3.19: A dose-dependant IgG4 regulation by rGITR and rGITRL.
$1x10^5$ Tr-TCC were incubated with $3x10^4$ $CD19^+$ autologous B cells and stimulated with αCD3/αCD28 (10/2.5µg/ml) in presence of increasing concentrations of either rGITR or rGITRL for 14 days. Thereafter, levels of IgG4 were measured by ELISA. The dotted line indicates IgG4 levels without recombinant proteins. Bars represent mean ± SD of IgG4 levels found in three independent experiments using 3-5 Tr-TCC. Asterisks indicate significant differences between the group indicated by the brackets, $**p<0.01$; $*** p<0.001$.

Thus we demonstrate that GITR and GITRL signalling together with IL-10 are tightly associated with the ability of Tr-TCC to induce autologous B cells to preferentially produce IgG4. However, it remains unclear whether GITR molecules on the Tr-TCC (Fig 3.5) directly activated its ligand on the B cells to induce IgG4, or whether GITRL on the B cells stimulates its receptor on T cells to increase IL-10 (as shown in figures 3.17, 3.18 and 3.19). The present data do not rule out the possibility that other factors may also be part of the induction of the IgG4 production by B cells or class switching of B cells to produce this immunoglobulin.

3.2.7 NO ROLE FOR A DIRECT REVERSE SIGNALLING THROUGH GITRL DURING IGG4 INDUCTION

It is hypothesized that regulatory T cells can inhibit the activation, differentiation and survival of pathogenic T cells through bidirectional communication (reverse signalling) between members of the TNF family on T lymphocytes and DC. This pathway is regulated through tryptophan catabolism which is initiated by the enzyme indoleamine 2,3-dioxygenase (IDO) and can be influenced by reverse signalling through GITRL. Since B cells are also APCs expressing GITRL and secrete IgG4, we postulated that a role for reverse signalling could be attributed through GITRL in the production of IgG4. To investigate this putative function of GITRL we established an *in vitro* system in which B cells, without T cells, were stimulated with CD40L/IgM and additional components believed to be implicated in the induction (rIL-10, rTGF-β) of IgG4 such as GITR, IL-10 and TGF-β). The results in figure 3.20 show that IgG4 production was not significant in presence of rGITR, IL-10 and TGF-β indicating that the IgG4 induction is intimately associated with the presence of T cells, at least in our *in vitro* system.

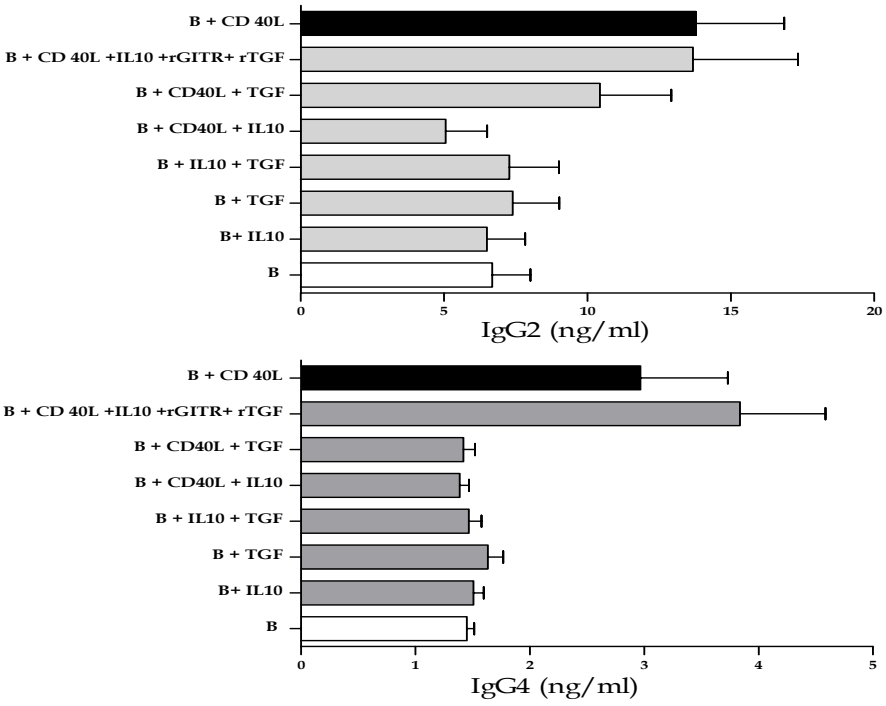

Figure 3.20: No role for reverse signalling through GITRL during IgG4 induction
B cells ($1x10^5$) were activated with 5µg/ml CD40L/IgM and rGITR, TGF-β, IL-10, have been added alone or in combination to the B cells. The cells were incubated at 37°C for 14 days and thereafter levels of IgG subclasses were then measured in the supernatants by ELISA. Bars represent means ± SD of IgG2 and IgG4 concentration in the supernatants from B cell cultures from 6 different donors.

3.2.8 IMPLICATION OF FOXP3 IN IGG4 EXPRESSION

As mentioned above (section 3.1), Tr-TCC were generated after multiple rounds of stimulation with TT-Ag/DD3 and a cloning procedure. Since the transcription factor Foxp3 was expressed by our generated Tr-TCC we tried to evaluate its functional relevance for these regulatory T cells, especially in the induction of IgG4. In addition, we were interested to know both the phenotype and functional activities of the TCL during their generation. In order to do this, we measured the levels of Foxp3 within the TCL after each round of stimulation and compared this to the levels of IgG subclass these cells could induce in our *in vitro* culture assays. Figure 3.21A-D shows the capacity of non-Tr-TCL (TT) and Tr-TCC (TT/DD3) in inducing IgG subclasses in autologous B cells. Interestingly, the induced IgG subclass altered after each round of stimulation and the subclass was dependent upon the stimulation type. In short, cell lines stimulated with TT alone induced increasing amounts of IgG3 (Fig. 3.21B) whereas those subjected to TT and DD3 predominantly induced IgG4 instead (Fig. 3.21D). Interestingly, the high levels of IgG1 are significantly reduced following the rounds of stimulation with TT/DD3 showing a reduction of this subclass as the cells become more specific for inducing IgG2 or 4. We also found that IgG4 was subjected to a similar modulation by GITR, IL-10 and TGF-β since production was significantly reduced when blocking Abs were applied (data not shown). Foxp3 expression within the re-stimulated Tr-TCL cultures (Fig. 3.21E) was also enhanced with increasing rounds of TT-Ag/DD3 stimulation and correlated with the induced levels of IgG2 and IgG4 (Fig. 3.21C and D). Interestingly, the phenotype of Foxp3 expressing Tr-TCL significantly correlated with the amount of IgG4 (Fig. 3.21F, R=0.98770) induced in T:B-cell cultures. In correlation, IgG1 levels decreased (Fig. 3.21A) with increasing Foxp3 expression in the Tr-TCL. These results show that upon continual activation in an Ag-specific manner, Tr-TCL up-regulated Foxp3 expression indicating a directed functional response.

Nevertheless, although there was a definite correlation between the levels of Foxp3 in the TCL and their increasing ability to induce IgG4 production in B cell co-culture assays, this did not confirm a functional role of Foxp3 in this process.

To investigate whether the expression of Foxp3 does play a direct role in the ability of Tr-TCC to produce IgG4, we used the technique of siRNA nucleofection to silence Foxp3 at the mRNA level in the Tr-TCC. The following sections describe the technical procedure and the results.

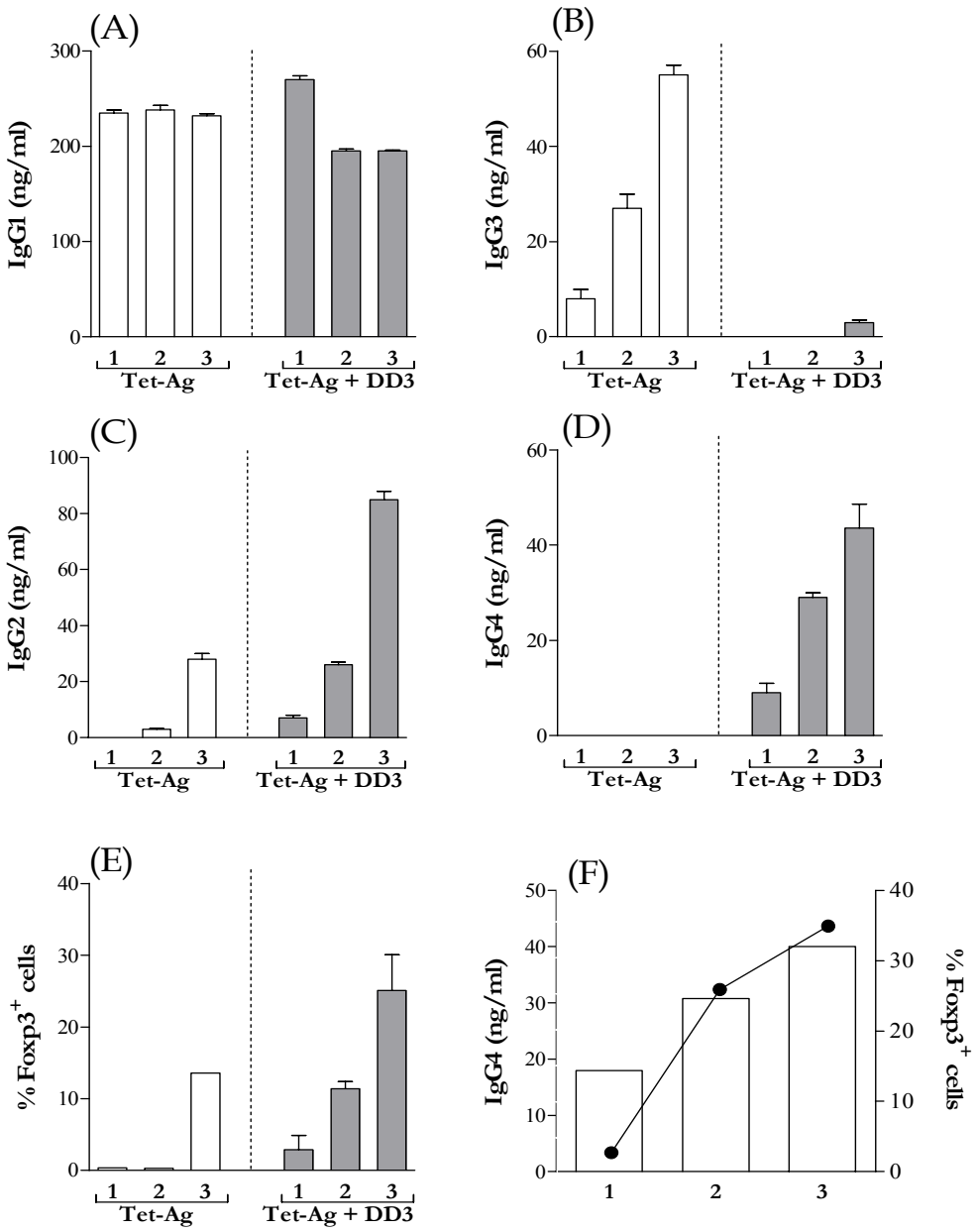

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Figure 3.21: Regulatory T cell lines (Tr-TCL) express increasing levels of Foxp3, which correlates with the amount of induced IgG4

After each round of stimulation (TT-Ag alone or TT-Ag/DD3), TCL ($1x10^5$) were activated with αCD3/αCD28 (10µg/2.5µg/ml) and assessed for their ability to induce (A) IgG1, (B) IgG3, (C) IgG2 and (D) IgG4 from co-cultured autologous $CD19^+$ B cells ($5x10^5$) after 14 days of culture. Bars represent mean ± SD of three pooled TCL generation experiments with 5-8 TT-Ag or TT-Ag/DD3 TCC per experiment. (E) The amount of $CD4^+Foxp3^+$ T cells within the two different TCL was also measured after each round of stimulation. In brief, TCL were activated with αCD3/αCD28 (10µg/2.5µg/ml) for 24 hours and measured for levels of intracellular Foxp3 using flow cytometric methods. Bars represent mean ± SD of Foxp3 levels in TT-Ag (18) vs. TT-Ag/DD3 (18) after each stimulation round. (F) Comparison of IgG4 levels induced by Tr-TCL after each round of stimulation (Fig. 5D) with the percentage of $Foxp3^+$ cells in the TT-Ag/DD3 generated TCL population (grey bars in E).

Selection of siRNA for the assay.

The first task for these experiments was to select from a pool of four Foxp3 specific siRNA provided by Qiagen the most suitable siRNA to continue our experiments. The silencing experiments were carried out using Tr-TCC that expressed significant levels of Foxp3 (section 2.4.5). To verify that nucleofection had occurred, three different controls were used: a control of non-treated Tr-TCC, these cells were simply cultivated in medium as normal, a control treated with siRNA with no specificity to a known gene, and third control with Tr-TCC that had been treated with siRNA specific to MAPK.

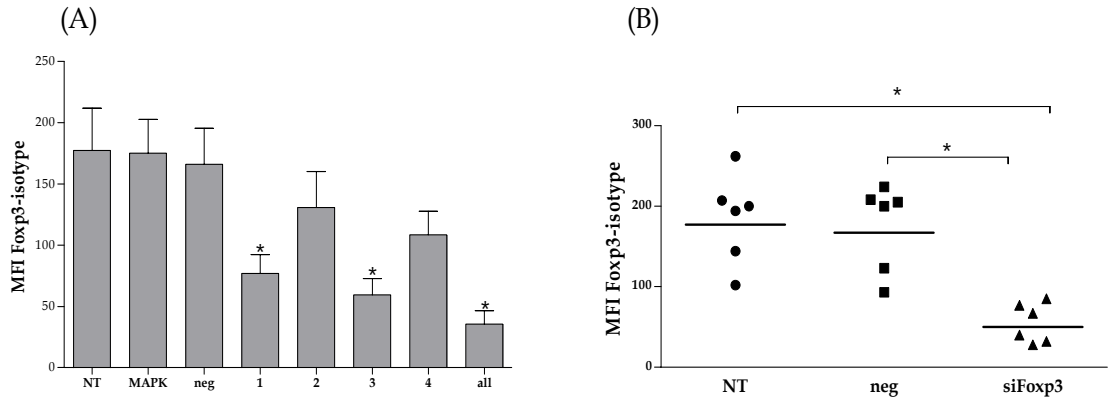

Figure 3.22: **Selection of Foxp3 specific siRNA for nucleofection studies**.
siRNA and transfection reagents from Qiagen were used to inhibit the expression of Foxp3 within Tr-TCC cultures growing in the presence of IL-2. (A) The expression of Foxp3 was assessed after 3 days. Bars represent the mean fluorescence intensities (MFI) of Foxp3-PE in the $CD4^+$ T cell population gates minus the MFIs in isotypes controls. The control NT (non-treated) represents the Foxp3 expression in cells without any treatment. Control MAPK and neg contl. represent the Foxp3 expression in cells treated siRNA targeting MAPK a non-specific siRNA respectively. siFoxp3-1 to 4 represents cells transfected with 4 different siRNA targeting Foxp3 mRNA. "all" shows the levels of Foxp3 expression after T cells were treated with a combination of all 4 siRNA. These data are representative of 3 independent experiments. (B) Suppression of Foxp3 using specific siRNA in 6 independent assays using active siRNA. Asterisks indicate significant differences between the indicated groups and the corresponding controls,* $p<0.05$.

Figure 3.22 shows the ability of the 4 different Foxp3 specific siRNA to inhibit Foxp3 in Tr-TCC. As one can see, the transfection procedure using the non-specific siRNA (neg ctlr) and transfection with MAPK (control MAPK) did not alter the levels of measurable Foxp3 in the cells: compare to levels of Foxp3 found in cultured Tr-TCC (control NT). In contrast, levels of Foxp3 in the cells were reduced after nucleofection with the specific siRNA to Foxp3 (Fig 3.22 bars 3-6). Some siRNA were better than others, for example, a more significant decrease in

Foxp3 was observed with siRNA 1 and 3 but the best reduction was achieved using all four siRNA together (Fig 3.22 bar 8).

However, although Foxp3 levels could be consistently reduced using siRNA, the same levels of reduction were not always highly reproducible using the same single siRNA. For example, our designated siRNA1 significantly suppressed levels of Foxp3 in 2 of 3 assays, whereas siRNA2 worked only once in two assays. This may be due to the instability of siRNA during the experiments or dependent on the levels of Foxp3 and thus the cell cycle of the growing Tr-TCC at the time of transfection. Since the combination of all siRNA demonstrated a more consistant inhibition, we used all four siRNA for the following described experiments. Finally, levels of TNF-α were measured in the culture supernatant of these above described assays after 24 hours. The principle of this task was to identify whether there were fundamental changes to the way in which the transfected cells behaved upon stimulation. However, some minor differences in TNF-production could be observed between the control untreated and the siRNA treated cells; indicating the existence of possible target off effects induced by the siRNA treatment (data not shown.

Foxp3, cytokines and regulatory properties

As mentioned above in section 3.1.2, the Tr-TCC generated in this work were selected on their ability to secrete high levels of IL-10. Following siRNA transfection, individual clones with specific and stable down regulation of Foxp3 expression were tested for their cytokine expression. Figure 3.23 shows the percentage of Foxp3 present within the cells at the onset of the experiment. Levels of Foxp3 in non-transfected (NT) cells were 68% and this value correlated to the cells transfected with non-specific siRNA (control si) and the cells transfected with MAPK (data not shown). However, when all siRNA (siFoxp3) were used levels of Foxp3 were dramatically reduced. Interestingly, in this experiment, siRNA nucleofection with siRNAFoxp3-1 and 2 did not alter the levels of Foxp3 in the cells whereas siRNAFoxp3-3 and 4 reduced Foxp3 levels by 40 and 20% respectively (data not shown). This correlates to the data described in the previous section which demonstrated that using all siRNA to Foxp3 produced the most consistent results. Following assessment of Foxp3 levels, nucleofected cells were re-stimulated with antiCD3/CD28 and their cytokine profiles were determined three days later. Figure 3.23 shows a representative experiment of cytokine levels in siRNA transfected cells. Surprisingly, successfully Foxp3-silenced Tr-TCC showed no reduction in their production of IL-10. Levels of

IFN-γ however were elevated. For example, levels of IFN-γ in non-transfected cells and non-specific siRNA were approximately equal. Whereas those in Foxp3 silenced cells were dramatically increased. TGF-β was also measured in these assays but not detectable levels could be observed (data not shown).

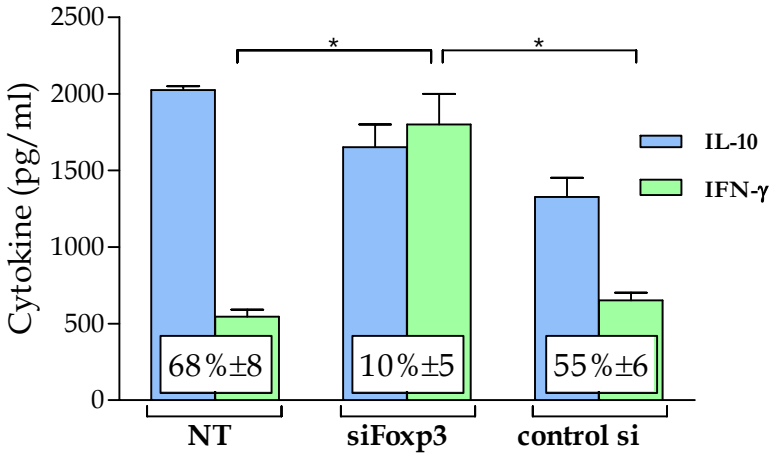

Figure 3.23: Foxp3 inhibition does not affect IL-10 production by transfected Tr-TCC.
Tr-TCC ($1x10^5$) were left either non-transfected (NT), or transfected with non-specific siRNA (control si), or Foxp3 specific siRNA (siFoxp3). After 3 days, levels of IL-10 (blue bars) or IFN-γ (green bars) were measured in the supernatant by ELISA. Data represents one of three independent experiments. Numbers in white boxes represent the percentage of $Foxp3^+$ T cells in Tr-TCC used in each experiment. Asterisks indicate significant differences between the groups indicated by the brackets,* $p<0.05$.

Due to the elevated status of IFN-γ in the siRNA Foxp3 Tr-TCC, we also tested whether these cells had lost their suppressive properties on effector T cells. Figure 3.24 shows that whereas non-transfected clones were able to reduce the proliferation of reporter T cells (*c.f.* bars 1 and 4), Tr-TCC with silenced Foxp3 failed to do so (*c.f.* bars 1 and 2). To demonstrate that this was an effect of the silenced Foxp3 gene, we also tested Tr-TCC which had been transfected with the non-specific siRNA, here Tr-TCC were still able to suppress effector T cells proliferation (bar 3). These results, suggest that the balance between pro-inflammatory cytokines (IFN-γ) and anti-inflammatory cytokines (IL-10) may play a role in the ability of Tr-TCC to suppress the proliferation of autologous non Tr-TCC and moreover, that this balance could be controlled by functional Foxp3.

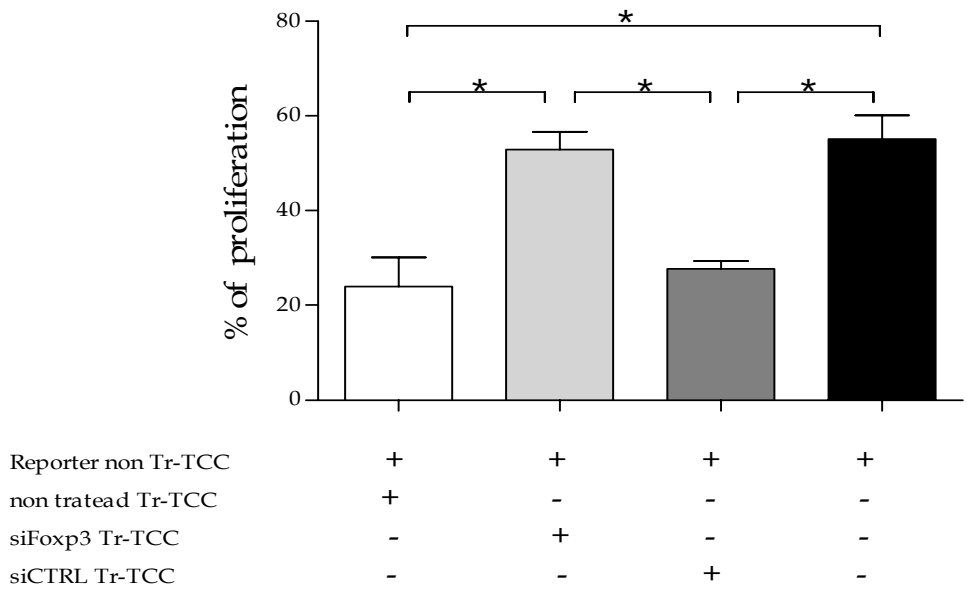

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Figure 3.24: si Foxp3 transfected Tr-TCC are no longer able to suppress non Tr-TCC proliferation.
$1x10^4$ Tr-TCC or siRNA-or control-transfected Tr-TCC were cultured in presence of $1x10^5$ CFSE labelled autologous non Tr-TCC. Cells were then stimulated with αCD3/CD28 expansion beads (Dynal). The dilution of CFSE was assessed by flow cytometry 3 days after stimulation. Percentage of proliferation was calculated according to the proliferation in the reporter cells alone stimulated with anti-CD3/CD28. Bars represent means ±SD of 3 independent experiments.

IgGs induction by Foxp3 silenced Tr-TCC

In the final set of experiments, we tested whether Tr-TCC with silenced Foxp3 could still induce B cells to preferentially produce IgG4. Using the same experimental setup as described for the other *in vitro* co-culture assays (section 2.3.7) we incubated $CD19^+$ B cells with the different transfected Tr-TCC for 14 days. Thereafter levels of IgG subclasses were measured in the supernatant. Figure 3.25 shows the data obtained for all four IgG subclasses and one can immediately see that Tr-TCC with silenced Foxp3 were no longer able to induce IgG4 production (Fig 3.25D). This finding correlates to their changes in cytokine profile (Fig 3.23) and lack of suppressive activity on effector T cells (Fig 3.24). Interestingly, instead of inducing IgG4, these Foxp3-silenced T cells induced IgG2 (Fig 3.25B). There were no alterations in the pattern of IgG1 and IgG3 (Fig 3.25 A and C respectively). Tr-TCC that had been transfected with control siRNA showed the same profile as those cells which were not transfected at all: preferential IgG4 production.

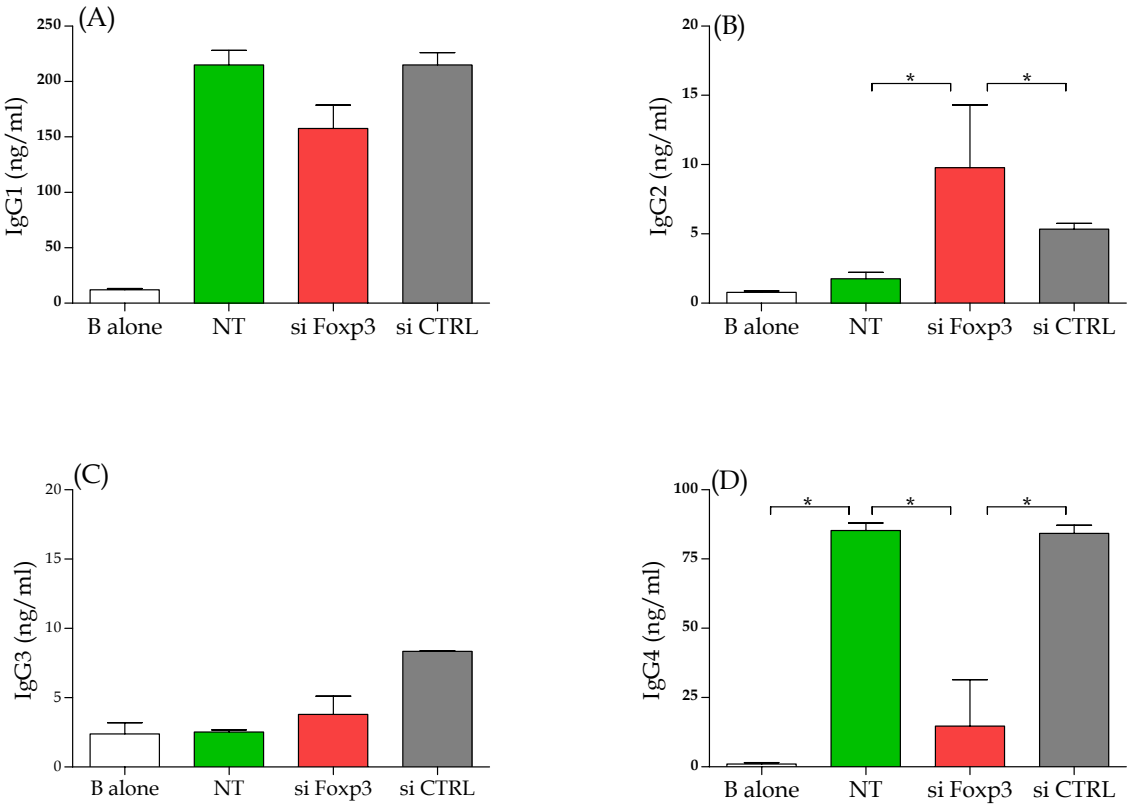

Figure 3.25: Blocking Foxp3 does not affect IgG1 but reduced IgG4 and simultaneously increases IgG2. $5x10^4$ $CD19^+$ MACS sorted B cells were co-cultured with $1x10^5$Foxp3-silenced anti-CD3 (10µg/ml) activated Tr-TCC: red bars(si Foxp3). As controls, Tr-TCC with functional Foxp3: green bars (NT) or a Tr-TCC that had undergone a transfection with a non-specific siRNA grey: bars (siCTRL) were also included. Levels of IgG subclasses were measured after 14 days of culture with ELISA. Bars represent the mean ± SD of each IgG subclass. Data are a representative of three independent experiments. Asterisks indicate significant differences between the indicated groups,** $p<0.01$.

3.3 Are natural occurring regulatory T cells also able to induce IgG4 expression by B cells?

3.3.1 Isolation and characterization of naturally occurring regulatory T cells

Since nTreg constitutively express Foxp3 and GITR, we assessed whether nTreg were also capable of inducing IgG4. In brief, nTreg ($CD4^+CD25^+$ T cells) were isolated via magnetic cell sorting from 6 healthy donors and placed in culture with appropriate stimulation and autologous B-cells. After 14 days, the levels of IgG2 and IgG4 were measured by ELISA. Interestingly, co-

cultures of nTreg and B cells enhanced IgG4 but not IgG2 (*c.f.* bar 3 on Figures 3.26A and B). Cultures were further subjected to anti-GITR or anti-IL-10 treatment. Similar to Tr-TCC (Fig 3.13), IgG4 induction by nTreg could also be reduced to background levels upon addition of αGITR or αIL-10.

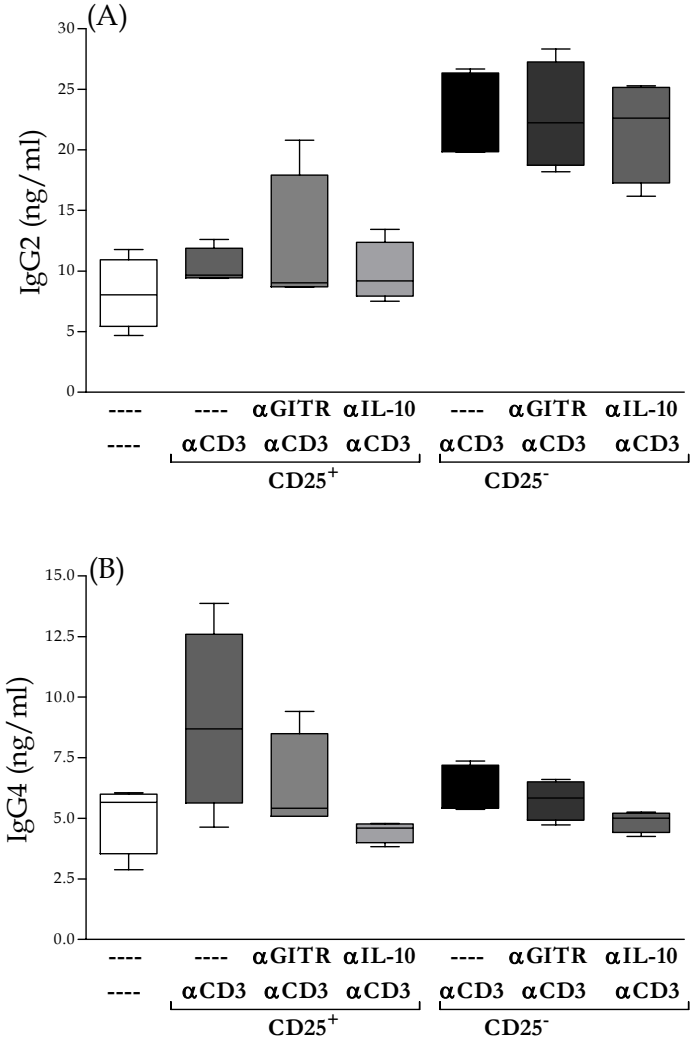

Figure 3.26*:* A role for GITR in the induction of IgG4 by nTreg.
nTreg ($CD4^+CD25^+$) or $CD4^+CD25^-$ T cells were isolated from 6 healthy individuals. $1x10^5$ nTreg or $CD4^+CD25^-$ T cells were co-cultured with autologous $CD19^+$ B-cells ($3\text{-}5x10^5$) and αCD3/αCD28 for 14 days. Thereafter, the levels of IgG2 (A) and IgG4 (B) were measured by ELISA. As depicted, cultures were also subjected to αGITR or αIL-10 antibody treatment. Bars represent the mean and SD of 6 independent results.

For comparison, populations of effector T cells ($CD4^+CD25^-$) were also tested for their ability to induce IgG2 or IgG4. $CD4^+CD25^-$ T cells were unable to induce IgG4 but could induce IgG2 and the latter IgG response was not affected by the addition of either αGITR or αIL-10. No differences in the levels of IgG1 or IgG3 could be observed (data not shown). In the same co-

culture system we compared the ability of $CD4^+CD25^+$ natural occurring regulatory T cells to elicit IgG4 production from autologous B cells with Tr-TCC (Fig 3.27). However, as clearly shown in figure 3.27, the amounts of IgG4 were much lower than those that we have observed with our high IL-10 producing Tr-TCC. Nevertheless, nTreg can also induce IgG4, and in addition preferentially to IgG2. Moreover, effector T cells (comparable to non-Tr-TCC) produced no IgG4 at all and were unaffected by the addition of blocking antibodies. This data also reflects the findings using siRNA in Tr-TCC since there, IgG4 production was abolished and Tr-TCC reverted to inducing IgG2 from B cells instead. Collectively, there is strong evidence from this work that IgG4 induction is specifically induced by IL-10 producing regulatory T cells through GITR/GITRL interactions and in addition these cells have overlapping characteristics with naturally occurring T cells.

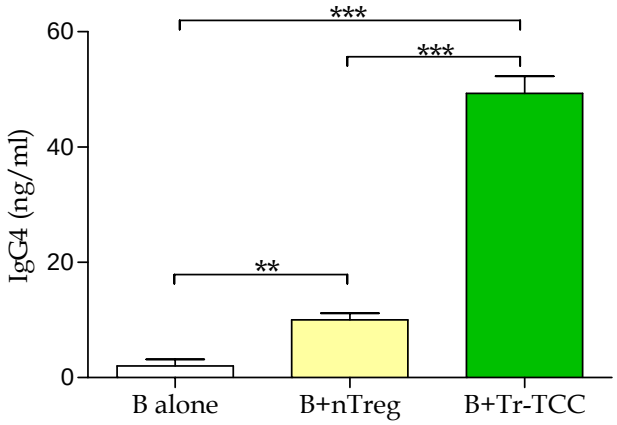

Figure 3.27: Naturally occurring Tregs induce IgG4 by B cells albeit weaker than Tr-TCC.
Comparison between naturally occurring Treg cells and Tr-TCC in their ability to induce IgG4. In brief, $1x10^5$ nTreg ($CD4^+CD25^+$) or Tr-TCC were incubated with autologous $CD19^+$ B-cells ($3\text{-}5x10^5$) and αCD3/αCD28 for 14 days. Thereafter, the levels of IgG4 were measured by ELISA. Bars represent the mean ± SD of 6 independent results. Asterisks indicate significant differences between the indicated groups $^{**}p<0.01$; $^{***}p<0.001$.

3.4 NAÏVE OR MEMORY B CELLS ARE PRODUCER OF IgG4?

Naïve B cells have been shown to induce the generation of regulatory T cells[32]. In our system, although we had an established IgG4 induction from B cells by activated Tr-TCC, it remained unclear whether this secretion originated from already differentiated memory B cells or whether Tr-TCC specifically induced the IgG4 switch in naïve B cells. Thus, to find out the B cell subpopulation responsible for the IgG4 production, we compared the IgG4 expression in co-culture of Tr-TCC and populations of B cells that had been separated according to their expression of the CD27 marker. CD27 is considered to be expressed on most memory B cells but

not on naïve populations. Using 6 individual donors, we found that the proportion of naïve ($CD27^-$) and memory ($CD27^+$) B cells within the total ($CD19^+$) B cell fraction was 61.2% ± 9.8% and 38.8% ± 9.8% respectively. Figure 3.28 below shows both the flow cytometry images of isolated B cells stained with anti-FITC-labelled CD27 antibody and the proportions of naïve and memory B cells from healthy donors.

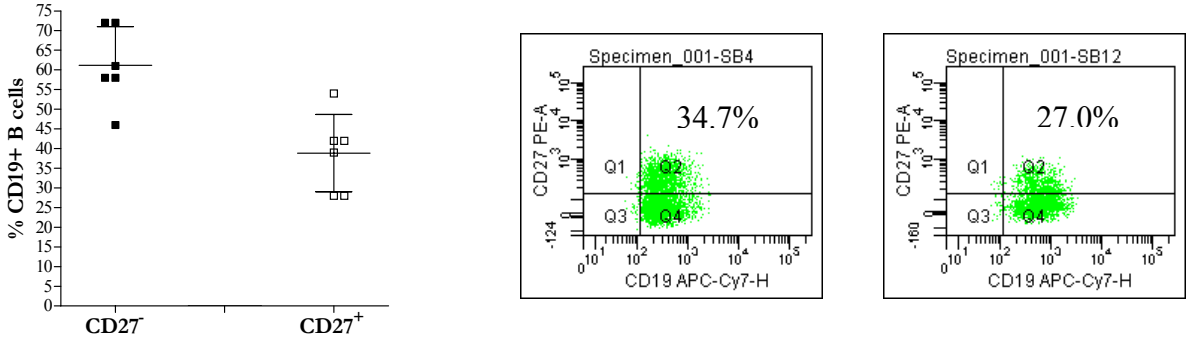

Figure 3.28 proportion of naïve and memory B cells in bulk B cells populations.
$CD19^+$ B cells were isolated microbeads and MACS technology. Thereafter, cells were stained with FITC-labelled anti-CD27 antibody. Naïve and memory B cell populations were then analyzed using FACS Diva Software.

After 14 days of co-culture with activated Tr-TCC, the preferential secretion of IgG-isotype by total, naïve or memory B cell fractions was analyzed. Here, we found that the most IgG4 is produced with all $CD19^+$ B cells and that naïve B cells produced slightly more IgG4 but much less IgG1 than memory B cells (Fig 3.29). Interestingly, upon comparison of IgG4/IgG1 ratio (Fig 3.29) we found that unlike memory cells, naïve B cells were comparable to the bulk cell population but it appears that both subsets are required for a complete response.

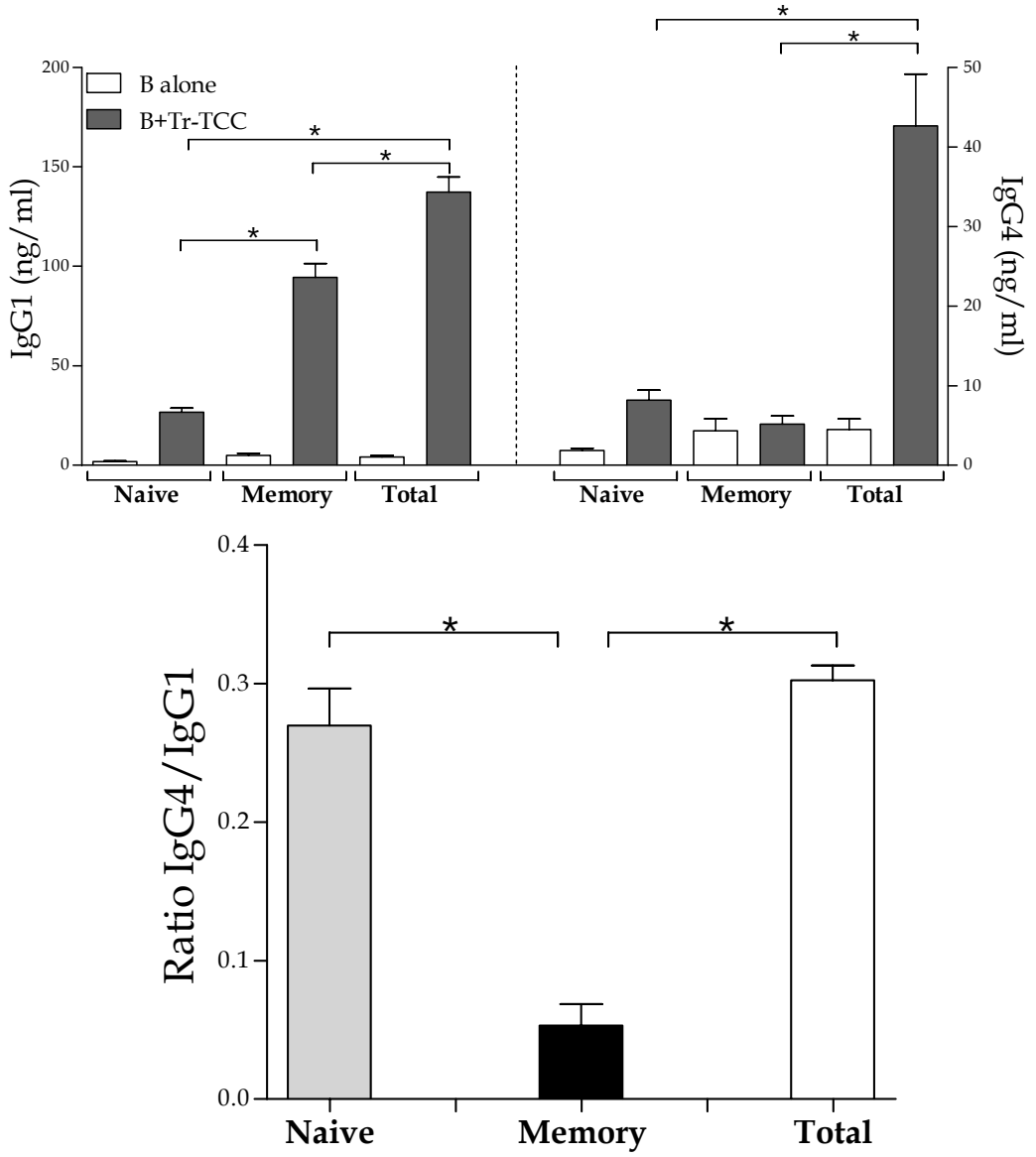

Figure 3.29 Tr-TCC induced IgG4 production by B cells requires both naïve and memory B cell populations. αCD3/αCD28 stimulated Tr-TCC ($1x10^5$) were cultured with either whole populations of autologous purified (> 98 %) $CD19^+$ B cells (total), or isolated subsets of naïve or memory ($CD27^+$) B cells. After 14 days of culture, levels of IgG subsets 1 and 4 were measured by ELISA. The ratio of IgG4/IgG1 was calculated by the amount of IgG subclass produced by the B cell subtypes. Bars represent the mean and SD of IgG subclass induction by B cells in two independent experiments using different Tr-TCC. Asterisks indicate significant differences between the indicated groups, *p<0.05.

3.5 ROLE FOR TLRS

TLR are known to initiate the innate immune response and to participate in the fine regulation of the adaptive immune response by influencing the maturation of antigen presenting cells. Also TLRs are known to be expressed on Treg and studies have shown that stimulation through the TLR can modulate their regulatory properties. However, since one member of this family of receptors, TLR2, has been shown to control expansion and function of regulatory T cells[170-172] we decided to investigate whether TLR stimulation affected the ability of our generated Tr-TCC to preferentially induce IgG4. Using our above described co-culture system (section 2.3.7) we primarily focused on two specific questions: does prior TLR stimulation on Tr-TCC affect their ability to induce IgG4 production in B cells and vice-versa, that is, does TLR stimulation of B cells over-ride the signals from Tr-TCC.

3.5.1 TR AND B CELLS EXPRESS TLRS

Before starting the co-culture experiments, we investigated the expression of different TLRs on Tr-TCC, B cells and for comparison on non-Tr-TCC control cells and natural-occurring Treg ($CD4^+CD25^+$ T cells). The data collected after FACS analysis showed that TLR are more prominently expressed on CD40L activated B cells (TLR2, 3, 4 and 9). In terms of regulatory T cells phenotypes, nTreg expressed high levels of TLR2 and TLR4 whereas Tr-TCC and Th1 cells display approximately the same TLR profiles (Fig 3.30).

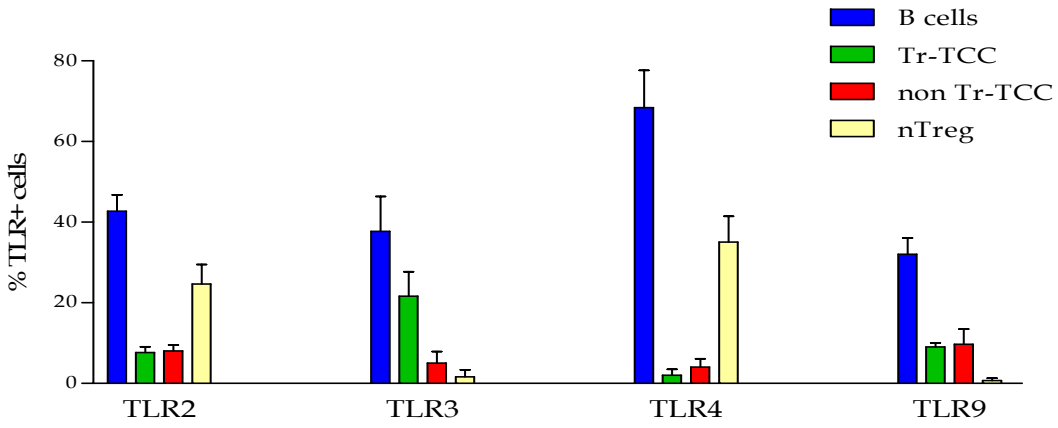

Figure 3.30 TLR expression on B cells, Tr-TCC, non Tr-TCC and nTreg
B cells, isolated nTreg, Tr-TCC and non Tr-TCC were analysed by FACS for their expression of TLR2, 3, 4 and 9 24 hours after stimulation with αCD3 (10µg/ml) and CD40L (5µg/ml). Bars represent the percentage of TLR^+ cells in two independent experiments using 5 different healthy PBMC preparations.

Surprisingly known intracellular TLRs like TLR3 were found to be expressed on Tr-TCC surface (Fig 3.30 and data not shown). However they were almost no intracellular TLRs expression in the intracellular compartments of all T cell types. In contrast, B cells displayed significant levels of intracellular TLR3 and TLR9 after permeabilization of the cellular membranes.

3.5.2 TLRs STIMULATION ON B AND T CELLS MODULATE IGGs EXPRESSION

Upon analysis of TLR distribution on the different cell populations we then evaluated the influence of TLR activation on the induction of IgG subclasses. In the first experiments, we cultured Tr-TCC with TLR stimulation for 5 hours, washed the cells and incubated them with B cells. No differences in expression patterns of IgG subclasses could be observed after 14 days of culture (data not shown).

In the second experiments we used the reverse approach. In short, B cells were pre-stimulated with LPS (TLR4), Pam3Cys (TLR2) or CpG (TLR9) for 24 hours. Thereafter, cells were washed and added to be cultured with activated Tr-TCC for a further 14 days. Levels of IgG1, IgG2, IgG3 and IgG4 were then analysed using ELISA (Fig 3.31). As expected and in correlation to the experiments described in the previous sections, activated Tr-TCC preferentially induced IgG4 in B cells. However, if the B cells had been prior stimulated with LPS or CpG, levels of this immunoglobulin were reduced to concentrations found with B cells alone. Interestingly, stimulation with the TLR2 activator Pam3Cys had no influence on the IgG4 production. Pam3Cys however, dramatically increased levels of IgG1 and IgG2 production and here, this stimulus correlated with the effects seen with CpG. LPS moderately up-regulated levels of IgG1 but appeared to have no effect on amounts of IgG2. In all of our previous experiments using this co-culture system, levels of IgG3 never exceeded background quantities. Surprisingly, addition of LPS or Pam3Cys to the B cell cultures strongly up-regulated this class of immunoglobulin. TLR9 ligation on the other hand failed to alter the induction pattern of IgG3.

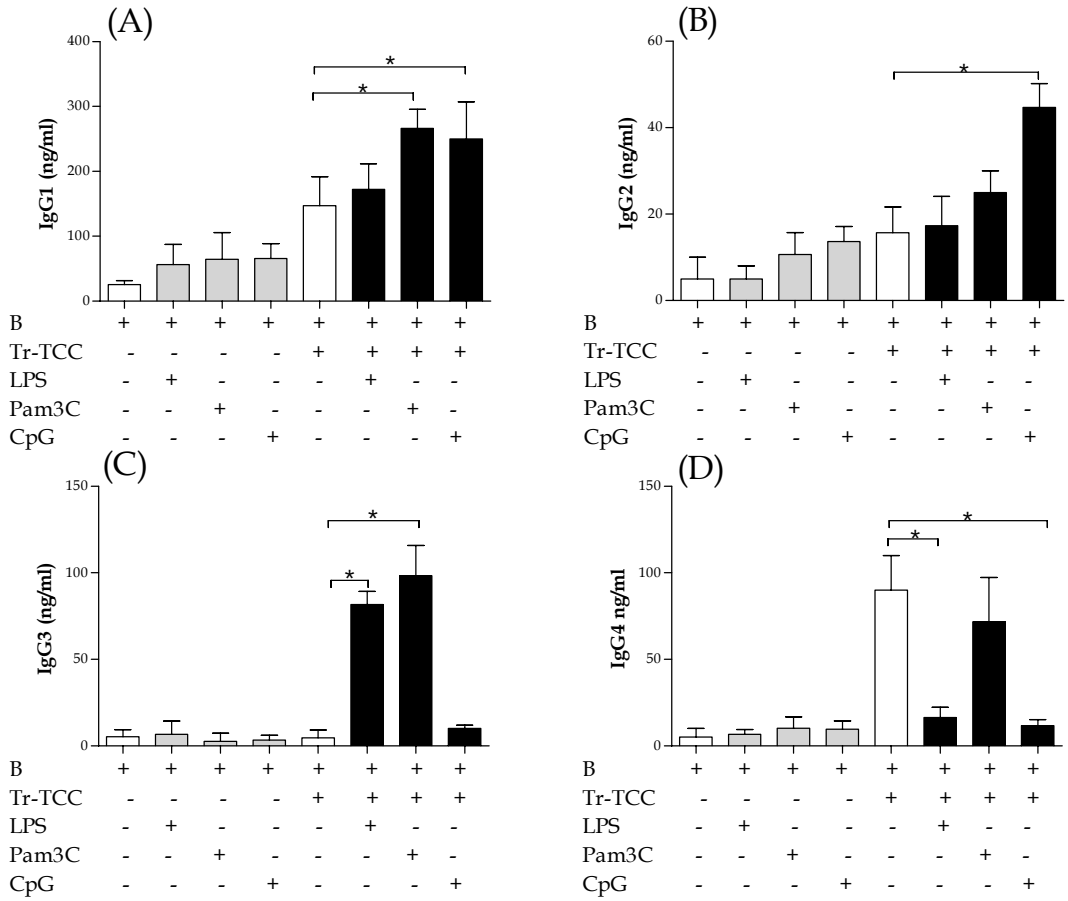

Figure 3.31: Effect of TLRL on IgGs production in T-B co-cultures.
B cells pre-stimulated with LPS (TLR4), Pam3Cys (TLR2) or CpG (TLR9) for 24 hours have been co-cultivated with αCD3/αCD28 activated Tr-TCC for 14 days as described previously. Thereafter, the levels of IgG1 (A), IgG2 (B), 3 (C) and 4 (D) were measured by ELISA. Bars represent the mean ± SD of 3 independent experiments. Asterisks indicate significant differences between the indicated groups *p<0.01.

4 DISCUSSION

The Th1-Th2 paradigm has been useful in explaining the mechanisms of immunity during infection[173-175]. Th1 cells through cytokines like IFN-γ, IL-12 and IL-6 have been shown to promote the clearance of intracellular pathogens but can also induce autoimmunity. In contrast, Th2 cells are responsible for the induction of the humoral immunity by using cytokines such as IL-4 and IL-5 to activate B cells which produce antibodies. This pathway can also lead to allergy due to B cells production of IgE. The reciprocal control of Th1 and Th2[174, 176] cells was believed to maintain the necessary balance for immune homeostasis. Disruption to this Th1-Th2 balance has provided an explanation for several immune pathologies like autoimmunity and allergy[177]. However, nowadays this paradigm is under intense revision[178] due to the discovery of "new" subtypes of T cells. In addition to Th1 cells, Th17 cells have recently been shown to trigger inflammation and autoimmunity[179, 180]. Furthermore, intense research into naturally-occurring regulatory T cells (nTreg) has confirmed their ability to control immune responses to self-antigens and thus preventing autoimmunity[180, 181]. Both adaptive and naturally-occurring Treg cells have also been shown to play an important role in limiting collateral damages during immune responses to pathogens[181]. In the regulation of humoral immune responses, one of the mechanisms proposed to explain control by Treg cells is the ability of these cells to induce of a preferential switch of antibody production in B cells to the non (or less) inflammatory immunoglobulin IgG4[143]. In this chapter, the findings presented in the chapter 3, will be related to important currently discussed topics like the in vitro generation of antigen-specific regulatory T cells (4.1), the identification of markers for regulatory T cells (4.2), the relevance of regulatory T cells in allergy and parasitic diseases (4.3 and 4.4) and the influence of TLRs on regulatory T cells functions (4.5).

4.1 *IN VITRO* GENERATION OF REGULATORY T CELLS

In vitro generation of regulatory T cells is a promising potential immunotherapy for fighting autoimmune disorders, allergic diseases and for prevention of allograph rejection. In this study, IL-10-producing regulatory T cells have been generated as previously described[97] using a repetitive stimulation with tetanus or O.v antigen and in combination with two immune suppressive drugs: dexamethasone and vitamin D3 (DD3). The active form of vitamin D3, 1,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], is a secosteroid hormone that binds to the vitamin D receptor (VDR), a member of the superfamily of nuclear receptors for steroid hormones, thyroid hormones and retinoic acid. VDR ligands are known to regulate calcium and bone metabolism but also known to control cell proliferation and differentiation, and exert immunoregulatory activities[182]. The combination of vitamin D3 and dexamethasone (DD3) has been demonstrated to have a synergistic effect on the maturation of DCs. Monocyte-derived DCs cultured with DD3 during LPS-induced maturation were shown to have low stimulatory effects on allogeneic T cells, a phenomenon comparable with that observed with immature DCs. However, in contrast to immature DCs, DD3 exposed DCs secrete IL-10 and show upregulation of the Ig-like inhibitory receptor (ILT4). Thus, DD3 is an effective immunosuppressive drug combination for the induction of APCs capable of eliciting effector T cell hypo-responsiveness[183] and expansion of regulatory T cells[97]. As previously described, our generated T cells were distinct from Th1 and Th2 cells since they produced low IFN-γ, no IL-4 and were able to suppress the proliferation of activated non-regulatory T cell clones *in vitro* in a IL-10 dependant manner (Figs 3.8 and 3.9). These characteristics were similar to those described for Tr-1-like T cells whose differentiation was driven by IL-10[96]. This T cell population was obtained after antigenic stimulation of PBMCs with DD3. Interestingly, these studies further showed that using these drugs individually did not allow for the generation of such regulatory T cells. It is also of interest to mention that blocking Th1 (IFN-γ) or Th2 (IL-4, IL-5) polarising cytokines did not influence the generation of these Treg cells, a finding that is in contrast with previous reports[184]. In confirmation of these findings, experiments performed here in the presence of neutralizing antibodies against IFN-γ or IL-4 did not significantly increase the IL-10 production in the DD3-cultured TCLs (Fig 3.2). Also in accordance with other studies[97], the data presented within this work also suggests that treatment with DD3 down regulates the expression of key transcription factors associated with Th1 and Th2 T cell differentiation: T-bet and GATA3 respectively[97] (Fig 3.5). However, after T cell cloning and further re-stimulation with

anti-CD3/CD28 in the presence of IL-2, the Tr-TCC recovered substantial Tbet expression which could be associated with their increased levels of IFN-γ (Fig 3.6). GATA3 levels on the other hand remained low in all tested Tr-TCC, suggesting only a minor role for this transcription factor in the differentiation of IL-10 producing regulatory T cells.

From the studies performed in this work, we now hypothesise that the presence of DD3 during naive T cell priming lowers the "danger" signal and in turns alters the direction of maturation towards a regulatory T cell phenotype. In essence, mimicking the events that normally occur when auto-reactive T cells encounter "self" antigen in the periphery under tolerogenic conditions. Furthermore, known APCs like macrophages have been shown to be able to synthesize vitamin D3 after activation in the presence of IFN-γ and LPS[185, 186] suggesting that upon triggering of APCs by microbial factors vitamin D3 can be synthesized and may constitute a potential mechanism for the generation of IL-10 producing cells *in vivo*. Furthermore, glucocorticoids released by the adrenal glands have been shown to profoundly dampen immune responses[187] confirming the role of glucocorticoids in immune suppression and in regulatory T cell generation *in vivo.*

4.2 CHARACTERISATION OF REGULATORY T CELLS

The identification of markers for regulatory T cells is of great interest. The IL-2 receptor alpha or CD25 has been associated with nTreg following the first description of $CD4^+CD25^+$ T cells by Sakaguchi[75]. However, the fact that activated effector T cells also transiently express significant levels of CD25 makes the use of this marker insufficient for the general characterization of Tregs. Other constitutively expressed markers such as GITR, $\alpha_E\beta7$ and CTLA-4 have also been proposed. Nowadays, the transcription factor Foxp3 is well accepted as the main marker for Tregs. However, certain subsets of regulatory T cells do not express Foxp3. Moreover, in humans, activated effector T cells were shown to also up regulate Foxp3 upon TCR engagement[188]. Despite these controversial aspects, the characterization of Tregs is possible and probably more accurate, by using all the known Treg makers together with cytokine profiles and *in vitro* or *in vivo* suppressive properties of the T cell population in question. In the second part of this work, we have used the expression of CD25, CD127, Foxp3, GITR, CTLA-4, IL-10 and TGF-β to characterize the DD3 generated Tr-TCC. Table 4.1 compares the expression different T cells markers on our generated Tr-TCC and other subset of T cells.

	Th1/2	Natural Tregs	Tr-1	Th3
Suppression mode	0	0	1	1
Resting Foxp3	0	1	0	?
CD25 high	0	0	1	?
CD127	1	0	?	?
CTLA-4	0	1	1	1
IL-10	0	0	1	0
TGF-beta	0	0	1	1
GITR	0	1	1	?
Proliferation/energy	0	0	1	0
Tr-TCC score	**1**	**3**	**7**	**3**

Table 4.1: Comparison of Tr-TCC with other T cells subtypes.

According to their expression of GITR, CTLA-4 and IL-10, Tr-TCC resemble to the well characterized Tr-1 cells but are simultaneously distinct from these cells because of their high levels of Foxp3 expression that makes them similar to natural Tregs. In addition, Tr-TCC are distinct from natural occurring Tregs because of their constitutively low expression of CD25. According to our scoring method, our generated Tr-TCC aligned most with Tr-1 cells. However, in opposition to previously described studies with glucocorticoid-derived IL-10 producing regulatory T cells our cells did not exclusively produce IL-10 since some IFN-γ was detectable aswell[189].

We show here that IL-10 producing $CD4^+$ T cells, generated in the presence of DD3, change their phenotype after limiting dilution cloning and re-stimulation in the absence of DD3 and produce significant levels of IFN-γ, described previously to be distinct from Tr-1 cells generated using IL-10[97]. This data suggests that the primary effect of DD3 was to down regulate IL-4 and IFN-γ production but after re-stimulation either with antigen or anti-CD3 in the absence of DD3, IFN-γ production was renewed and seems to play a role in their ability to retain significant proliferation capacities despite their regulatory properties and IL-10 production (Fig 3.3). Most interestingly, excess recombinant Th1 and Th2 polarizing cytokines where able to down regulate the number of IL-10–producing regulatory T in the presence of DD3 providing evidence that regulatory T cell development and function can be influenced by ongoing activities of Th1 and Th2 cells and may have significant implications for the physiological balance between these T cell subset during the course of an immune response.

The ratio of Treg to T effector and T effector cytokines (IFN-γ, IL-4, IL-5, IL-6) to regulatory cytokines (IL-10, TGF-β) may also contribute to the outcome of the ensuing immune response.

Our present findings describing the generation and cloning of populations of IL-10–producing regulatory T cells has allowed us to determine that there are more similarities between these cells and other regulatory T cell populations. Furthermore, it has also demonstrated that these previously described distinct populations are not completely defined populations and that there is considerable overlap between regulatory subsets[190]. In addition, these data have provided us with an insight on how better to understand the mechanisms underlying IL-10 positive Treg development. Our data also show similarities of the generated Tr-TCC to natural regulatory T cells in terms of the expression of surface or intracellular markers.

4.2.1 CD25, CD127, FOXP3

Regulatory T cells are distinguished from other T cell subsets by their expression levels of specific surface and intracellular markers. For example, natural occurring regulatory T cells are defined by their constitutive expression of the IL-2 receptor alpha (CD25) and the transcription factor Foxp3; both markers have been shown to play a critical role in their development as well as in their function[191]. However these markers are not exclusive for regulatory T cells. Activated effector T cells for example also up-regulate and express significant levels of both CD25 and Foxp3[188]. Thus only constitutive high CD25 expression and constitutive Foxp3 expression seem to be specific for regulatory T cells. A more controversial point is that not all regulatory T cell populations express these markers. High CD25 expression for example has been shown to be not relevant for IL-10 producing regulatory T cells (Tr-1). In both rodent and human studies, Foxp3 expression was not found in these Tr-1 cells on the protein or mRNA level[192]. Nevertheless, whether Tr-1 and IL-10 producing regulatory T cells express Foxp3 remains a controversial point especially when other reports have attested to up-regulation of Foxp3 upon activation[193]. Taken together, these considerations make the use of these markers to characterise regulatory T cells problematic. Recently, the expression of the IL-7 receptor (CD127) has been described to discriminate regulatory T cells from effector T cells[194]. Regulatory T cells have been shown to express lower levels of CD127 in comparison to effector T cells. Thus, levels of CD127 and CD25 can distinguish $CD4^+CD25^+$ regulatory T cells from activated effector T cells that also express significant levels of CD25.

During the generation of the Tr-TCC used in this study, it became apparent that after each round of stimulation there was an increase in the level of intracellular Foxp3 (Fig 3.21). Moreover, this induction of a natural regulatory T cell phenotype also corresponded with their ability to induce IgG4 in B cells. After cloning, and dependent on the individual Tr-TCC, these generated clones possessed not only the classical Tr-1 phenotype but displayed high levels of Foxp3 in their resting state. An explanation for the constitutive and high levels of IL-10 and Foxp3 in the generated Tr-TCC is that, unlike freshly isolated PBMCs which possess perhaps "non-primed" IL-10 producing Tr-1 cells, the cells described here are "directed" into becoming more antigen-specific and in addition to retaining their IL-10 phenotype adopt features of classical natural Treg. Further studies would have to be performed to elucidate whether Tr-1 cells in the periphery can be driven towards a classical regulatory phenotype upon repetitive antigen stimulation. Despite the Foxp3 expression, the generated Tr-TCC are further associated with Tr-1 like cells since they retain their ability to suppress effector cells in a cell contact independent manner: probably due to their IL-10 production (Figs 3.2.and 3.8). Moreover, whereas one-time stimulated cells induced hardly any IgG4 from B cells, multiple-stimulated cells or clones elicited predominantly IgG4.

In this study, the regulatory T cells that we have generated express intermediary levels of Foxp3 when compared to CD25-high nTreg isolated for PBMCs (Fig 3.4) and in concordance to what has been previously described, high levels of CD25 was expressed only upon activation. More surprisingly, our generated Tr-TCC express significant levels of the IL-7 receptor differentiating them from the natural or antigen-induced $Foxp3^+$ regulatory T cells[194] that express lower levels of CD127. Taken together these observations lead us to conclude that the Tr-TCC generated in this study were phenotypically different from the well-known $CD25^{high}$ $Foxp3^+$ regulatory T cells. We also investigated the expression of other additional markers which are used to characterise regulatory T cells and the findings of these results are described in the following sections.

4.2.2 GITR, CTLA-4 AND ICOS

The glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), also known as Tumor Necrosis Factor Receptor Super Family member protein 18 (TNFRSF18) or AITR (Activation-inducible TNFR family receptor)[195, 196], is a type I transmembrane protein

with high homology to other members of the family, like 4-1BB, CD27, and OX40[197]. As with most of the members of this superfamily GITR is known to regulate diverse biological functions, including cell proliferation, differentiation, and survival. In both human and murine models stimulation of T cells through GITR is shown to induce NFκB activation through a TRAF2–NIK signalling pathway[196]. Nevertheless, it is well acknowledged that GITR is constitutively expressed on regulatory T cells[198, 199] even though its actual function remains undetermined[200]. The regulatory T cells we generated here constitutively expressed GITR and up-regulated its expression upon TCR ligation suggesting a functional meaning for the expression of this protein on these regulatory T cells. However, as mentioned above, GITR is not exclusive for regulatory T cells since it is also found on effector T cells, and its stimulation renders the latter resistant to regulatory T cell activity[200]. Apart from GITR, Foxp3 and CD25, Treg cells express high levels of other markers that have also been shown to be important for their function. CTLA-4 for example is known play a role in terminating effector T-cell responses and is important in the induction of self-tolerance[201]. The inducible co-stimulator ICOS is not a specific Treg marker but is thought to modulate the function of regulatory T cells[202].

4.2.3 ANTIGEN SPECIFICITY OF THE GENERATED T CELL CLONES

Despite their critical role in the control of inflammatory and autoimmune diseases, a potential exploitation of natural Treg in immunotherapy is limited by their lack of "known" antigen specificity. In contrast, antigen induced regulatory T cells get activated only when they encounter the appropriate antigen presented by APCs[201]. The Tr-TCC we generated were activated upon antigen-specific stimulation but not following incubation with non-specific antigen as shown by their differential upregulation of CD69 after specific and non specific antigen challenge (Fig 3.7 A). However the Tr-TCC proliferate only mildly upon antigen stimulation and depending on individual TCC. This lack of proliferation could be do to the high levels of immunoregulatory cytokine IL-10 produced after stimulation in presence of APCs loaded with their specific antigen(Fig 3.7 B and C)

4.3 LIMITING DILUTION T CELL CLONING

The limiting dilution microculture T-cell cloning technique was developed by Keystone *et al*., in 1993 and allows one to obtain single growing T cell clones from a polyclonal population. The principle consists of making a serial dilution from a known cell suspension to theoretically obtain 1 cell per well and to let this cell proliferate after stimulation. Although the principle is intuitive, the method is time consuming and there is no guarantee of single cell clones as shown in several studies[203]. However it is one of the best available methods to obtain and study homogenous cell populations. Here we used a modification of this technique to generate antigen-specific T-cell lines and antigen-specific regulatory T cell clones (Tr-TCC).

4.4 MOLECULAR MECHANISMS OF IGG4 INDUCTION BY TR-TCC

One of the mechanisms used by adaptive type-1 regulatory T cells to control an excessive immune response in allergy and chronic infections is the induction of non-inflammatory immune responses through IgG4 production. The aim of this study has been to decipher the molecular mechanisms that are involved in IgG4 induction.

Using a combination of tetanus toxoid or *Onchocerca volvulus* antigens with DD3 we continually stimulated PBMCs so that they would develop a Tr-1 like phenotype. After cloning, we employed this "antigen-primed or TCR-activated" Tr-TCC to explore the requirements of regulatory T cells to induce IgG4 production from B cells. We found that specific IgG4 responses from B cells after Tr-TCC activation were completely abolished upon neutralization of IL-10 and TGF-β. The same outcome occurred when GITR was blocked on the Tr-TCC. Further investigations showed that α-GITRL antibody had the same effect on IgG4 production but addition of recombinant GITR and GITRL to the system actually enhanced the levels of produced IgG4. GITR has been shown to be a regulator of immune responses by co-stimulating T-effector cells and abrogating the suppressive effects of Treg cells. As mentioned previously, nTreg cells and Tr-1 cells display a panel of specific surface markers which are now well-established with the regulatory T cell phenotype. However, the absolute necessity of these markers, especially during infection, remains unclear. It is hypothesised that Treg cells can inhibit the activation, differentiation and survival of pathogenic T cells through bidirectional communication (reverse signalling) between members of the TNF family on T lymphocytes and

DC. This pathway is regulated through tryptophan catabolism which is initiated by the enzyme indoleamine 2,3-dioxygenase (IDO). Although initially described for CTLA-4 and B7 molecules[204], Grohmann *et al.,* have now demonstrated that GITR/GITRL pathways are also influenced by this pathway. This study showed that reverse signalling through GITRL in pDCs resulted in non-canonical NF-kB activation and the onset of IDO-dependent immune regulation. We showed here that during each round of stimulation, the Tr-TCL have an increasing propensity to induce IgG4 production from B cells but this is entirely dependent on the presence of dexamethasone since its absence initiated IgG3 induction instead. In addition, blocking GITR or IL-10 prevented the production of this IgG subclass. Thus, we now hypothesize that in the initial stimulation of PBMCs, the steroid has synergistic effects on GITR/GITRL interactions which may modulate IDO activation. Repetitive stimulation then directs the IL-10 producing cells to develop a Tr-1 phenotype. Since the pDCs non-canonical NF-kB and IDO function have already been associated in controlling inflammatory pathology and allergy we conclude that this mechanism could also induce Tr-1 cells which have the ability to induce IgG4, and thus control arising immunopathology. In support of this theory, an experimental model of allergic bronchopulmonary aspergillosis has demonstrated that Treg cells, pDC and tryptophan catabolism are all required to mediate protection[205, 206]. Studies using this model showed that application of dexamethasone inhibited Th2 responses but enhanced Foxp3 expression in the $CD4^+$ T cells. Furthermore, although IgG4 levels were not measured, blockade of IDO in this model enhanced both IgE/IL-5 levels but significantly decreased IL-10 and TGF-β[206].

These data also suggest a possible signal through GITRL working as a receptor and activated by GITR acting in this model as ligand. Since these two molecules are also implicated in the model proposed in this thesis, we tested the possibility of a direct role for a signal through GITRL expressed on the B cells that could lead to IgG4 induction. For this purpose, we used a T-independent system in which B cells were stimulated with 5µg/ml CD40L/IgM.(BD pharmingen) In this system, addition of recombinant GITR with or without the presence of recombinant IL-10 and/or TGF-β did not induce B cells to significantly increase IgG4 production. These results indicate that T cells (Tr-TCC) are absolutely required in the system and suggest a critical role for a signal through GITR molecules on the Tr-TCC in their ability to induce IgG4 production by B cells.

In other studies the presence of induced IgG4 appears to be associated with reduced pathology and protection. Just recently, studies using venom immunotherapy showed an expansion of circulating regulatory T cells ($Foxp3^+$) which significantly correlated with the increased allergen-specific IgG4 and reduced IgE levels[207]. In association with that study, studies from patients with paediatric asthma showed a significant increase in $CD25^{hi}CD4^+$ T cells after corticotherapy both in the peripheral blood and in the bronchoalveolar lavage fluid[208]. Classically, both allergic and parasitic diseases induce a strong Th2 response with dominant IgE levels. It is now well-established that in several chronic infections such as helminth infections, a regulatory T cell response is generated, which may permit parasite survival within the immune-competent host[209-211]. Patients that develop a more regulatory response to parasite antigens also possess regulatory T cells which secrete IL-10 and TGF-β[211]. All of these features are currently assumed to explain the absence of Th2-related autoimmune disease in patients suffering from parasite infections. In onchocerciasis, the hypo-responsive form of the infection is characterized by a strong prevalence of IgG4, low IgE and elevated Treg numbers. This phenotype is of course much better for the host, but how it actually arises and is maintained remains unclear. Taken together, these data highlight the connection between glucocorticoids, Treg and IgG4 in maintaining immunologic tolerance and implicate that therapeutic approaches aimed at boosting this population should be explored as a strategy to control allergic and parasitic diseases.

4.4.1 THE ROLE OF FOXP3

We show here a novel function for Foxp3. Since our Tr-TCC express significant levels of Foxp3 in resting as well as in activated stages, we supposed that this molecule may play a key role in their function and we could show that the Foxp3 expression is tightly correlated with the ability of the Tr-TCC cells to induce B cells to specifically produce IgG4. We could also block the expression of Foxp3 in already differentiated Tr-TCC at the mRNA level using siRNA technology. However the method was not fully reproducible and most of the T cells do not survive transfection. The few Tr-TCC that were successfully transfected display after inhibition of Foxp3 a completely different and relatively stable profile: they up-regulate their IFN-γ expression without significantly changing their expression of IL-10, IL-4 was not measurable and the cells loose their ability to induce B cells to specifically induce IgG4. They elicit IgG2 production instead. The loss of IgG4 capacity seems to be due to the up-regulation of the other cytokines, suggesting that a balance in the expression of the cytokine milieu is critical for the

signals that lead to IgG4 production. These data were however in contrast to previous studies on the expression and function of Foxp3 in glucocorticoid induced IL-10 producing regulatory T cells: that is, the absence of this transcription factor and lack of functional role[97]. Our data reveal a new role for Foxp3 in the stability of the regulatory functions of IL-10 producing regulatory T cells. Foxp3 seems also to play a functional role through the control of the cytokines expressed by the T cells.

4.4.2 THE LOOP GITR, IL-10, TGF-β AND CTLA4

Signalling through GITR on regulatory T cells has been described to inhibit their suppressive effect and to induce development of autoimmunity presumably due to immunoregulatory T cell inhibition[199, 212]. However, Igarashi *et al*., recently showed that GITRL engagement increases proliferation and IL-10 production in regulatory T cell populations during CD3 stimulation and in the presence of exogenous IL-2. This is concordant to our data showing an increase of IL-10 production by Tr-TCC after engagement of GITR with its natural ligand[200]. We postulate that this is one of the most important signals necessary for the induction of IgG4, and we could block IgG4 production by B cells using neutralizing GITR antibody which in turn could be reversed by the addition of excess recombinant IL-10. However, other signals also seem to be necessary, the blockade of TGF-β in our co-culture system for example completely reverses the production of IgG4 suggesting a key role for this molecule in this process. Nevertheless an excess of recombinant TGF-β could not recover the IgG4 expression after GITR blockade suggesting an independent but additional and indispensable role for TGF-β in the signals leading to IgG4 production. Our data also suggests that the TGF-β implicated in this process may be membrane bound since it was not possible to measure TGF-β in culture supernatants, and furthermore, using flow cytometry it was possible to detect TGF-β on the Tr-TCC, without permeabilization. Our data are in correlation with the fact that TGF-β and IL-10 often cooperate in anti-inflammatory processes[213].

4.4.3 NAÏVE AND MEMORY B CELLS

Surprisingly, the use of isolated naïve ($CD27^-$) or memory ($CD27^+$) B cells separately in co-culture with Tr-TCC showed completely different results to that obtained using bulk B cells populations. Naïve B cells alone produced more IgG4 than the memory ones. Moreover, the

collective amount of IgG4 produced by naïve and memory B cells separately was also much lower than that the one produced by bulk B cells, suggesting a possible cooperation between the two B cell populations in this process. However, memory B cells produce higher amount of total IgG in comparison to naive B cells. These findings corroborate with observations showing that subpopulations of $CD27^+$ B cells produce larger amounts of immunoglobulin than $CD27^-$ B cells in presence of activated $CD4^+$ T cells[214]. These differences were shown to be due to the interaction of CD27 on the B cells with the CD27L (CD70) on the T cells[214, 215]. Furthermore, triggering of CD27 on B cells by CD70 was shown to induce an increase in the number of plasma cells in the presence of stimuli such as IL-10[162] and IL-4 plus CD40 signalling[216]. On the other hand, a preferential differentiation of $CD27^-$ B cells into IgG4 capable plasma cells could justify the higher amount of IgG4 produced by $CD27^-$ B cells in the Tr-TCC-B cells co-culture system. However there is no confirmation of collaboration between naïve and memory B cells in the literature, our data are in favor of a direct or indirect cooperation between the two B cell subpopulations at least in the Tr-TCC dependant IgG4 production (Fig 4.1).

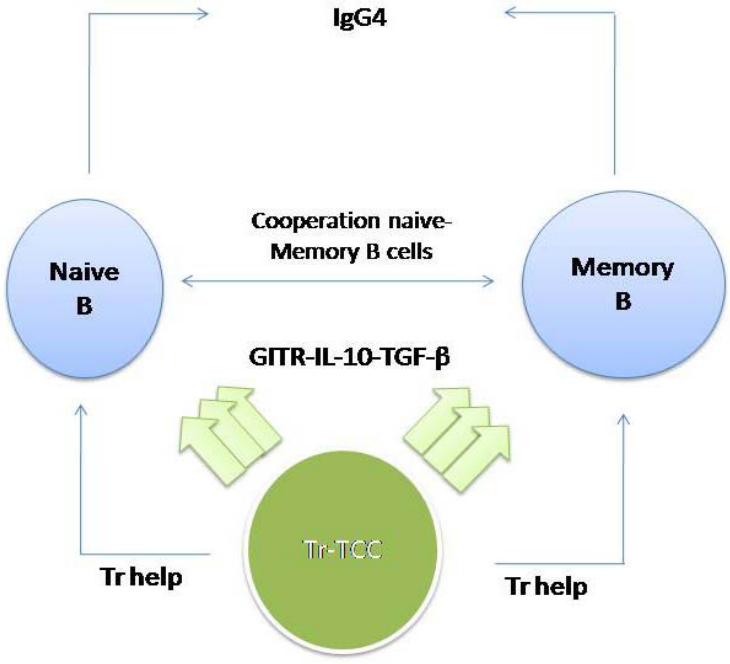

Figure 4.1: **Hypothetical cooperation of memory and naïve B cells.**
Tr-TCC induce signals through GITR-GITRL, IL-10 and TGF-β in association to additional signals from Tr-TCC (Tr help) stimulate both naive and memory B cells. Naive and memory B cells in the presence of these regulatory signals might then collaborate to preferentially secrete IgG4. This collaboration might be through direct B-B-cell interactions and/or cytokines produce by B and/or T cells present in the system.

4.5 TLR AND IGG4 PRODUCTION

TLRs are used by cells of the innate immunity to recognize invading pathogens. Signalling through TLRs induces the so-called "danger" signal, which ultimately leads to rapid clearance of infection[6]. TLRs are known to influence the outcome of the adaptive immune responses.

Regulatory T cell function has also been shown to be modulated through TLR activation[170, 172]. In the studies described in section flow cytometric analysis of PBMCs revealed that there was generally a high expression of TLRs on B cells. In addition, natural Treg expressed significantly higher levels of TLR2 than Tr-TCC and Th1 cells. In fact, generated Tr-TCC and Th1 cells displayed approximately the same TLR profile in terms of TLR expression (Fig 3.30). Surprisingly, known intracellular TLRs like TLR3 were found to be expressed on the surface of Tr-TCC. However, there was almost no TLR expression in the intracellular compartments of all T cell types. In contrast, B cells displayed significant levels of intracellular TLR3 and TLR9 after permeabilization of the cellular membranes.

We could show that Pam3Cys, CpG and LPS stimulations promote the induction of IgG1 and 3 by acting directly on the B cells. In contrast, the production of IgG4 was inhibited by the presence of either LPS or CpG. Pam3Cys stimulation however did not significantly affect IgG4 induction by Tr-TCC. Thus, TLRs stimulation redirects the IgG expression in the direction of a pro-inflammatory antibody production. This showed that, in case of an additional bacterial infection, B cells can overcome the control by Treg cells and redirect the IgG production to clear infection. These observations could explain the different pathological outcomes during parasitic infection. In Onchocerciasis for example, there are two possible immunopathological consequences during the development of infection: the generalized onchocerciasis usually associated with parasitemia, high IL-10 levels and IgG4 expression and the hyper-responsive form or "sowda" which shows few microfilariae but intense dermal pathology. In the latter case, additional bacterial infection such as LPS could initiate the development of the latter form due to too strongly stimulated B cells and the consequent production of other more inflammatory Ig molecules such IgG1, 2, 3 or IgE. Helminth antigens which are known to elicit IgG4[141] production can dampen TLR expression on dendritic cells[217]. Our data show that TLR stimulation in counterpart can also modulate the immunoregulatory effect of regulatory T cells.

Furthermore, vitamin D3 was shown to down-regulate monocyte TLR expression and triggers hypo-responsiveness to pathogen-associated molecular patterns[218]. All together, TLR pathways and immune regulatory mechanisms seem to be tightly interconnected.

4.6 NATURAL TREGS AND IgG4 INDUCTION

In experiments performed using isolated nTreg ($CD4^+CD25^+$ T cells) we were also able to show that these cells preferentially induce IgG4 in B cells whereas $CD25^-$ effector T cells do not, the latter actually induced IgG2 instead. These observations correlate with the data obtained using Tr-TCC and additionally to the experiments using Foxp3-silenced Tr-TCC since the latter could no longer induce IgG4 in B cells but elicited IgG2 instead. Therefore, it appears that Foxp3 is a vital component in directing B cells. Furthermore, IgG4 production induced by isolated nTreg cells was also dependent on GITR since adding αGITR to the co-cultures dampened the ability of those cells to make IgG4. Since we have shown that IL-10, TGF-β and Foxp3 play a critical role in the IgG4 induction and nTreg express all these markers, it was predictable that their effect on B cells were similar to the ones observed with Tr-TCC. However, the total amount of IgG subclasses induced by isolated $CD4^+CD25^+$ Tregs was much lower than the levels induced by Tr-TCC. These differences are certainly due to the activation status of the T cells and their levels of cytokines. For example, Tr-TCC produce much larger amounts of cytokines (chiefly IL-10) than $CD4^+CD25^+$ T cells and due to the multiple rounds of antigen-specific stimulation these cells are possible more "active" in their actions. In summary, the data suggest that $CD4^+CD25^+$ Tregs and IL-10 producing antigen-specific regulatory T cells use the same GITR dependant mechanism to induce IgG4 production by B cells.

4.7 CONCLUDING REMARKS

The data accumulated during this thesis procure one working hypothesis where at least three signals are indispensable for Tr-TCC to induce preferential IgG4 production by B cells (Fig 4.2.A). The first signal might go through the TGF-β receptors on B cells and provide the first instruction to B cells for a switch in favor of IgG4. In support of this aspect, blocking this signal using neutralizing antibodies against TGF-β in Tr-TCC:B cell co-culture assays abrogated the secretion of IgG4 (Fig 4.2B). The second signal may then be provided through GITR on the Tr-TCC that could be activated by connecting to GITRL expressed on the B cells. Blocking this

signal in Tr-TCC-B cells co-culture experiments, reduced the number of IL-10 producing T cells and thus the secretion of IgG4 (Fig 4.2C) indicating a link between IL-10 production, GITR-GITRL signalling and IgG4 induction. Tr-TCC:B cell co-cultures in an IL-10 deficient environment (anti-IL-10 mAb) failed to produce IgG4 (Fig 4.2D) and the excess addition of IL-10 recovered IgG4 production even when GITR signalling was blocked. Thus, confirming the key role of IL-10 which constitutes the third most direct signal necessary for IgG4 production. In addition, the TGF-β pathway might be independent from the GITR-GITRL-IL-10 signalling since addition of recombinant TGF- β did not recover IgG4 production when GITR is blocked (see section 3.2.5).

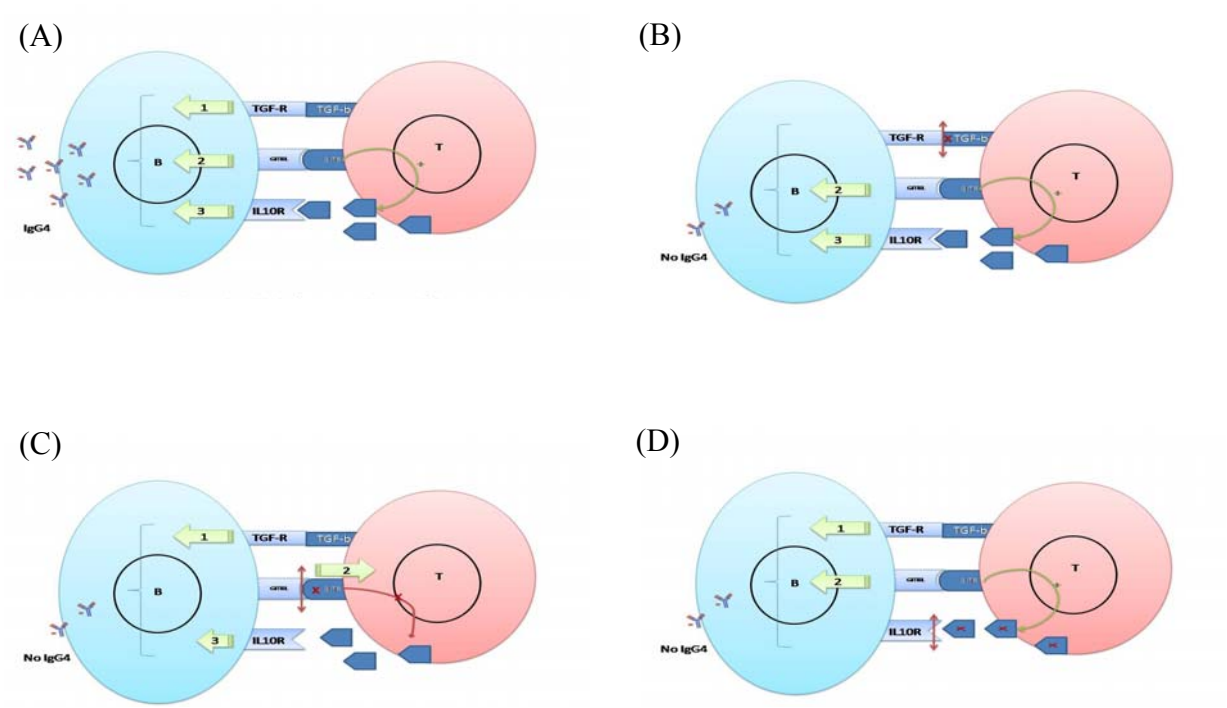

Figure4.2: Three signals are indispensable for the IgG4 induction by IL-10 producing regulatory T cells. Three signals seem to be indispensable for Tr-TCC to induce preferential IgG4 production by B cells. The first signal is mediated by TGF-β and provides the first instruction to B cells for a switch in favor of IgG4 (A). Blocking this signal with mAbs against TGF-β in Tr-TCC:B cell co-cultures abrogated the secretion of IgG4 (B). The second signal is provided through GITR-GITRL interaction respectively expressed on Tr-TCC and B cells. This second signal elicits IL-10 production (C) that in turn provides the third and more direct signal necessary to induce IgG4 secretion. In Tr-TCC:B cells co-culture experiments, blocking GITR or GITRL reduce the number of IL-10 producing T cells and thus the secretion of IgG4 (C) indicating a link between IL-10 production and GITR-GITRL signaling. The key role of IL-10 is confirmed by the fact Tr-TCC:B cell co-cultures in an IL-10 deficient environment failed to produce IgG4 (D) and that addition of IL-10 recovers IgG4 production when GITR signaling is blocked.

According to our data and the literature, we can propose a mechanism that leads to IgG4 production where the three signals described above are provided by a specialised T cell population expressing Foxp3 and IL-10. Although the data reported in this thesis provide

evidence that IgG4 is induced by Foxp3$^+$ IL-10$^+$ Tr-1 like cells, this IgG class switching is probably also modulated by additional signals given by conventional Tregs that may play a positive role in the system. In contrast, this pathway might be negatively controlled by activated effector Th1 cells through pro-inflammatory cytokines like IFN-γ and inflammatory responses from TLR-activated innate cells. These strong pro-inflammatory responses would then initiate other immunoglobulins in detriment of IgG4. Understanding the mechanisms that lead to the production of IgG4 may provide useful information for understanding and in addition the treatment of parasitic, autoimmune and allergic diseases.

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APPENDIX A: EQUIPMENT

Automatic pipettes (10-1000µl)	Eppendorf AG, Hamburg, Germany
BD FACSCanto™ flow cytometer	BD™ Biosciences, Heidelberg, Germany
BD FACS Diva™ flow cytometer	BD™ Biosciences, Heidelberg, Germany
Casy® TT Cell Counter	Schärfe Systems, Reutlingen, Germany
Centrifuge (Eppendorf 5415 R)	Eppendorf AG, Hamburg, Germany
Centrifuge (Multifuge 4KR)	Heraeus Holding GmbH, Hanau, Germany
ELISA Plate reader (Spectra Max 340pc^{384})	Molecular Devices, Sunnyvale, USA
Freezer (-20°C)	Bosch GmbH; Stuttgart, Germany
Freezer (-80°C)	Heraeus Holding GmbH, Hanau, Germny
Fridge	Bosch GmbH; Stuttgart, Germany
Gamma Cell irradiator	Atomic Energie of Canada, Ottawa, Canada
Glass pipettes (1-20ml)	Brand GmbH & Co.KG, Wertheim, Germany
Glassware	Schott AG, Mainz, Germany
Ice machine (Scotsman AF 80)	Gastro Handel GmbH, Wien, Austria
Incubator	Binder GmbH, Tuttlingen, Germany
Liquid scintillation counter(LS6500)	Beckman Coulter Inc, California, USA
Microscope (Leica DM IL)	Leica Microsystems GmbH, Wetzlar, Germany
Neubauer counting chamber	LOLaboroptik GmbH, Friedrichsdorf, Germany
PH meter	Mettler Toldo GmbH, Giessen, Germany
Pipetboy (pipetus®-akku)	Hirschmann Laborgeräte, Eberstadt, Germany
Rotorgene PCR machine	Corbett research, Sydney, Australia
Strip PCR tubes 0.1ml	Corbett research, Sydney, Australia
Thermo magnetic stirrer	IKA® GmbH & Co.KG, Staufen, Germany
Vortex mixer (Minishaker)	IKA® GmbH & Co.KG, Staufen, Germany
Water bath	VWR Lab Shop, Batavia, USA
Weighing machine	Sartorius AG, Goettingen, Germany

APPENDIX B: CHEMICAL AND REAGENTS

Advanced Protein Assay™	Cytoskeleton, Inc., Denver, USA
Alamar Blue	Sigma-Aldrich GmbH, Munich, Germany
Ammonium chloride	Merck KGaA, Darmstadt, Germany
BSA	Sigma-Aldrich GmbH, Munich, Germany
$CD25^+$ Treg isolation kit	Miltenyi Biotech, Bergisch Gladbach, Germany
CpG	Sigma-Aldrich GmbH, Munich, Germany
Dexamethasone	Sigma-Aldrich GmbH, Munich, Germany
Disodium hydrogen phosphate	Merck KGaA, Darmstadt, Germany
DMSO	Merck KGaA, Darmstadt, Germany
EDTA	Merck KGaA, Darmstadt, Germany
Ethidium Bromide	Biomol GmbH, Hamburg, Germany
Ethanol	Merck KGaA, Darmstadt, Germany
FCS	PAA Laboratories GmbH, Pasching, Austria
Fc Block (anti-human CD16/32)	eBiosciences, Inc., San Diego, USA
Ficoll, density 1.077	Seromed Biochrom KG, Berlin, Germany
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich GmbH, Munich, Germany
Ionomycin	BD Biosciences, Heidelberg, Germany
Liquid scintillator cocktail	Fisher Scientific, Pennsylvania, USA
Liquid nitrogen	University Clinic Bonn
LPS	Sigma-Aldrich GmbH, Munich, Germany
Monosodium phosphate	Merck KGaA, Darmstadt, Germany
Pam3Cys	Sigma-Aldrich GmbH, Munich, Germany
Paraformaldehyde	Merck KGaA, Darmstadt, Germany
PHA	Remel Europe, Dartford, United Kingdom
PBS (endotoxin free)	PAA Laboratories GmbH, Pasching, Austria
PMA	BD Biosciences, Heidelberg, Germany
PMSF	Merck KGaA, Darmstadt, Germany
Potassium chloride (KCl)	Merck KGaA, Darmstadt, Germany
Potassium dihydrogen phosphate	Merck KGaA, Darmstadt, Germany
Recombinant human IL-2	eBiosciences, San Diego, USA
Recombinant human TGF-β	eBiosciences, San Diego, USA

RPMI-1640	PAA Laboratories GmbH, Pasching, Austria
siRNA starter kit	Qiagen, Hilden, Germany
Sodium chloride (NaCl)	Merck KGaA, Darmstadt, Germany
TMB	Sigma-Aldrich GmbH, Munich, Germany
Tris	Merck KGaA, Darmstadt, Germany
Trypan Blue	Sigma-Aldrich GmbH, Munich, Germany
Tween 20	Sigma-Aldrich GmbH, Munich, Germany
Vitamin D3	Sigma-Aldrich GmbH, Munich, Germany

APPENDIX C: BUFFERS, MEDIA AND SOLUTIONS

20x phosphate buffer saline (PBS):

360g NaCl

8.0g KCl

46.4g Na_2HPO_4

8.0g KH_2PO_4

Volume was then adjusted to 1 liter of distilled water.

For 1x phosphate buffer saline (PBS): 50 ml of 20x PBS solution was diluted in 900 ml distilled water. The pH was adjusted to 7.3 and the solution was topped up to 1 liter and autoclaved.

T cell culture medium (TCM)

T cell culture medium was prepared using:

10% FCS

100 Unit/ml IL-2

2.5% amphotericinB

RPMI 1640 qsp 50ml

SOC medium

Bacto-tryptone	20g
Bacto-yeast extract	5g
NaCl	0.5g
1M KCl	2.5ml
ddH2Oqsp	1L

adjust pH to 7.0 with 10N NaOH, autoclave to sterilize, add 20 ml of sterile 1 M glucose immediately before use

FCS: Fetal calf serum, (PAA)

FCS used for medium supplementations was heated for 30 minutes at 56°C to inactivate the complement factors. Aliquots were then stored at -20°C until required.

ELISAs Solutions and buffers:

Coating solution: 0.1 M $NaHCO_3$, pH 9.6

Washing buffer: PBS-0.05% Tween 20 (Sigma)

Blocking solution: PBS-1%BSA

Substrate buffer: 0.1 M $NaH_2PO_4.2H_2O$, pH 5.5

Substrate: 3,3', 5,5' Tetramethylbenzidine (Roth, Karlsruhe, Germany), dissolved to a concentration of 6 mg/ml in DMSO (Sigma)

Substrate solution: 12 ml substrate buffer

200 µl substrate

1,2 µl 30% H2O2 (Sigma)

Stop solution: 4N H_2SO_4(Merck, Hohenbrunn, Germany)

Cells culture reagents

Freezing medium: 80% FCS, 20% Dimethylsulfoxid (DMSO), Sigma

Loading dye (6x)

0.25 % bromophenole blue

0.25 % xylencyanole FF

15 % ficoll

in ddH2O

TBE (Tris-borate-EDTA)

108 g Tris-base

55 g borate acid

9.3 g Na_2EDTA

in 1000 ml ddH2O

Working solution: 0.5 x stock solution

APPENDIX D: SUPPLEMENTS

DMSO	PAA Laboratories GmbH, Pasching, Austria
FCS	PAA Laboratories GmbH, Pasching, Austria
GMCSF	PAA Laboratories GmbH, Pasching, Austria
L-glutamine	PAA Laboratories GmbH, Pasching, Austria
Penicillin/streptomycin	PAA Laboratories GmbH, Pasching, Austria
Gentamicin Sulfate	BioWitttaker®, Walkersville, USA

APPENDIX E: SOFTWARE

BD FACSDiva™ software

Flow cytometry software obtained from BD™ Biosciences, Heidelberg, Germany.

GraphPad Prism 4

Analyze, Graph and Organize Software obtained from GraphPad Software, Inc., La Jolla, USA.

SoftMax Pro

Microplate Data Acquisition and Analysis Software obtained from Molecular Devices, Sunnyvale, USA

ERKLÄRUNG

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbst und ohne jede unerlaubte Hilfe angefertigt habe, daß diese oder eine ähnliche Arbeit noch keiner anderen Stelle als Dissertation eingereicht worden ist.

Bonn, 10.08.08

_____

Gbèdomidji Tomabu Adjobimey

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