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Regluation der terminalen Differenzierung von T-Zellen durch Blimp-1

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Abbreviations

7-AAD	7-Amino-actinomycin D
Ag	Antigen
Amp	Ampicillin
APC	Antigen presenting cell
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
CIITA	MHC class II transactivator
DMSO	Dimethylsulphoxide
dNTPs	Deoxynuleosidetriphosphates
DTT	Dithiothreitol
E. coli	Escherichia coli
EB	Ethidium bromide
EBV	Epstein-Barr virus
EDTA	Ethylene-diaminetetraacetic acid
FACS	Fluorescence-activated cell sorter
Fc	Fragment of crystallizable (antibody)
FCS	Fetal calf serum
FITC	Fluorescein-5-isothiocyanate
FSC	Forward scatter
GCG	Genetics computer group
YFP	Yellow-fluorescent protein
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLA	Human histocompatibility antigen
HRP	Horseradish peroxidase
Ig	Immunoglobulin
Jak1	Janus kinase 1
LB	Luria-Broth
lck	Lymphocyte-specific protein tyrosine kinase
mab	Monoclonal antibody
MHC	Major histocompatibility complex
M-MuLV	Moloney murine leukemia virus
O/N	Overnight
ORF	Open reading frame
PB	Sodium phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
RNase	Ribonuclease
SSC	Side scatter
SV40	Simian virus 40
TAE	Tris-EDTA-acetate

TBE	Tris-EDTA-borat
TCR	T-cell receptor
TE	Tris-EDTA
Tris	Tris-(hydroxymethyl)aminomethane

1. Introduction

1.1. B and T Cell Development

1.1.1. Distinct Stages of B Cell Development

The stages in B cell development are classified according to the sequential rearrangement and expression of heavy- and light-chain immunoglobulin (Ig) genes (Fig. 1). Briefly, the earliest B-lineage cells are known as pro-B cells which are progenitor cells and derived from hematopoietic stem cells in the bone marrow. Rearrangement of D_H to J_H in the Ig heavy chain locus takes place in early pro-B cells. V_H to DJ_H joining is followed at the later pro-B cells stage. A successful VDJ_H rearrangement leads to the expression of μ chain at the large pre-B cell stage, which forms the pre-B cell receptor in combination with a surrogate light chain. The large pre-B cells give rise to small pre-B cells, in which light chain rearrangements occur. Upon successfully assembling a heavy and light chain gene, the pre-B cells become immature B cells that express a complete IgM molecular on the surface. The immature B cells migrate to the peripheral lymphoid tissues and further undergo differentiation to be mature B cells (naïve B cells) that express IgD besides IgM. These naïve B cells have the potential to be activated by foreign antigen, thus, the humoral immune response is initiated with assistance of helper T cells. At the second phase of the primary B-cell immune response activated B cells migrate to follicles and proliferate to form germinal centre. The B cells with mutations that improve affinity for antigen are selected by the process of somatic hypermutation, terminally differentiate into either memory B cells or antibody secreting B cells (plasma cells) which are end-stage effectors of the humoral immune response (Janeway, 2001).

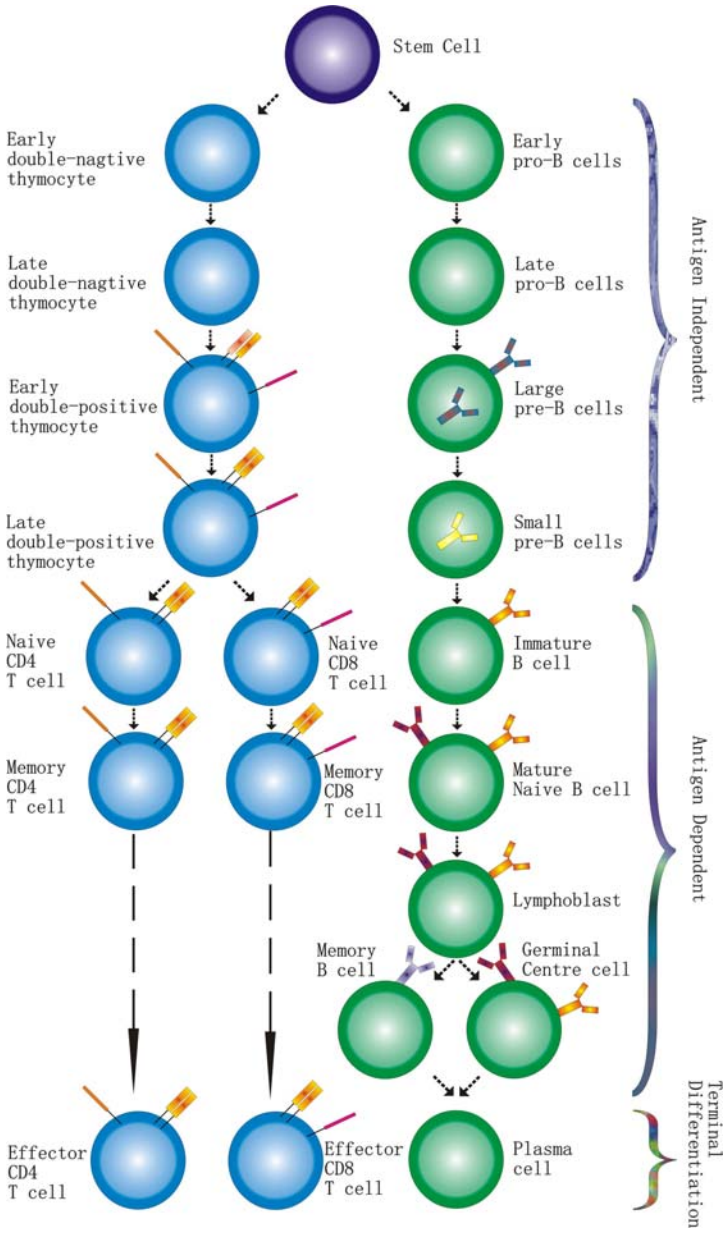


Fig.1. Schematic models of T and B cell developments.

1.1.2. Distinct Stages of T Cell Development

T cells originate from a common lymphocytes precursor in the bone marrow, but all the important events in their development occur in the thymus. The earliest cell population in the thymus is that of 'double-negative' (DN) thymocytes which do not express CD4 and CD8. This precursor with a pre-T cell receptor (β and surrogate pre-T α chain) continues to undergo rearrangement at the α chain locus and express CD4 and CD8, resulting in

CD4+CD8+'double-positive' (DP) T cells with an α:β T cell receptor. Most cells (approximately 95%) undergo apoptotic death by negative selection as engagement between TCR and a peptide-MHC ligand occurs with high affinity. Positive selection (approximately 5% DP cell population) occurs if TCR of DP cells engage a peptide-MHC ligand with low affinity, leading to the transduction of survival and differentiation signals. Engagement of a peptide-MHC class I ligand positively selects for MHC-I restricted, CD8+, cytotoxic T cells, whereas recognition of a peptide-MHC class II ligand allows to develop MHC-II restricted, CD4+, T helper cells (Janeway, 2001).

1.2. Regulation of T and B Cell Differentiation

Antigen stimulation can result in divergent responses that range from the deletion of antigen specific lymphocytes and tolerance to generation of huge numbers of effector cells, followed by establishment of normal immunological memory. These responses indicate that immune responses have to be regulated properly. The mechanism that accounts for the precise relationship between signal strength and T and B cell fate is uncertain. However, it has become quite clear that the decision for differentiation involves the regulation of transcriptional processes that control the cell cycle, response to cytokines, effector functions and susceptibility to activation-induced cell death (AICD). For instance, naïve T cells accumulate and integrate signals from the TCR, co-stimulatory molecules (presented by APC) and cytokine receptors. Once the threshold of signal strength is reached, naïve T cells proliferate, acquire survival capability and responsiveness to homeostatic cytokines, acquire effector function and peripheral-tissue-homing capability, generate memory T cells that are arrested at intermediate stages of differentiation and die by AICD (Reviewed by (Lanzavecchia and Sallusto, 2002)). In particular, regulatory T cells can suppress self-reactive T cells to maintain self-tolerance (Read et al., 1998; Thornton and Shevach, 1998). On the other hand, memory B cells and plasma cells are intermediate and terminally differentiated cells that are responsible for memory and immediate immune reactions, respectively. The decision between memory B cells and plasma cell differentiation is under control of several transcriptional factors. Stimulation through CD40 ligand and IL-4, sustained expression of Pax5 and BCL-6,

prevent terminal differentiation. By contrast, in the absence of CD40 stimulation, IL-2, IL-6, IL-10 result in reduced expression of BCL-6 and induction of expression of Blimp-1 that control plasma cell differentiation (Reviewed by (Lanzavecchia and Sallusto, 2002)). Thus, we have to elaborate the possible regulatory mechanisms on T and B cell development, particularly on B cell terminal differentiation as well as regulatory T cells to fully understand the relationship between these phenomena.

1.2.1. Transcriptional Control of B Cell Terminal Differentiation

1.2.1.1. Blimp-1 (B-Lymphocyte-Induced Maturation Protein 1)

Blimp-1 is qualified as "master regulator' of plasma cell development as Blimp-1 is found in all plasma cells and is sufficient to trigger terminal differentiation in activated splenic B cells, resulting in generation of plasma cells (Calame et al., 2003; Knodel et al., 2001; Schliephake and Schimpl, 1996; Turner et al., 1994). Furthermore, an inhibitory form of Blimp-1 (TBlimp) blocked plasmacytic differentiation of primary splenic B cells after LPS stimulation (Lin et al., 2002; Messika et al., 1998; Shaffer et al., 2002). Because of the early embryonic lethality of Blimp-1 knock-out embryos, definitive proof that Blimp-1 was required for plasma cell differentiation was long missing. However, Blimp-1 conditional knock-out mice were recently generated by crossing mice expressing CD19-driven Cre recombinase with mice whose Blimp-1 locus was flanked by loxP sites. Numbers of IgM secreting cells and CD138+ cells were significantly decreased in Blimp-1 conditional knock-out mice, consistent with lower levels of serum Ig of all isotypes. Moreover, Blimp-$1^{-/-}$ B cells stimulated with LPS also failed to differentiate into CD138+ plasma cells, and as a consequence, were unable to generate wild-type levels of IgM and IgG3 (Shapiro-Shelef et al., 2003). These data strongly suggest that Blimp-1 plays an essential role in plasma cell differentiation.

Blimp-1 is a 98-kDa transcriptional repressor that contains five zinc finger motifs conferring DNA binding ability. It is encoded by the PRDI-BF1 gene in human. A proline-rich region N-terminal to the zinc finger motifs is required for transcriptional repression, which associates with histone deacetylases and hGroucho (Yu et al., 2000). A 12 bp consensus binding site, GTAGTGAAAGTG, has been determined for Blimp-1. In

addition to all plasma cells, Blimp-1 is expressed in a subset of germinal centre B cells (5%–15%) but not found in memory B cells (Angelin-Duclos et al., 2000). Blimp-1 is also expressed in differentiating myeloid lineage cells (Chang et al., 2000) and transiently during mouse embryonic development (Chang et al., 2002). These studies showed that Blimp-1 is much more widely expressed than original expected; however, the precise role of Blimp-1 in these cells is still unknown.

A recent microarray study showed that Blimp-1 down-regulates more than 225 genes and up-regulates more than 30. Among those named genes regulated by Blimp-1, more than 10% were transcription factors, suggesting a cascade of gene regulation initiated by Blimp-1 (Shaffer et al., 2002). Blimp-1 directly represses transcription factors, for example, Pax5, Spi-B, Id3, CIITA and c-myc. Pax5, is a transcription factor critical for commitment and maintenance of the B-lineage (Mikkola et al., 2002; Nutt et al., 1999) and for B cell function through out the GC stage (Nutt et al., 2001). Blimp-1 binds in the Pax5 promoter and represses Pax5 transcription (Lin et al., 2002). Repression of Pax5 by Blimp-1 is required for IgM secretion by LPS-treated splenocytes. Another direct target of Blimp-1 repression is the promoter II of CIITA, a transcriptional co-activator that is critical in regulating the expression of class II MHC (Chang and Flavell, 1995; Piskurich et al., 2000; Steimle et al., 1993). In addition, Blimp-1 binds to evolutionarily conserved sites in the regulatory sequences of both Spi-B and Id3, which are required for effective BCR signalling (Garrett-Sinha et al., 2001; Pan et al., 1999). It suggests that Spi-B and Id3 are direct repression targets of Blimp-1. Also, Blimp-1 has previously been described to repress the c-myc promoter, indicating a requirement for c-myc repression and cessation of cell cycle in plasma cell development.

Overall, these studies showed that genes regulated by Blimp-1 comprise three programs: proliferation, Ig secretion, BCR signalling (Shaffer et al., 2002). The proliferation program repressed by Blimp-1 involves repression of c-myc. On the other hand, Blimp-1 up-regulates mad-4, thus, leading to a shift in the ratios between c-myc and mad4 (Knodel et al., 1999). The anti-apoptotic gene A1 is also down-regulated by Blimp-1. All these alterations are consistent with cell cycle arrest and cell death of terminally differentiated B cells. Secondly, Blimp-1 induces expression of genes required for Ig secretion including XBP-1, J chain and HSP70. Finally, genes encoding BCR signalling

components, such as BLNK, btk, PKC, lyn, syk, CD19, and CD45 are repressed by Blimp-1 (Shaffer et al., 2002). Consistent with the expression pattern of Blimp-1 in plasma cells, BCR signalling inhibits plasma cell formation, involving repression of Blimp-1 (Knodel et al., 2001; Schliephake and Schimpl, 1996). Blimp-1 also down-regulates BCL-6, which is a master regulator for GC development. Therefore, it is reasonable to speculate that the ratio between BCL-6 and Blimp-1 is critical to control the fate of B cells at the late stages of development. Interestingly, Blimp-1 also represses genes required for isotype switch recombination and somatic hypermutation, such as AID, Ku70, Ku86, and DNA-PKcs. Obviously, inhibition of BCL-6, Pax5, AID, c-myc, CIITA and other genes critical for GC function ensures that plasmacytic differentiation initiated by Blimp-1 is irreversible.

Other factors involved in plasma cells differentiation, for example, XBP-1, IRF4, NF-ATc, Octamer proteins and their relationship to Blimp-1 expression are still poorly understood.

1.2.2. Regulatory T Cells

Sakaguchi (Sakaguchi et al., 1995) rekindled interests in the concept of T-cell-mediated suppression in the mid-1990s by showing that a minor population (10%) of CD4+ T cells, which co-expresses the interleukin-2 receptor (IL-2R)α-chain (CD25), is crucial for the control of autoreactive T cells *in vivo*. Subsequent *in vitro* studies by several groups showed that CD4+CD25+ T cells are both hyporesponsive and suppressive (Read et al., 1998; Thornton and Shevach, 1998). CD4+CD25+ T cells were discovered originally in mice, but a population with identical phenotypic and functional properties has been defined in humans.

In addition to CD4+CD25+ T cells that are best termed 'naturally occurring suppressor cells', several *in vitro* and *in vivo* treatments have been shown to generate a spectrum of suppressor T cells (Tab. 1). The naturally occurring CD4+CD25+ T cells might develop in the thymus during T cell development (Fig. 2). Fluorochrome labelling showed that CD25+ T cells emigrate from the thymus to populate the periphery. One possibility is

that CD25 expression and suppressor function is acquired during positive selection on the cortical epithelial cells. Another possibility is that these cells might be educated on medullary dendritic cells (DCs) during the process of negative selection. CD25+ T cells in the thymus might recognise self-antigens presented by medullary DC with an intermediate affinity, which is insufficient to lead to deletion, but high enough to allow them to stay alive and to receive a signal that renders them anergic and suppressive (Shevach, 2002) (Fig.2). Recently, such suppressor T cells have been referred to as regulatory T cells (Tr).

Table.1. Characteristics of regulatory T cells

	CD4+CD25+	Tr1	Tr2	CD8+Tr
Surface Marker				
CD25	+	+	+	?
$CD45RB^{low}$	+	+	?	?
CD45RO	+	+	+	+
CTLA-4	+++	-	++	-
T1-ST2	?	++	?	?
Cytokine secreted				
IL-10	+/-	+++	+	++
TGF-β	+/-	+	+++	+/-
Differentiation factors	Foxp3	IL-10, IFN-α	IL-4, TGF-β	?
Suppressor mechanism				
in vitro	Cell contact	IL-10	TGF-β	IL-10,TGF-β
in vivo	Cell contact, IL-10,TGF-β	IL-10	TGF-β	?

The symbols – to + correspond to the relative surface marker expression or cytokine production by different Tr subtype; +/- corresponds to cytokine production, shown by some but not other studies;? corresponds to unknown.
Abbreviation: IFN, Interferon; IL, Interleukin; TGF, transforming growth factor; Tr T regulatory

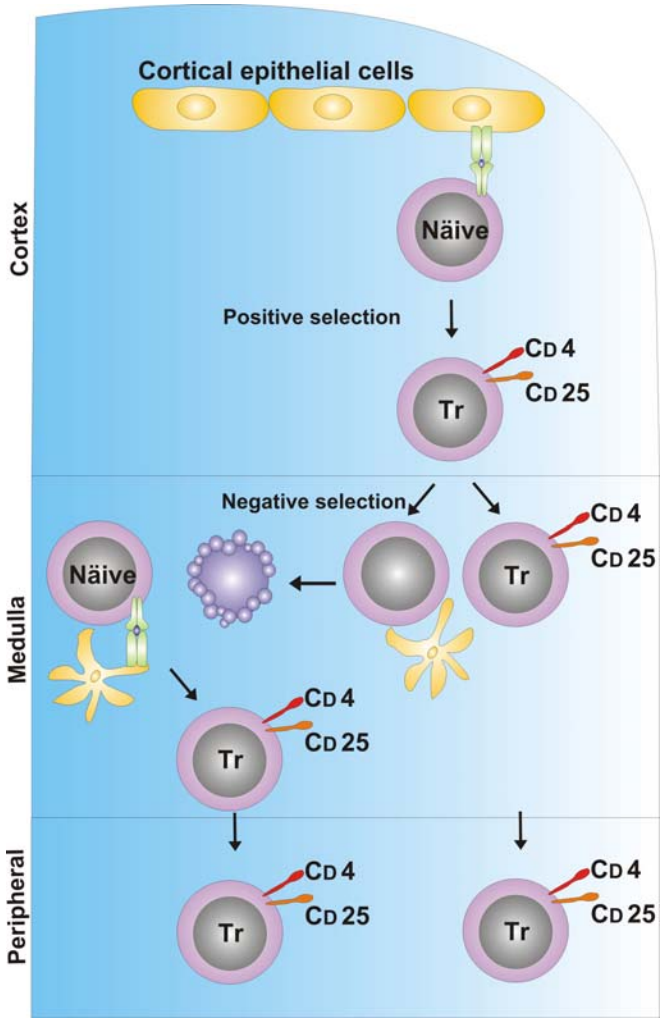


Figure.2. CD4+CD25+ T cells differentiate through positive or negative selection in the thymus.

1.2.2.1. CD4+CD25+ T Cell Mediated Suppression in vitro

1.2.2.1.1. Suppression By Means of a Cell-Contact-Dependent Mechanism.

In vitro proliferation of CD25- T cells induced by CD3-specific antibodies has been shown to be inhibited at a ratio of one CD25+ T cell to four CD25- T cells. Suppression occurred only when the CD25+ T cells were activated via their T-cell receptor (TCR) (Thornton and Shevach, 2000). The main mechanism of suppression appeared to be inhibition of the transcription of IL-2 in CD25- responder cells. Suppression could be

abrogated by the addition of exogenous IL-2 or by enhancing endogenous IL-2 production in responders through anti-CD28 antibody. This antibody mimics the potent stimulus for IL-2 production normally induced by the interaction between CD28 on T cells with CD80 (B7.1) and/or CD86 (B7.2) on antigen-presenting cells (APCs) (Takahashi et al., 1998; Thornton and Shevach, 1998). The CD4+CD25+ T cells might act on APC to inhibit the upregulation of expression of co-stimulatory molecules which are required for activation of responders, and thus indirectly lead to suppression of IL-2 in CD25- T cells. Although cell contact between suppressors and responders is required (Takahashi et al., 1998; Thornton and Shevach, 1998), it is not yet clear whether the CD25+ T cells exert their suppressive effects by means of targeting the responder CD25- T cells or APCs.

In favour of the notion that CD25+ T cells target on responder T cells rather than on APCs, argues that CD4+CD25+ T cells have been found to suppress directly the proliferation of CD8+ T cells and their effectors cytokine production (Piccirillo and Shevach, 2001). CD8+ T cells can be activated readily by peptide–MHC tetramers in the complete absence of APCs. When CD8+ T cells from a TCR-transgenic mouse were stimulated with their target peptide–MHC tetramer, marked suppression of both proliferation and cytokines (IL-2 and IFN-γ) production was seen in the presence of the CD25+ T cells. The results from this experiment show conclusively that CD25+ T cells can mediate suppression via a T-cell–T-cell interaction, and that APCs are not required directly for the delivery of the suppressive signal to the responding CD8+ T cells (Fig. 3). However, this result does not rule out the possibility that CD25+ T cells might also exert inhibitory effects on APCs, or use the APC surface as a platform on which the suppressor cells interact physically with CD4+ or CD8+ responder cells *in vivo*.

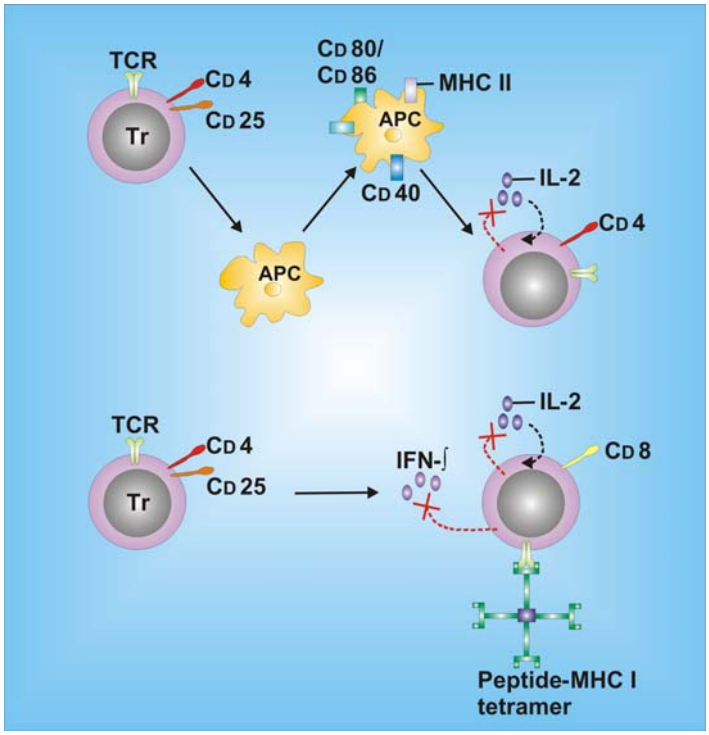


Figure.3. Possible schematic models for suppression of CD4+CD25+ T cells in vitro. CD4+CD25+ T cells might act on APC or directly target CD4+CD25- T cells, resulting in inhibition of IL-2 production and proliferation of CD4+CD25- T cells.

1.2.2.1.2. A Role of IL-2 for CD4+CD25+ T Cells Survival and Maintenance

It was noted first that CD4+CD25+ T cells were absent from the periphery and from the CD4+CD8- thymocyte pool of IL-2-/- mice characterised by the presence of an autoimmune syndrome (Papiernik et al., 1998). This finding suggested that IL-2 is required for the differentiation and/or survival of the CD25+ T cells even if CD25+ T cells never produce IL-2. The CD25+ T cell population might control autoimmunity in an IL-2-dependent manner, either preventing activation or by mediating ACTIVATION-INDUCED CELL DEATH (AICD) of autoreactive T cells. In addition, IL-2 receptor -chain (*IL-2rb*)-/- mice also develop an autoimmune syndrome and lack CD25+ T cells. Selective expression of the IL-2R β chain in the thymus (but not the periphery) prevented autoimmunity and rescued CD25+ T cells (Malek et al., 2000; Malek et al., 2002). These results indicate that IL-2R signalling in the thymus is required to regulate the development of CD25+ T cells and raises the question whether IL-2 is only required for

the development of CD25+ T cells in the thymus but not for their maintenance in the periphery. However, it is possible that CD25+ T cells may be usually maintained in the periphery by IL-2 but that other cytokines, such as IL-4, IL-15 or IL-21, the production of which is dependent on co-stimulatory signals can partly overcome the requirement for IL-2 (Papiernik et al., 1998).

1.2.2.1.3. Possible Molecular Pathways Mediating Suppression

The main issue in the studies of CD25+ T-cell-mediated suppression is to identify the responsible molecular pathways. Engagement of the tumour-necrosis factor/tumour-necrosis-factor receptor (TNF/TNFR) superfamily might result in the inhibition of cytokine production and cell growth similar to that mediated by CD25+ T cells. However, antibodies that are specific for several members of this family have failed to reverse suppression when added to co-cultures of CD25+ and CD25- T cells (McHugh et al., 2002). One member of the TNFR family (the glucocorticoid-induced TNF receptor, GITR, also known as TNFRSF8) has been shown recently to play an important role in the induction of the suppressor function of CD4+CD25+ T cells. It is predominantly expressed on CD4+CD25+ T cells. Moreover, stimulation of GITR abrogated CD4+CD25+ T cell-mediated suppression. In addition, removal of GITR-expressing T cells or administration of a monoclonal antibody to GITR produced organ-specific autoimmune disease in otherwise normal mice (McHugh et al., 2002; Shimizu et al., 2002). A second candidate mechanism would be the engagement of a cell-surface molecule on the CD25- responders that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) by a ligand on the CD25+ suppressor resulting in the activation of phosphatases that could mediate suppression (Sinclair, 2000).

The cytotoxic T-lymphocyte antigen 4 (CTLA4) is constitutively expressed only on CD4+CD25+ T lymphocyte subpopulation in mice. However, the question that arises is whether expression of CTLA4 merely is consistent with the activated phenotype of CD25+ T cells, or whether CTLA4 plays an important functional role. It has been shown that the addition of anti-CTLA4 antibody or its Fab (fragment of antigen binding) reverses suppression in co-cultures of CD4+CD25+ and CD4+CD25- T cells, (Takahashi et al., 2000). These results indicate that the engagement of CTLA4 by its ligands, either

CD80 or CD86, is essential for the induction of suppressive function. Under some circumstances, the engagement of CTLA4 on the CD4+CD25+ T cells by antibody might lead to inhibition of the TCR-derived signals that are required for the induction of suppressor activity. Furthermore, antibody-mediated blockade of the interaction of CD80 or CD86 with CTLA4 might raise the threshold that is required for CD4+CD25+ T cells to mediate suppression.

There are, undoubtedly, other potential molecules that might also be involved in suppression.

1.2.2.2. CD4+CD25+ T Cells Induced Suppression

The relationship between induced regulatory T cell populations and the naturally occurring regulatory population is unclear. Probably, the most intriguing question that must be addressed is whether CD4+ T cell in the normal peripheral lymphoid environment can develop into regulatory cells? Several different *in vitro* protocols have been described over the past few years that result in the generation of regulatory T cells (Tab. 1). The activation of human or mouse CD4+ T cells *in vitro* in the presence of IL-10 or TGF-β has been shown to result in the generation of Tr1 or Tr2 clones (See Tab.1). However, what are the factors that promote the differentiation of CD4+CD25+ T cells?

1.2.2.2.1. The Role of Foxp3

Foxp3 was identified as a novel member of the forkhead/winged-helix family of transcriptional regulators (Brunkow et al., 2001). In addition to the forkhead domain, the protein (referred to as scurfin) also contains a single C2H2 Zinc finger and an apparent leucine-zipper motif. The gene is highly conserved in humans and appears to have a similar function since mutations within Foxp3 result in a severe autoimmune syndrome referred to as IPEX (Bennett et al., 2001; Wildin et al., 2001). Furthermore, the phenotype of scurfin deficient mice resembled that of animals deficient in either CTLA4 or TGF-β.

Although little is known about the biochemistry of scurfin, it can act as a transcriptional repressor as shown by using an IL-2 promoter based reporter assay *in vitro* (Schubert et al., 2001). However, the actual in vivo targets of scurfin have yet to be defined.

The association of Foxp3 mRNA expression with Tr cells indicated a potential functional linkage. Using either retroviral transduction (Fontenot et al., 2003; Hori et al., 2003) or transgenic animals (Khattri et al., 2003), overexpression of Foxp3 lead to the acquisition of in vitro Tr activity by non-Tr cells. Consistently, the "non-Tr cells" that expressed Foxp3 obtained the capability of inhibiting disease *in vivo*. Similar to naturally-occurring Tr cells, a substantial portion of Foxp3-bearing cells also expressed both CD25 and GITR constitutively. Finally, it was demonstrated that all CD4+25+ T cells that developed in mixed bone marrow chimeras originated from Foxp3+ cells. Thus, the development of CD4+25+ T cells is under control of Foxp3.

CD4+25+ T cells develop during thymic selection and Foxp3 mRNA could be controlled directly by the affinity of TCR interactions during selection. However, whether a Foxp3+ subset of Tr cells is also generated in peripheral tissues has not yet been determined. On the other hand, expression of Foxp3 during thymic development alone is insufficient to prevent Foxp3 null animals from disease (Khattri et al., 2001). This suggests that continued Foxp3 expression within peripheral tissues is necessary, either for the maintenance of functional Tr cells or for other unknown functions of scurfin. CD4+ T cells that are induced to overexpress scurfin only obtain suboptimal suppressive activity, possibly due to their hyporesponses to stimulation as well as defects in their cytokine production.

Foxp3 null and CTLA4 null mice have a very similar phenotype. Furthermore, CTLA4 null mice overexpressing Foxp3 display a dramatic delay of the lethality and partial prevention of disease, possibly accounted for by the presence of CD4+CD25+ T cells (Khattri et al., 2003). This suggested that there could also be a direct link between CTLA4 signalling and Foxp3.

Overall, scurfin plays a primary role in the generation and potentially the maintenance of naturally occurring CD4+25+ T cells, representing a distinct T cell lineage.

1.3. Expression of Blimp-1 in T Cells

To determine whether Blimp-1 is expressed in T lymphocytes, Santner-Nanan and colleagues in Wuerzburg first investigated mRNA and protein expression of Blimp-1 in human and mouse T cells and found that Blimp-1 was expressed in purified primary CD3+ T cells and CD19+ B cells from human peripheral blood as well as in Jurkat cells, a human T cell line. Although Blimp-1 was found in all T cell preparations, cells with the CD3+CD4+CD45RA- phenotype, representing human memory T-helper cells, exhibited a more intense Blimp-1 signal than primary CD4+ T cells or memory CD8+ T cells. In parallel, Blimp-1 mRNA was found in mouse T cell (subpopulations) generated in vitro. Non-polarized Th0, Th1 and Th2 populations all expressed Blimp-1 but its expression was always higher in Th2 populations, particularly following re-stimulation. Intriguingly, when enriched CD4+CD25+ and CD4+CD25- T cells were analyzed for Blimp-1 expression before and after 6 hours of activation with PMA/Ionomycin (P/I), CD4+ CD25+ T cell subset expressed about 2.5 times more Blimp-1 mRNA than the CD25- T cell subset both before and after stimulation with P/I by RNase protection assays (Fig.4).

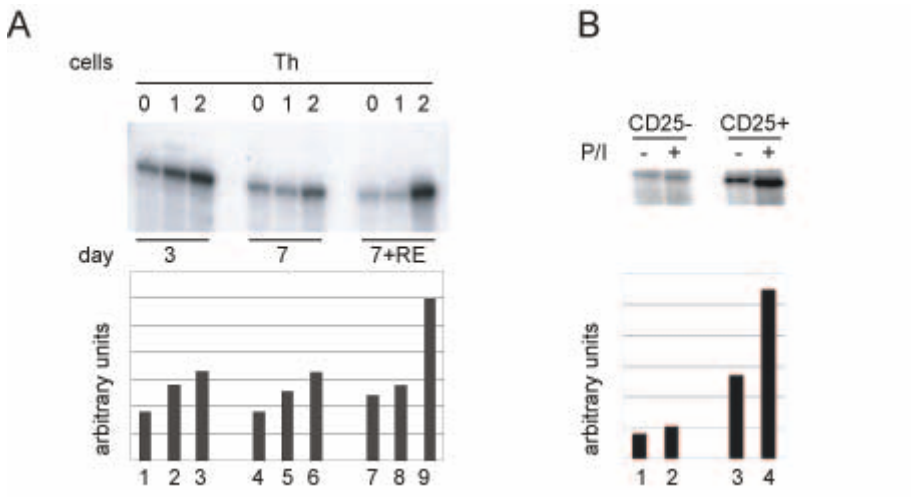


Figure.4. Blimp-1 expression in T0, Th1, Th2 and Tr cells. A: Mouse CD4+ T cells were cultured under Th0 (lanes 1,4,7), Th1 (lanes 2,5,8) and Th2 polarizing conditions (lanes 3,6,9). RNA was prepared after 3 and 7 days. On day 7, polarized T cells were harvested and further cultured either in medium or restimulated with immobilised anti-CD3 and soluble anti-CD28 for 6 hours. B: Freshly isolated CD4+ cells were enriched for CD25+ and CD25- cells. RNA was prepared from CD4+CD25+ and CD4+CD25- cells either directly (lanes 1,3) or following activation by P/I (lanes 2,4) (Santner-Nanan et al., 2004).

2. Objectives

Blimp-1 serves as a master regulator in terminal differentiation, cell cycle arrest and death. The most intriguing question that we addressed was whether CD4+ T cells can develop into a CD4+CD25+ T cell *in vitro* by means of Blimp-1 which promotes the terminal differentiation of B cells. Can Blimp-1 orchestrate terminal differentiation of T cells *in vitro*? The answers to these questions might shed light on the so far puzzling observation on Blimp-1 expression in T cells.

3. Materials and Methods

3.1. Materials

3.1.1. Antibiotics

Zeocin	Invitrogen, Merelbeke, Belgium

3.1.2. Antibodies

3.1.2.1 Antigen Presenting Cell Isolation

Anti-CD8 (monoclonal Rat IgM-AK 3.168.1)	Gift from R, McDonald, Lausanne
Anti-CD4 (monoclonal Rat IgM-AKRL172.4)	Institute for Virology and Immunobiology, Wuerzburg
13-4 (Anti-Thy 1.2, monoclonal IgM)	Institute for Virology and Immunobiology

3.1.2.2. CD4 Cell Isolation

Anti-CD8 (monoclonal Rat IgM-AK 3.168.1)	Gift from R, McDonald, Lausanne
Anti-HSA (J11D)	$ATCC_{15}$

3.1.2.3. CD25 Cell Isolation

Anti-CD25 Biotin	(Clone 7D4)	BD, PharMingen, Hamburg

3.1.2.4. Lymphocyte Stimulation

Anti-CD3	(Clone 145-2C11)	BD,PharMingen
Anti-CD28	(Clone 37.51)	BD, PharMingen
Anti-CTLA-4	(Clone UC10-4F10-11)	BD, PharMingen

3.1.2.5. Cytometry

Rat-anti-mouse-Fc-receptor	(2.4G2) (FC Block)	Institute for Virology and Immunobiology
Anti-CD4 FITC	(Clone H129.19)	BD,PharMingen
Anti-CTLA-4 PE	(Clone UC10-4F10-11)	BD, PharMingen
Anti-CD103-PE	(Clone M290)	BD, PharMingen
Anti-IL-2-PE	(JES6-5H4)	BD, PharMingen
Anti-IL-4-PE	(Clone BVD4-1D11)	BD, PharMingen
Anti-IFN-γ-PE	(Clone XMG1.2)	BD, PharMingen
Anti-mGITR/TNFRSF18-Biotin		R&D Systems, Wiesbaden
Rat IgG2a, κ-PE	(R35-95)	BD, PharMingen
Rat (Lewis) IgM, κ-Biotin	(R4-22)	BD, PharMingen
Amenian Hamerster IgG1, κ-PE	(A19-3)	BD, PharMingen
Rat IgG1, λ-PE	(A110-1)	BD, PharMingen
Amenian Hamerster IgG2,λ-Biotin	(Ha4/8)	BD, PharMingen
Mouse (BALB/C) IgG2b, κ-Biotin	(MPC-11)	BD, PharMingen
Streptavidin Cy		BD, PharMingen
Anexin V, PE		BD, PharMingen

3.1.3. Buffers

PBS (Phosphate buffer saline)
8,00 g NaCl
0,20 g KCl
1,15 g Na2HPO4
2,00 g KH2PO4
1,67 g CaCl2
0,10 g BSA

FACS-Buffer
1 x PBS

0,1 % BSA
0,02 % Natriumazid

BSS (Balanced salt solution)
Working solution:
1 Vol. BSS 1 (10X)
+ 1 Vol. BSS 2 (10X)
+ 8 Vol Water

BSS 1 (10X):
10,0 g Glucose
0,6 g KH_2PO_4
2,3 g $Na_2HPO_4 \times 2H_2O$
0,1 g Phenolrot
Dissolved in 1L H_2O und filter sterilize

BSS 2 (10X):
1,86 g $CaCl_2 \times 2H_2O$
4,00 g KCl
80,0 g NaCl
2,00 g $MgCl_2 \times 6H_2O$
2,00 g $MgSO_4 \times 7H_2O$
Dissolve in lL H_2O und filter sterilize

3.1.4. Cell lines

293T	Human embryonic kidney cells (SV40 large T antigen)	Gift from Dr. Berberich, Wuerzburg

3.1.5. Chemicals

7-AAD (1 mg/ml)	Calbiochem, Deisenhofen

Agrose	Sigma, Deisenhofen
Brefeldin A (1mg/ml)	Sigma
Polybrene (13.2 µg/ml)	Sigma
SNAF-1 carboxylic acid (1 µg/ml in DMSO)	Molecular Probes, Leiden, Netherland

3.1.6. Complement

Guinea pig complement	Institute for Virology and Immunobiology

3.1.7. Enzymes

Taq-Ploymerase	MBI-Fermentas, St. Leon-Rot
RNase-Inhibitor	MBI-Fermentas
M-MulV Reverse Transcriptase	MBI-Fermentas

3.1.8. Interleukins

Interleukin-2 (P30-IL2)	Institute for Virology and Immunobiology

3.1.9. Media

RPMI 1640 Medium with L-Glutamin	Invitrogen
Supplement with	
MEM (non ess. AA 1%)	Invitrogen
Natrium-Pyruvat (1%)	Invitrogen
β-Mercaptoethanol (0,05mM)	Invitrogen
L-Glutamin (0,07%)	Fluka, Buchs (CH)
Penicillin (0,0025%)	Grünenthal, Aachen
Streptomycinsulfat (0,0025%)	Fatol, Schiffweiler

FCS (5%), 30 min at 56°C inactive	PAN Systems, Aidenbach
Dulbecco´s MEM with Glutamax-1 (293T cells)	Invitrogen
Supplement with	
10% FCS	PAN Systems
Penicillin (1%)	Grünenthal
Streptomycinsulfat (1%)	Fatol
DUTCH modified RPMI^{+}	
With supplementary as above + 10% FCS	Invitrogen
X-Vivo	
With supplementary as above + 10% FCS	Bio Whittaker/Cambrex, Verviers, Belgium

3.1.10. Mice

BABL/C	Animal Facility, Institute for Virology and Immunobiology

3.1.11. Nucleic Acids and Nucleotides

10mM dNTP Mix	MBI-Fermentas
Oligo $(dT)_{18}$	MBI-Fermentas

Primers

Blimp seq-3

5’-TAA ACT TGG CAG GGC ACA C-3’

β-actin

Sense: 5’-CCA GGT CAT CAC TAT TGG CAA CGA-3’

Antisense: 5’-GAG CAG TAA TCT CCT TCT GCA TCC-3’

A1

Sense: 5’-ATG GCT GAG TCT GAG CTC ATG-3’

Antisense: 5’-CTC TTT CTC CTC AAG TAA-3’

Blimp-1 and isoform

Sense: 5’-GAA GAA ACA GAA TGG CAA GA-3’

Antisense: 5’-AAG ACA CTT TCA GAC TGG T-3’

c-Myc,

Sense: 5’-GGG CCA GCC CTG AGC CCC TAG TGC-3’

Antisense: 5’-ATG GAG ATG AGC CCG ACT CCG ACC-3’

Foxp3

Sense: 5’-CAG CTG CCT ACA GTG CCC CTA G-3’

Antisense: 5’-CAT TTG CCA GCA GTG GGT AG-3’

Mad4

Sense: 5’-CTC GAG AAT TCC ATG GAG CTG AAC TCT CTG CT-3’

Antisense: 5’-CTC GAG AAT TCG GAT CCC TAC GAA AGG CCA GGG CAG CCA-3’

3.1.12. Radioactivity

^{3}H-Thymidin (6.7 Ci/mmol)	NEN Life Science, Dreieich

3.1.13. Standards

100bp DNA ladder	MBI-Fermentas
1.0kb DNA ladder	MBI-Fermentas

3.1.14. Serum

BSA (Bovine serum albumin)	Boehringer/Roche, Penzberg
FCS (Fatal calf serum)	PAN systems

3.1.15. Other Reagents Used and Kits

ABI PRISM Big Dye terminator cycle sequencing kit	Perkin-Elmer, Rodgau-Juegesheim
First stand cDNA sythesis kit	MBI-Fermentas
Mouse Th1/Th2 cytokine cytometric bead assay (CBA) kit	BD, PharMingen
Mouse CD4 cell recovery column kit	Cedarlane Laboratories/Biozol, Eching
MACS streptavidin microbeads	Miltenyi Biotec, Bergisch-Gladbach
MACS positive separation column(LS^+/VS^+)	Miltenyi Biotec
QIAEX II gel extraction kit	QIAGEN, Hilden
Trizol-reagent (RNA isolation)	Invitrogen

3.1.16. Computer programs

Sequence analyses were performed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystem). The analysis of DNA sequences, restriction maps, alignment of sequences, primer selection were operated through LASERGENE 99 or GCG sequence analysis software package from Wisconsin Package Version 10.2, Genetics Computer Group (GCG), Madison, Wisc. All data acquisition, storage and analysis by FACS were accomplished with Cell Quest software, version 1.0. Mouse Th1/Th2 Cytokine expression by cytometric beads assay was operated through BD CBA software.

3.2. Methods

3.2.1. Cellular Methods

3.2.1.1. Isolation of Lymph Node Cells

Mice were euthanized by CO_2 gas and sterilised with 70% ethanol. Lymph nodes were taken and put into plates with BSS/BSA, ground into single cell suspensions with plunger in a sterilised filter. The cell suspension was filtered through a filter into a 50 ml tube, filled with BSS/BSA up to 50 ml, centrifuged at 1200 rpm for 10 minutes, The supernatants were discarded and the cells were washed with BSS/BSA once more, suspended in X-Vivo$^+$ media and used for further treatment.

3.2.1.2. Enrichment of CD4+ T Cells

Cedarlane's rat T cell recovery column kit was applied to enrich T cells. By a process of negative selection, virtually all B cells are removed from a population of lymphocytes, resulting in enriched T cells in the column eluant (Cedarlane, Hornby, Canada).

Preparation of columns was as described in the company's protocol, allowing the antibody (Polyclonal goat anti-rat/mouse cross reactive IgG (H+L)) in 1.5 ml BSS/BSA to run into the bed of the column. The liquid level was kept about 1cm higher than the top of the column bed. The column was allowed to sit at room temperature for at least 1h, but no more than 10h. Before applying the sample, the column was washed with 20 ml BSS/BSA.

Cell pellets (1.0 to 1.5 x 10^8/per column) were resuspended in 3 ml antibody mixture (1 ml Anti-CD8, Gk1.1 and 2 ml Anti-HSA, J11D) and 2 ml BSS/BSA per column and kept on ice for 30 minutes. After washing two times, cells were applied to the column; a total of 15 ml of eluant was collected. Thus, HSA-positive B and CD8+ T cells were fixed to the anti mouse/rat Ig antibodies on the glass-matrix and only CD4+ T cells passed through the column.

CD4+ T cells were analysed by FACScan procedure (see 4.2.1.7.1). For each experiment, a purity of at least 93% CD4+ T cells was achieved.

3.2.1.3. Enrichment of CD4+CD25+T Cells

MACS streptavidin microbeads were developed for the positive selection of cells in suspension which are labelled with biotinylated antibodies. Cells were labelled with biotinylated antibodies and subsequently magnetically labelled with streptavidin microbeads and separated on the column, which was placed in the magnetic field of a MACS separator. The unlabelled cells run through. After removal of the column from the magnetic field, the magnetically retained cells in the column can be eluted as positively selected fraction (Miltenvi Biotec, Germany).

Cells were labelled with biotinylated Anti-CD25 antibody (7D4) (1:100, 1 X 10^7 cells /100 µl). Typically, staining for 5 minutes was sufficient. Cells were washed carefully by adding 10-20X the volume of labelling buffer (0.1% BSA in PBS), centrifuged at 1200 rpm for 10 minutes and the supernatant was removed completely. The cell pellet was resuspended in 90 µl of labelling buffer per 10^7 total cells. 10 µl of MACS streptavidin microbeads per 10^7 total cells were added and mixed well, incubated for 15 minutes at 6-12°C. Cells were washed carefully with 50 ml separation buffer and the cell pellet was resuspended in 3 ml of separation buffer (BSS/BSA free of air) per 10^8 total cells.

The column (LS^+/VS^+) was prepared as described by the manufacturer. Cell suspensions were applied in 3 ml separation buffer, the negative cells let run through, rinsed with 3 X 3 ml separation buffer (collecting the drops in a clean, sterile 15 ml tube on ice). The column was removed from the separator, placed on a suitable collection tube, firmly flushed out with 5 ml of separation buffer. The positive fractions were washed carefully and the cell pellets were resuspended in appropriate volume of X-Vivo media.

CD4+CD25+ T cells were analysed by FACScan procedure (see 4.2.1.7.1). For each experiment, a purity of at least 90% CD4+CD25+ T cells was achieved.

3.2.1.4. Preparation of Antigen Presenting Cells (APC) from Mouse Spleen

CD4+ and CD8+ T cells from spleen were depleted by complement after incubation with corresponding antibodies and splenic APC isolated.

Spleens were taken, ground into pieces with a plunger in a sterilised cell strainer, all suspensions allowed to go through, filled with BSS/BSA up to 50 ml, centrifuged at 1200 rpm for 10 minutes, washed with BSS/BSA once, cell pellets were resuspended into 5 ml

TAC solution/per spleen and kept on ice for 5 minutes, washed with BSS/BSA again. Anti-CD4 0.5 ml, Anti-CD8 0.5 ml and Anti-Thy (1.2) 1 ml /per spleen were added and the cells were incubated on ice for 30 minutes. After one wash with BSS/BSA, cell pellets were resuspended in 3.3 ml BSS/BSA, mixed well with 1.05 ml guinea pig serum as a source of complement /per spleen, incubated for 45 minutes at 37°C. After washing with BSS/BSA twice, cells were resuspended in 1 ml X-Vivo medium, irradiated (γ-irradiated, 2000 rad) and used as APC.

3.2.1.5. Enriched Dendritic Cell (DC) Preparations

Three spleens were ground in 30 ml Dutch modified RPMI (10% FCS), the suspension centrifuged at 1200rpm for 10 min, the supernatant discarded and the cell pellets were resuspended in 8 ml medium (up to 2 million cells). After culture overnight (37°C, 5%CO_2), the cultured cells were overloaded on the surface of 2 ml 14.5% metrizamid solution very slowly and carefully, centrifuged at 1800rpm for 10 minutes at room temperature without brake. Cells at the interface were collected, washed with 10 ml medium, resuspended in 1 ml X-Vivo medium, irradiated (γ-irradiated, 2000 rad) and used as DC.

3.2.1.6. Cell Activation

3.2.1.6.1. Coating 24-Well Plate with Anti-CD3 Antibody

500µl Anti-CD3 (145.2C11) antibody solution in coating buffer pH 9.5 (2 µg/ml) were pipetted into each well of a 24 well plate, incubated 1 to 2h at 37°C or overnight at 4 °C. Prior to usage, wells were washed very carefully with BSS/BSA (1-2 ml) twice, then 1-2 ml BSS/BSA were added for 5 minutes or 1h at room temperature. BSS/BSA was discarded and cell suspensions were applied to each well.

3.2.1.6.2. Stimulation of CD4+CD25- and CD4+CD25+ T Cells with Anti-CD3 and Anti-CD28 Antibody

1x 10^6 CD4+CD25- and CD4+CD25+ T cells in 1 ml culture media were applied to each well (Anti-CD3 antibody coated 24-well plate) separately, anti-CD28 antibody was added (5 μg/ml), cultured for 72h at 37°C, 5% CO_2.

3.2.1.6.3. Infection of Primary T Cells with Retrovirus

The stimulated T cells (5x10^5, 24 h after stimulation) were centrifuged for 3 hours in 1 ml infectious supernatant (1,000g, 32°C) and replaced with the original culture medium. On day 3 of culture cells were sorted by FACSDiva**.** Purity of sorted populations was always > 93% upon re-analysis.

3.2.1.6.4. Restimulation of Cells

For restimulation (cytokines expression) cells were harvested, washed twice with balanced salt solution, resuspended in fresh medium and stimulated for 6 h on plate bound anti-CD3 (10 μg/ml) in combination with soluble anti-CD28 (5 μg/ml). Two hours before cell harvest, brefeldin A (10 μg/ml) was added.

To measure cytokine production at the later stages of cell culture, 4x10^4 sorted T cells together with 1x10^5 APC (irradiated) were cultured in the presence of 0.5 μg/ml anti-CD3 mAb (200 μl) at 37°C, 5% CO_2 and the culture supernatant was harvested after 24 h of culture.

3.2.1.7. FACS Analysis

3.2.1.7.1. FACS Analysis of Molecules on the Surface of Cells

Cells (1x 10^5-10^7) were washed with 4 ml FACS buffer, centrifuged at 1300rpm for 7 minutes (megafuge), the supernatants were discarded, the cell pellets were resuspended in 25 μl FC block (Anti-FcR II/III (2.4G2) solution (1:25), kept for 10 minutes at 4°C, 75 μl fluorescence conjugated antibody solution (optimal concentration) was added and kept for 15 minutes at 4°C in dark, washed with 4 ml FACS buffer once. For biotinylated antibody, the cell pellets were resuspended in 25 μl Streptavidin-Cy-chrome solution (1:200), kept for 10 minutes at 4°C in dark, washed with 4 ml FACS buffer. Cells were fixed with 200 μl 4% PFA and 200 μl FACS buffer, ready for FACScan analysis.

3.2.1.7.2. FACS Analysis of Molecules Inside Cells (Intracellular staining)

Cells ($2x10^5$) were harvested and washed with ice-cold FACS buffer, cell pellets were resuspended in 50 µl Fc block solution (1:50) for 10 minutes at 4°C, 50 µl 4% Formaldehyd was added and cells were incubated for at least 20 minutes at room temperature. After one wash with 4 ml FACS buffer, the cell pellets were resuspended in 1 ml 0.1% Saponin buffer, incubated for 10 minutes at room temperature, centrifuged and the supernatant discarded, cell pellets were resuspended in 25 µl fluorescence conjugated antibody solution (optimal concentration), vortexed and incubated for 15 minutes at room temperature in the dark. Cells were washed with 1 ml 0.1% Saponin buffer, once with 4 ml FACS buffer and analyzed by FACScan.

3.2.1.8. Analysis of Cell Proliferation

^3H Thymidin is integrated in the replicated DNA in proliferating cells. All proliferation tests were performed in 96-well, round bottom plates (total volume 0.2 ml/per well).

The freshly-prepared CD4+CD25-T cells ($2x10^4$/50 µl/well), T cell–depleted spleen cells ($2x10^5$/50 µl/well, irradiated), anti-CD3 antibody solution (50 µl) (0, 1, 3, 10 µg/ml, respectively), indicated numbers of stimulated CD4+CD25+ T cells or stimulated Th0 after infection with retrovirus ($2x10^4$/50 µl/well) were cultured for 72 h at 37°C, 5% CO_2. Cultures were pulsed with 25 µl ^3H-Thymidin (0.25 µCi) each well for the last 8 h of culture. Cells were harvested with a Beta-Plate-Harvester (Pharmacia) and incorporated ^3H were measured in a Beta counter.

3.2.1.9. Cytokine Bead Assay

The expression of levels of the cytokines IFN-γ, TNF-α, IL-5, IL-4 and IL-2 was determined by using a new cytokine bead array (BD Biosciences San Diego CA, USA).

The assays consist of a two-site sandwich immunoassay. The particles are covalently coupled with an antibody (Ab) against each of the 5 cytokines. Each particle population of a given intensity level represents a discrete population for constructing an immunoassay for a single cytokine. To configure the assay for each cytokine, the fluorescent phycoerythrin (PE) conjugated "detector" antibody is used to complete the

sandwich. The fluorescence intensity measurement of PE is proportional to the concentration of the cytokine in the sample. Briefly, the assay features Ab-bead mix, detector Ab-PE mix and calibrator mix for 5 cytokines. The paneled assay is run at once with 50 µl sample on a flow cytometer after incubation with cytokine capture beads and PE detection reagent, respectively. At the end of the experiment 5 separate results from each sample are simultaneously available. Custom software is provided to assist the data analysis by generating calibration curves and calculating sample concentration of the cytokines.

3.2.2. DNA Techniques

3.2.2.1. Determination of DNA Concentration

The DNA solutions were diluted from 1:10 to 1:200 (Readings should be taken at the wavelengths of 260 nm and 280 nm). The reading at 260 nm allowed the calculation of the concentration of nucleic acids in the samples (Beckman DU 640 spectrophotometer).

The concentration of DNA was calculated as follows:

[DNA] =OD_{260} x dilution factor x 50 µg/ml

The ratio between the reading at 260 nm and 280 nm (OD_{260}/ OD_{280}) provided an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD_{260}/ OD_{280} values of 1.8. If there was a contamination with protein, the OD_{260}/ OD_{280} were significantly less than the value given above. If there was contamination with RNA, the OD_{260}/ OD_{280} were close to 2.0.

3.2.2.2. Extraction of DNA with Phenol-Chloroform

Phenol-chloroform extraction is a common technique used to purify a DNA sample from protein contamination.

An equal volume of phenol-chloroform was added to the DNA sample contained in a 1.5-ml microcentrifuge tube. The mixture was vortexed vigorously for 15-30 seconds until an emulsion formed, then centrifuged (14000 rpm) at room temperature for 5 minutes to separate the phases. About 90% of the upper, aqueous layer was removed to a clean tube, carefully avoiding the protein precipitates of the aqueous: phenol interface. At this stage,

the aqueous phase could be extracted a second time with an equal volume of phenol-chloroform, but this additional extraction usually was not necessary if care was taken during the first phenol extraction. After the extraction was repeated, DNA was concentrated by ethanol precipitation.

3.2.2.3. DNA Precipitation with Ethanol

Solution: 3M sodium acetate

10:0.1 TE buffer (10 mM Tris-HCl, pH 7.6-8.0, 0.1 mM EDTA)

Most nucleic acids may be precipitated by addition of monovalent cations, recovered by centrifugation and dissolved in an appropriate buffer at a desired concentration.
2.5 volumes of ethanol and 1/10 volume 3M sodium acetate were added to the DNA sample contained in a 1.5 ml microcentrifuge tube. The mixture was inverted several times and incubated in an ice-water bath for at least 10 minutes. It was possible to place the sample at -20°C overnight at this stage. The mixture was centrifuged at 4°C, 12,000 rpm for 15 minutes and the supernatant was decanted by inverting the tube on a paper towel. 70% ethanol (corresponding to about two volumes of the original sample) was added; the mixture was incubated at room temperature for 5-10 minutes and centrifuged again for 10 minutes. The supernatant was decanted as above. The DNA pellet was dried in a Savant Speed-Vac and dissolved in 10:0.1 TE buffer.

3.2.2.4. Agarose Gel Electrophoresis

Solution: 1x TAE: 40 mM Tris-Acetat, 10 mM EDTA (50x TAE: 242 g Tris, 57.1 ml acetic acid, 100 ml 0.5M EDTA pH 8.0, ad 1000 ml H2O)

Agarose gel electrophoresis was employed for example to check the progression of a restriction enzyme digestion, as well as to quickly determine the yield and purity of DNA isolations or PCR reactions. Electrophoresis was used to separate molecules based on their size and charge. DNA has a negative charge in an appropriate buffer solution, so it migrates to the positive pole in an electric field. In agarose gel electrophoresis, DNA was forced to move through a sieve made of agarose. The result was that the large pieces of

DNA moved more slowly than small pieces of DNA. Ethidium bromide was included in the gel matrix to enable fluorescent visualization of the DNA fragments under UV light (λ=302 nm). Ethidium bromide molecules intercalate into double strands of DNA and emit orange fluorescence light (λ=590 nm) upon excitation by UV light.

Dried agarose was dissolved in the appropriate volume of 1 x TAE buffer by heating and 1/10000 volume of ethidium bromide (10 mg/ml) was added to the warm gel solution (60˚C). Then the gel was poured into a mold, which was fitted with a well-forming comb. The percentage of agarose in the gel could vary between 0.5% and 2.5%, depending on the expected size of the DNA fragments to be separated. The agarose gel was submerged in electrophoresis buffer within a horizontal electrophoresis apparatus. The DNA samples were mixed with loading buffer and loaded into the sample wells. Electrophoresis usually was performed at 1-5 V/cm at room temperature, depending on the desired separation. Size markers were also loaded with DNA samples to aid in fragment size determination. Two types of size markers were used, 1 kb ladder markers (Pharmacia) and 100 bp-ladder markers (GibcoBRL). After electrophoresis, the gel was placed on an UV light box and pictures of the fluorescent ethidium bromide-stained DNA separation pattern were taken with a video camera.

3.2.2.5. Elution of DNA Fragments from Agarose Gels

The QIAquick (Qiagen) purification procedure removed primers, nucleotides, enzymes, mineral oil, salts, agarose, polyacrylamide, ethidium bromide, dyes, detergents and other impurities from DNA samples.

The QIAquick system uses a simple bind-wash-elute procedure. DNA fragments were purified using low-melting temperature agarose gels and the band of interest was excised with a scalpel under UV illumination. The gel slices were mixed with the appropriate binding buffer and then applied to the spin columns where the DNA bound to the silica-gel membrane. The impurities were washed away and the pure DNA was eluted in a small volume of low-salt elution buffer. The purified DNA was ready for use in any subsequent application (Modified from Qiagen gel extraction kits).

3.2.2.6. Cycle-Sequencing

The sequences of PCR products and plasmids were obtained by the cycle sequencing method (Rosenthal and Charnock-Jones, 1992) with a ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (PE Biosystems). In the ready reaction format, thermally stable AmpliTaq DNA polymerase, modified deoxynuleoside triphosphates (dNTP) and a set of dye terminators labelled with high-sensitivity dyes were provided. Once a sequence-specific primer was designed, the sequencing could be carried out using this kit.

The cycle-sequencing reaction system was set up as follows: 1 µl template DNA (less than 1 µg), 6 µl terminator ready reaction mix; 1 µl (5 pmol/µl) primer; 12 µl ddH2O, 20 µl total volume in a thin-wall tube. The cycle sequencing was performed in the GeneAmp PCR systems 9700 (Perkin Elmer).

The sequencing program was as follows: 96˚C for 2 minutes, 25 cycles with: 96˚C for 10 seconds, 50˚C for 5 seconds, 60˚C for 4 minutes.

The reaction mixture was transferred to a microcentrifuge tube. 2µl 3M sodium acetate (pH 4.6) and 55µl 100% ethanol were added. The solution was kept at room temperature for 15 minutes and centrifuged at 14000 rpm for 20 minutes. The supernatant was discarded, 150 µl 70% ethanol was added, the mixture was centrifuged at full speed for 10 minutes, the 70% ethanol washing was repeated once, then the DNA pellet was dried in a vacuum centrifuge for 15 minutes. Capillary electrophoresis and data collections were performed on the ABI PRISM 310 Genetic Analyzer (PE Biosystems).

3.2.2.7. Transient Transfection of 293T Cells Mediated by Calcium Phosphate

Solution: 2 M $CaCl_2$

2x HBS: 50 mM Hepes, 10 mM KCl, 12 mM Dextrose, 280 mM NaCl,

1.5 mM Na2HPO4, pH 7.01

Calcium phosphate mediated DNA coprecipitation was used for the transfer of foreign DNA into cells. The appearance of calcium phosphate crystals strongly depended on the pH and the total amount of DNA (Graham and van der Eb, 1973).

The day before the transfection, 1.5 x 10^6 293T cells were seeded in 60 mm dishes with 5 ml DMEM containing 10% FCS to reach 50% to 70% confluence before transfection. 2h before transfection, media were replaced with 4 ml fresh culture medium. pBLIMP-1F or pEYZ 8µg and 5 µg packaging DNA, 500 µl of 2 x transfection buffer (HBS) (pH 7.01), 425 µl water as well as 65µl of 1M aqueous $CaCl_2$ were added to a 4-ml polystyrene tube. The mixture was vigorously pipetted up and down several times to make air bubbles. Afterwards, the mixture was equally applied to the cultured cells with 5 ml medium. Cells were incubated for 24h at 37°C, 7.5% CO_2. The transfection medium was replaced with 4 ml of DMEM containing 10% FCS. The supernatant was harvested at 48 and 72 h after transfection. The harvested culture supernatants were filtered through 0.45 µm filters to remove cellular debris, supplemented with polybrene (1:1000 dilution, 13.2 µg/ml) (Sigma, Deisenhofen, Germany) and stored at -70°C.

PS: pBLIMP-1F vector obtained Blimp-1, EYFP and Zeocin open reading frame. pEYZ vector had a similar structure except for the omission of Blimp-1.

3.2.3. RNA Technology

3.2.3.1. Isolation of RNA from Eukaryotic Cells (TRIZOL Method)

Solution: Chloroform, RNase-free

Isopropanol, RNase-free

70% (v/v) Ethanol in DEPC-H2O

DEPC-H2O: H2O, 1:1000 with DEPC (Diethylpyrocarbonat) stock solution

The total RNA of eukaryotic cell was isolated by using Trizol reagent, following the manufacturers' protocol. The whole procedure was conducted in RNase free plastic ware and solution.

1 x 10^6-1 x 10^7cells were washed with 1 X PBS, spun down at 1300 rpm for 7 minute, excess PBS was removed and 1 ml Trizol reagent was added, vortexed and inverted, kept at room temperature for 10 minutes (should see stringy-like material), centrifuged at 14000 rpm for 10 minutes at 4° C. The supernatant was removed to a fresh RNase free Eppendorf tube and 200 µl of chloroform was added, vortexed for 15 seconds and kept at

room temperature for 3 minutes, centrifuged at 12000 rpm for 15 minutes at 4°C. The top layer (clear) was carefully removed into a new RNase free Eppendorf tube and 500 µl of isopropanol was added, inverted to mix, kept for 10 minutes at room-temperature and centrifuged at 12000 rpm for 10 minutes at 4°C. (RNA was seen as a clear white pellet). The pellet was washed with 100 µl of 75% ethanol (made by diluting into DEPC-treated water), centrifuged at 13000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet was drained in air for 10 minutes. The pellet was dissolved in 25 µl of DEPC-treated water, heated for 10 minutes at 60°C to dissolve RNA. RNA was stored at -20°C.

3.2.3.2. Measurement of RNA Concentration

The RNA solutions were diluted 1:70 in microcuvette (70 µl volume) and readings were taken at the wavelengths of 260 nm and 280 nm. The reading at 260 nm allowed the calculation of the concentration of RNA in the samples (Ultro spectrophotometer Plus 4045, Pharmacia).

The concentration of RNA was calculated as follows:

C (µg/µl) = C (µg/ml) x dilution factor / 1000

3.2.3.3. RT-PCR

3´-poly(A)-end eukaryotic mRNAs were hybridized with Oligo$(dT)_{18}$ primer, and then reverse transcribed by M-MuLV reverse transcriptase.

Synthesis of first strand cDNA suitable for PCR amplification:

Total RNA	1-5 µg
Oligo$(dT)_{18}$ primer (0.5 µg/µl)	1 µl
Deionised water	up to 11 µl

Components were mixed gently and spun down for 3-5 sec in a microcentrifuge, incubated at 70°C for 5min, chilled on ice and drops collected by brief centrifugation. Tube was placed on ice and the following components were added in the indicated order:

5 X reaction buffer	4 µl
Ribonuclease inhibitor (20 U/µl)	1 µl
10mM dNTP mix	2 µl

Mixture was spun down for 3-5 sec in a microcentrifuge, incubated at 37°C for 5 min, M-MuLV reverse transcriptase (20 U/μl) 2μl was added, incubated at 37°C for 60 min, stopped by heating at 70°C for 10 min, chilled on ice.

To relatively quantify cDNA, each sample was first normalized after semi-quantitative PCR for β-actin. Reaction mixtures (25 μl) contained: 2 mM MgCl2, 0.2 mM of each dNTP, 5 pmol forward and reverse primers, and 0.6U Taq DNA polymerase in the supplier's buffer. PCRs were performed in a PE9600 (Perkin Elmer). For β-actin amplification, PCR consisted of 2 min at 94°C denaturation step followed by 22 cycles of 30 sec at 94°C, 30 sec at 62°C, 30 sec at 72°C. For Foxp3, reaction was conducted as described above except that the annealing temperature was 57°C and the number of cycles 32. For Blimp-1 and its isoform, the reaction was conducted as follows: 2 min at 94°C denaturation step followed by 30 cycles of 60 sec at 94°C, 60 sec at 55°C, 60 sec at 72°C. To detect A1 gene expression, reaction was conducted as follows: 2 min at 94°C denaturation step followed by 40 cycles of 60 sec at 94°C, 60 sec at 62°C, 120 sec at 72°C. For the Mad4 gene, reaction was conducted as follows: 2 min at 94°C denaturation step followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C, 45 sec at 72°C. For c-Myc reaction was conducted as follows: 2 min at 94°C denaturation step followed by 40 cycles of 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C.

4. Results

Terminally differentiated, antibody-secreting plasma cells are the end-stage of mature B cells. Blimp-1 has been referred to as a 'master regulator' of this process. Blimp-1 is a 98 KDa zinc finger-containing protein which functions as a transcriptional repressor. Expression of Blimp-1 is sufficient to trigger terminal differentiation of B lymphocytes. Blimp-1 is also required for terminal differentiation of monocyte/macrophages. Recently, we have identified that Blimp-1 is also expressed in subsets of T cells, including the CD4+ CD25+ T cell subset which expressed about 2.5 times more Blimp-1 mRNA than the CD25- T cell subset both before and after stimulation with P/I as measured by RNase protection assays. A small number of CD4+ T cells constitutively express CD25 (5–10% of total CD4+ T cells), are generated in the thymus, and migrate into the periphery to regulate normal immune responses and maintain self-tolerance. CD4+ CD25+ regulatory T cells regulate the balance between immunity and tolerance to safeguard the host against autoimmunity and immunopathology. Although Blimp-1 is expressed in T cells subsets, virtually nothing is known about both regulation of Blimp-1 and the function of Blimp-1 in T cells. Therefore, we wanted to investigate whether Blimp-1 also triggers terminal differentiation of T cells or whether Blimp-1 even drives primary T cells to differentiate into CD4+CD25+ T cells.

4.1. Characteristics of Blimp-1 mRNA Isoforms

Murine Blimp-1 genomic DNA spans ~33 kb and contains eight exons. The identified domains are encoded within separate exons except the PR domain which is encoded in exon 4 and 5 and the zinc finger domains which are encoded in exon 6,7 and 8. Three major Blimp-1 mRNA are synthesized by different polyadenylation, ~5.7, 4.3 and 3.6 kb respectively (Tunyaplin et al., 2000). However, all these major mRNA are translated into the same protein. Of particular interest, a minor splice variant is generated by using alternative splice sites, lacking part of exon 7. Partially losing exon 7 leads to an

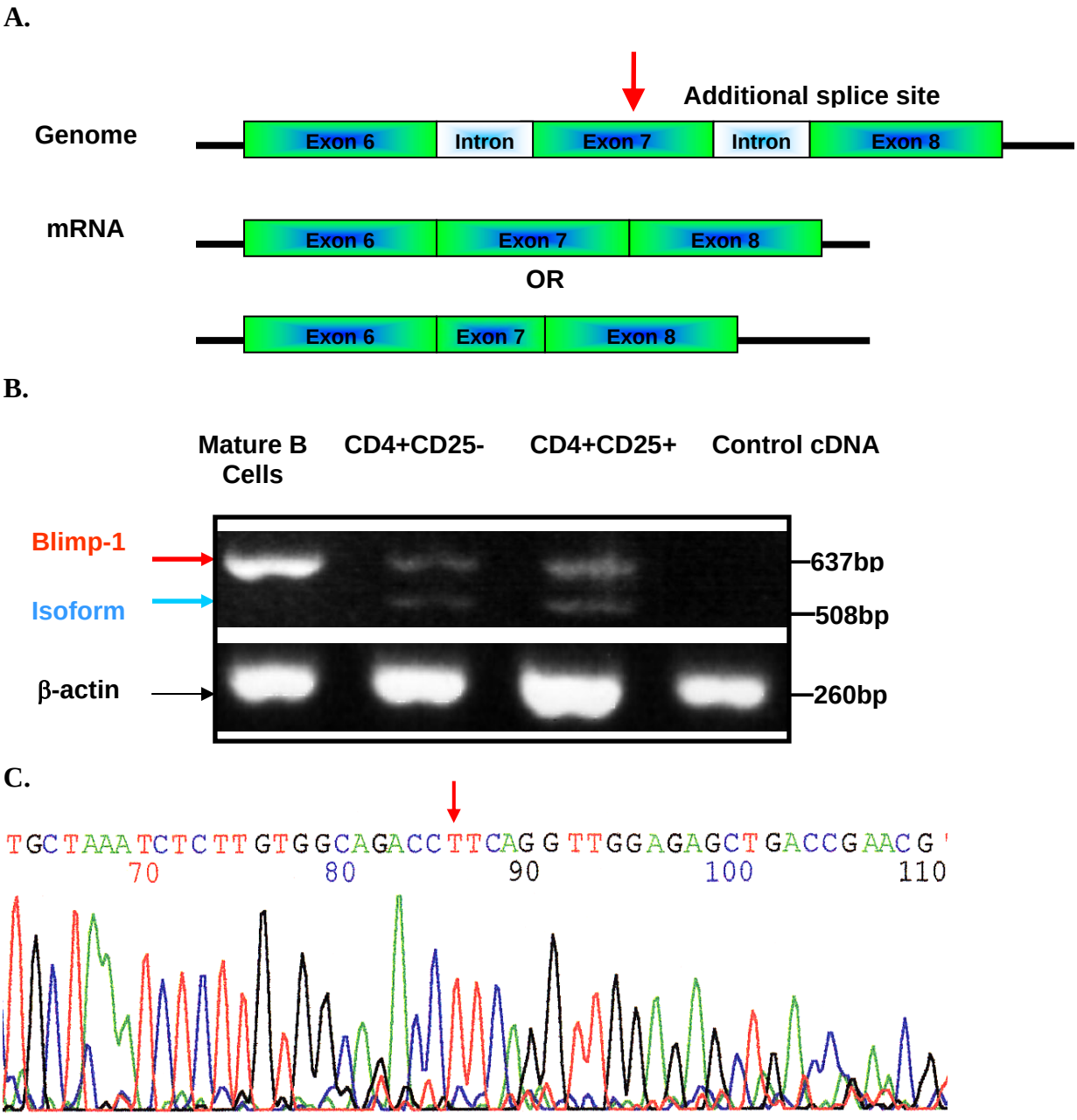


Fig.5. A. Schematic model of Blimp-1 isoform generated by the usage of an additional splice site in Exon 7. B. Isoforms of Blimp-1 were detected by RT-PCR in different mouse T cell subsets. C. The additional splicing site in Exon 7 was verified by sequencing the shorter Blimp-1 RT-PCR product. The red arrow indicates the junction between part of Exon 7 and Exon 6. As a down stream primer was used, the bottom strand of partial Exon 7 is located on the left of the arrow, the right side of the arrow stands for Exon 6 (bottom strand). The standard junction between Exon 6 and 7 is not shown.

alternatively spliced mRNA detected by RT-PCR with a ~120 bp smaller product. The protein encoded by this exon 7 isoform lacks zinc finger 2 and parts of zinc finger 1 and 3, is supposed to bind DNA inefficiently. Thus, this isoform might have an influence on Blimp-1's function.

In order to understand the regulation of Blimp-1 in T cells, primers for detection of exon 7 isoforms were utilized. Full length Blimp-1 was expected to yield RT-PCR products with 637 bp, while RT-PCR products with 508 bp would be generated due to a Blimp-1 isoform RNA. Intriguingly, besides a product predicted for the full length Blimp-1 mRNA, a ~128 bp smaller RT-PCR product was detected in freshly isolated CD4+CD25- and CD4+CD25+ T cells. Sequencing (Fig.5) showed that the complete coding part of Exon 7 was missing in this isoform. As exon 7 is crucial for encoding the whole zinc finger 2 and part of zinc finger 1 and 3, it is reasonable to speculate that Blimp-1's function in T cells might be affected, given the two types mRNA are translated equally. In other words, the truncated protein encoded by exon 7 isoform might be competitive to its functional protein and eventually act as a dominate negative Blimp-1 in T cells. Only full length Blimp-1 was detected in mature, activated B cells, indicating that this isoform might be sufficient to play a key role in terminal differentiation of B cells.

4.2. Effects of Ectopic Expression of Blimp-1 in Primary T Cells

Although Blimp-1 is found in naïve and CD4+CD25+ T cells and increased after stimulation, the question was whether primary T cells can be induced to be CD4+CD25+ Tr cells *in vitro* under certain circumstance, for example, by Blimp-1. To answer this question, Blimp-1 was introduced into primary T cells to be expressed ectopically. The retroviral system is a useful tool for gene transfer, especially for lymphocytes which are hardly transfected by most other methods. The full length Blimp-1 and control viral substrates with an EYFP transfection marker were prepared as described (Knodel et al., 2001). An IRES was inserted between the Blimp-1 and EYFP cDNA, therefore, the transcriptional copies of EYFP correlated well with those of the Blimp-1. As a result, EYFP was set as both a transduction marker and an indicator of Blimp-1 ectopic

expression. Primary T cells were infected with retrovirus 24h after stimulation with anti CD3 under neutral non-polarizing conditions. On day 3 of culture, yellow fluorescent

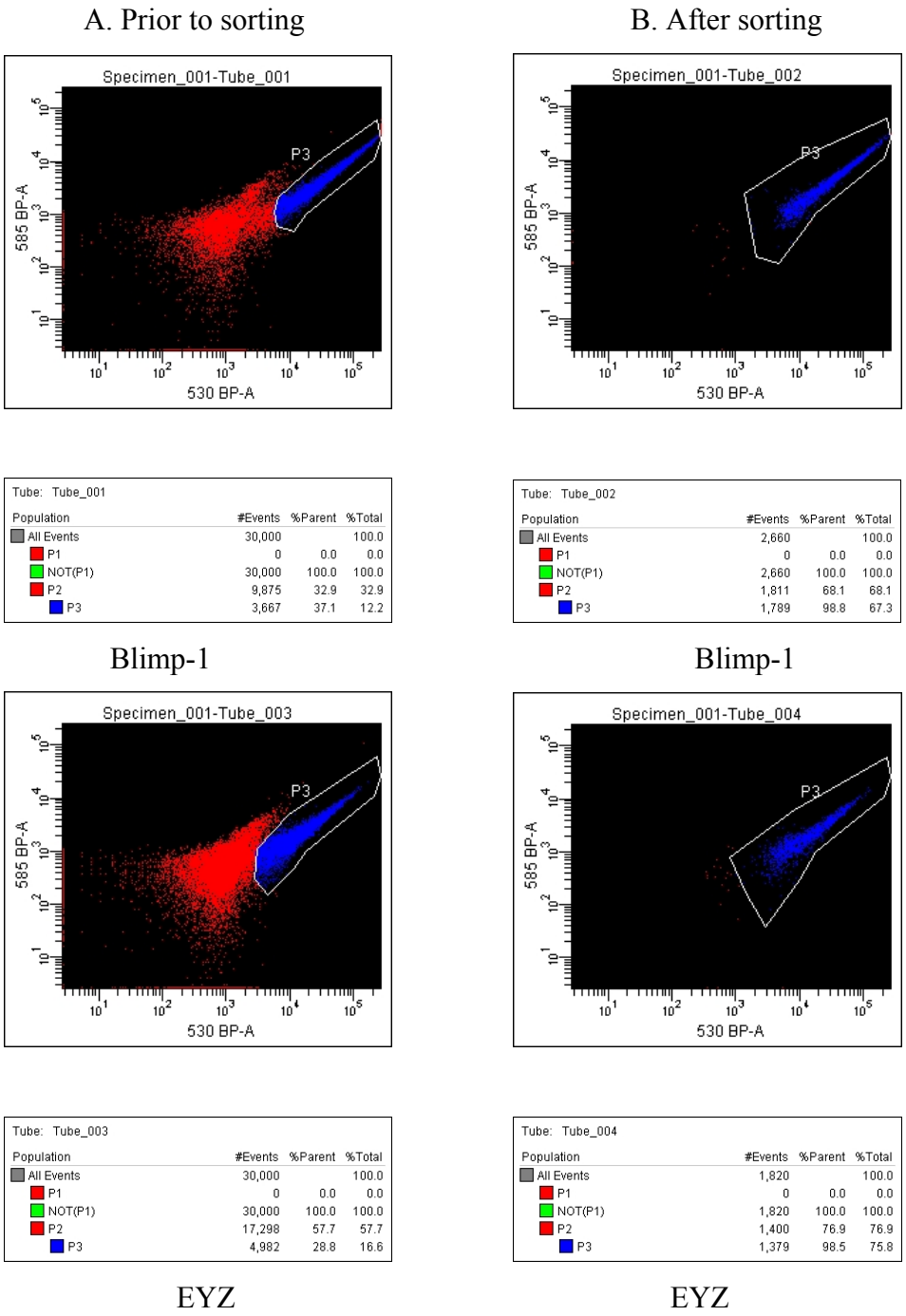


Fig.6. Flow cytometer analysis of T cells transduced with Blimp-1 and EYZ prior to and after sorting.

cells were sorted on a FACS Vantage (DB Bioscience, CA). Before sorting, about 30-50% cells among total populations were yellow fluorescent cells for Blimp-1 transduced T cells, while 98% cells were EYFP positive after sorting (Fig 6). Cultures treated with the control EYZ showed almost the same result. This experiment was repeated many times, with almost identical results. The sorted yellow fluorescent cells were restimulated and used for intracellular fluorescence, cytokine profiling or ^{3}H-Thymidine incorporation as given in Materials and Methods.

4.3. Characterization of Surface Molecules on Blimp-1 Transduced T Cells

Surface and secreted molecules are critical to characterize the phenotypes of functionally different T cell subsets. For instance, several molecules have been implicated in the suppressor mechanisms underlying the immunoregulatory function of CD4+CD25+ Tr cells, including cell surface molecules such as CTLA4 and glucocorticoid-induced TNFR, or secreted molecules such as TGF-β1, IL-10. CD25 is a characteristic surface marker of CD4+CD25+ Tr cells isolated in vivo; however, its expression can be activated after stimulation with anti-CD3 antibody in vitro. Compared with the EYZ transduced T cells, there is no significant difference in CD25 expression when Blimp-1 was transduced in vitro activated primary T cells.

TGF-β1 signalling of target cells was also demonstrated somewhat more indirectly by the fact that CD25- target cells cocultured with CD4+CD25+ T cells were induced to express CD103 (α_E integrin), an integrin previously shown to be regulated by TGF-β1 (Nakamura et al., 2004). The Blimp-1 transduced T cells expressed CD103 at a low level, similar to that of the EYZ transduced T cells.

It has been shown that the addition of anti-CTLA4 antibody or its Fab (fragment of antigen binding) reverses suppression in co-cultures of CD4+CD25+ and CD4+CD25- T cells (Takahashi et al., 2000). These results indicate that the engagement of CTLA4 by its ligands, either CD80 or CD86, is essential for the induction of suppressive function. Under some circumstances, the engagement of CTLA4 on the CD4+CD25+ T cells by antibody might lead to inhibition of TCR-derived signals that are required for the

induction of suppressor activity. Furthermore, antibody-mediated blockade of the interaction of CD80 or CD86 with CTLA4 might raise the threshold that is required for CD4+CD25+ T cells to mediate suppression. However, our results showed that the percentage of cells in Blimp-1 transduced anti-CD3 stimulated T cell populations and vector control cells expressed CTLA4 similarly.

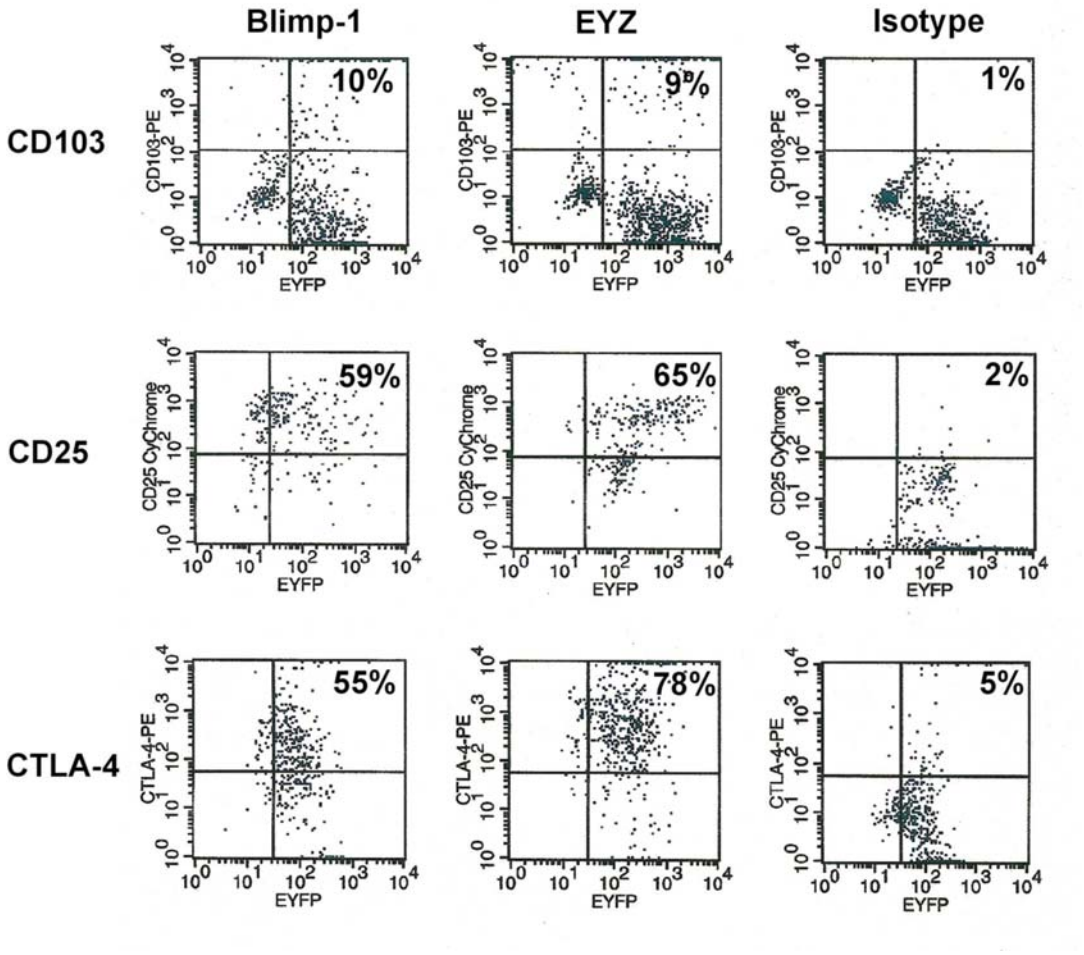


Fig.7. Analysis of surface molecules on Blimp-1 transduced T cells and EYZ vector controls. CTLA4 was measured by intracellular fluorescence.

4.4. Profile of Cytokines in Blimp-1 Transduced Primary T Cells

The cytokine milieu is crucial for determining the outcome of an immune response or T and B cell development. T or B cells produce a dynamic profile of cytokines depending

on the strength, timing and nature of stimulation they receive. Cytokines, such as IL-10 or TGF-β provide an environment conductive to Tr differentiation. Stimulation of naïve T cells in presence of IL-10 or TGF-β leads to the generation of Tr1 or Tr2, respectively. Therefore, a profile of cytokines is essential and crucial for development of Tr. Particularly, analysis of cytokine expression pattern might open avenues to understand the phenotypes of Blimp-1 transduced primary T cells.

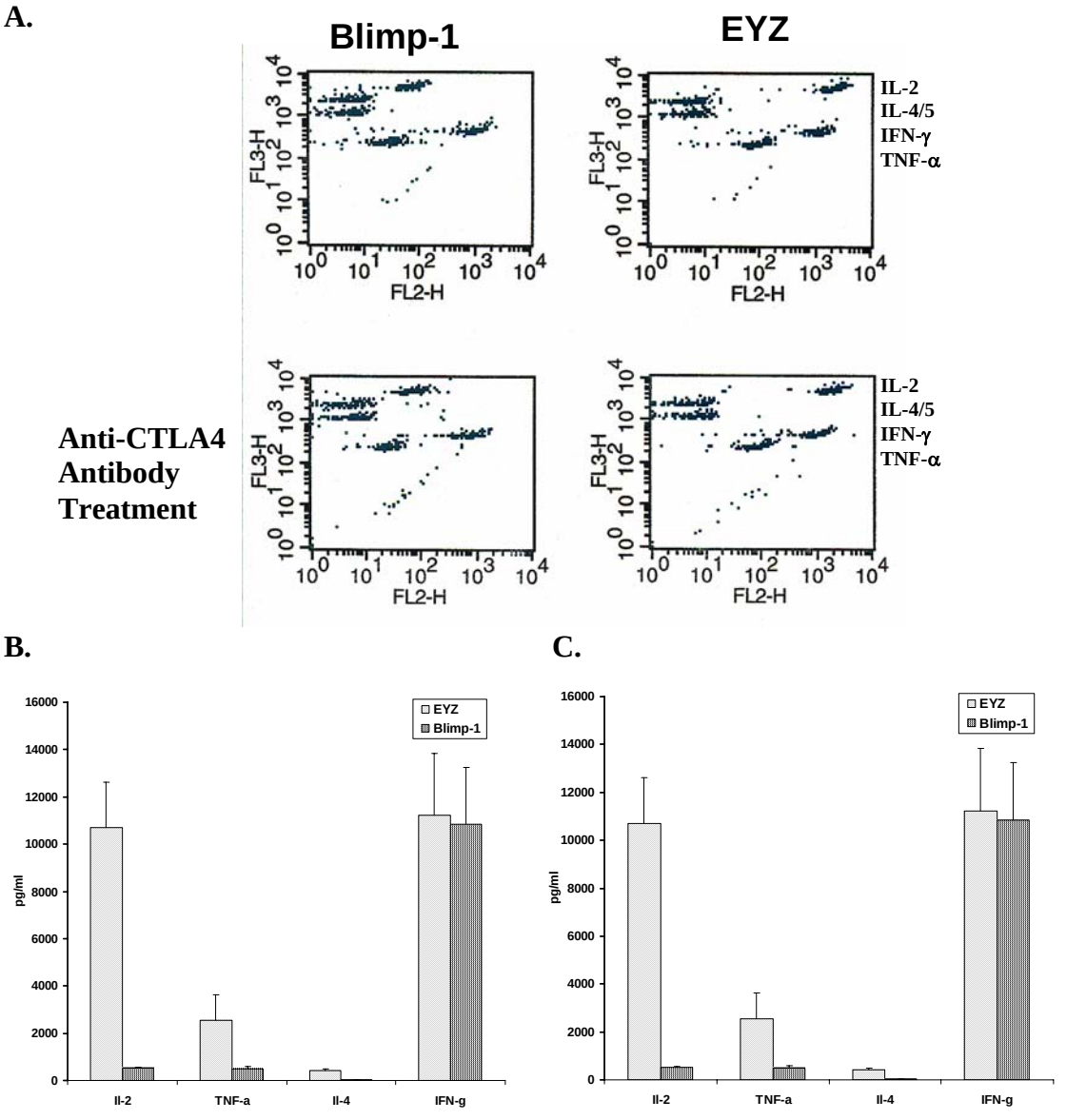


Fig.8. Profile of cytokines in Blimp-1 and EYZ transduced primary T Cells. A. Expression patterns of cytokines were tested by CBA. Quantification of cytokines in the absence (B) and presence (C) of anti CTLA4.

To measure cytokine production at the later stage of cell culture, $4x10^4$ sorted T cells together with $1x10^5$ APC (irradiated) were cultured in the presence of 0.5 μg/ml anti-CD3 mAb (200 μl) at 37°C, 5% CO_2 and the culture supernatant was harvested after 24 h of culture. The CBA assay was used to quantify cytokines produced.

CBA is a two-site sandwich immunoassay to detect cytokine expression (Carson, 1999). In the commercial kit used, the expression levels of the cytokines IFN-γ, TNF-α, IL-5, IL-4 and IL-2 could be determined. A dramatic decrease in IL-2 levels was detected in Blimp-1 transduced; activated primary T cells compared with EYZ transduced T cells. In all three independent experiments, we observed an about 20 fold reduction in Blimp-1 transduced T cells. The reduction in IL-2 production could not be reversed when Blimp-1 transduced T cells were treated with anti-CTLA4 antibodies. TNF-α level was also significantly decreased in Blimp-1 transduced T cells, while the difference in IFN-γ secretion was variable, ranging from no to a 5 fold decrease in Blimp-1 transduced T cells. Under the neutral conditions used for stimulation, IL-4 and IL-5 were poorly detected in both Blimp-1 and EYZ transduced primary T cells. Theses results were consistent with those after treatment with anti-CTLA4 antibody (Fig.8). As IL-10 is crucial for development of Tr, we also analyzed secretion of IL-10 by intracellular staining, but there was no increase in IL-10 production following induction of Blimp-1 (data no shown).

4.5. Gene Expression in Primary T Cells Following Blimp-1 Induction

In order to gain some information on functional characteristics of T cells following Blimp-1 induction, several genes which are known to be involved in Tr development, cell cycles progression as well as cell survival were determined by RT-PCR.

At first, ectopic expression of Blimp-1 transduced T cells was confirmed; As Foxp3 plays a primary role in the generation and potentially the maintenance of naturally occurring CD4+25+ T cells, we also investigated the expression of this molecule. Foxp3 was not enhanced following Blimp-1 induction; the most marked effect was on Mad4, which was expressed at higher levels in Blimp-1 transduced primary T cells; c-Myc mRNA levels

were lower in Blimp-1 transduced T cells than in EYZ controls (Fig.9), A1 was barely affected.

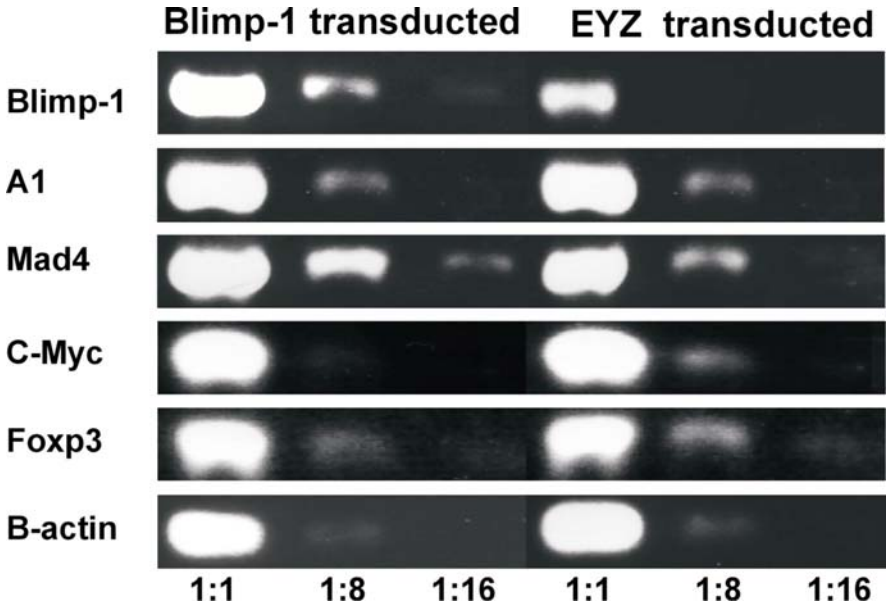


Fig.9. Gene expression following Blimp-1 introduction into primary T cells.

4.6. Effect of Blimp-1 on T Cell Viability

It has been shown previously that, in B cells, Blimp-1 expression controls a check point that decides between cell death and differentiation. We therefore analyzed viability of Blimp-1 transduced T cells. 7-AAD is very useful to distinguish dead and living cells. Compared with control, EYZ transduced T cells, Blimp-1 transduced T cells dramatically lost their viabilities after 2-day in culture. Almost 70% cells were dead in Blimp-1 transduced T cells. In contrast, more than 50% cells were still alive when EYZ was transduced. Particularly, viabilities were significantly decreased in Blimp-1 transduced primary T cells after 3 days of culture. This experiment was highly reproducible and indicates that ectopic expression of Blimp-1 leads to death of activated primary T cells (Fig.10).

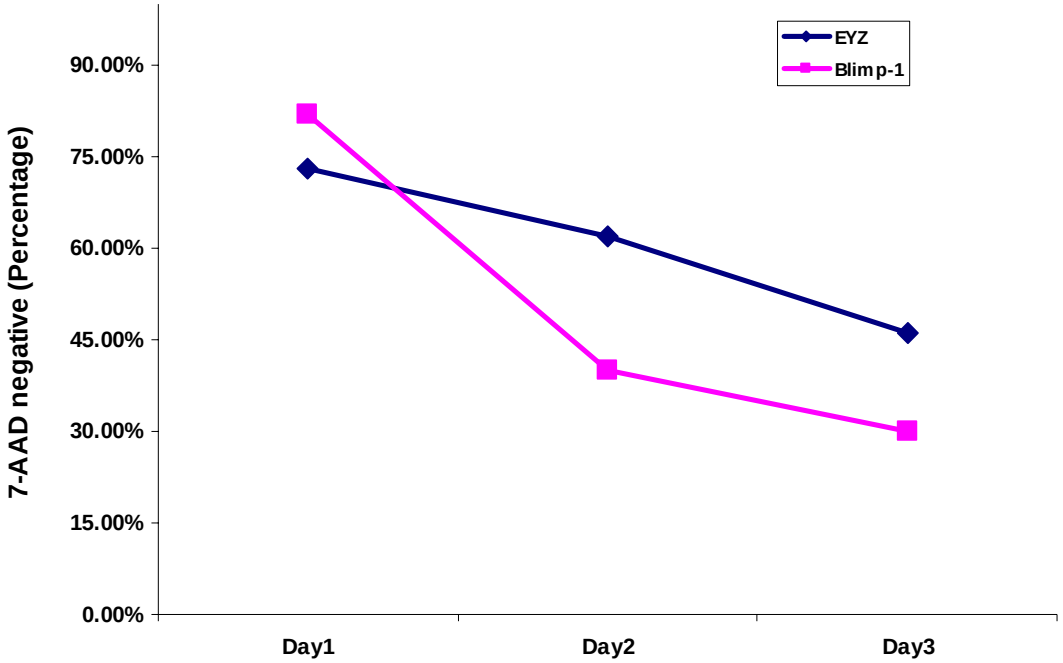
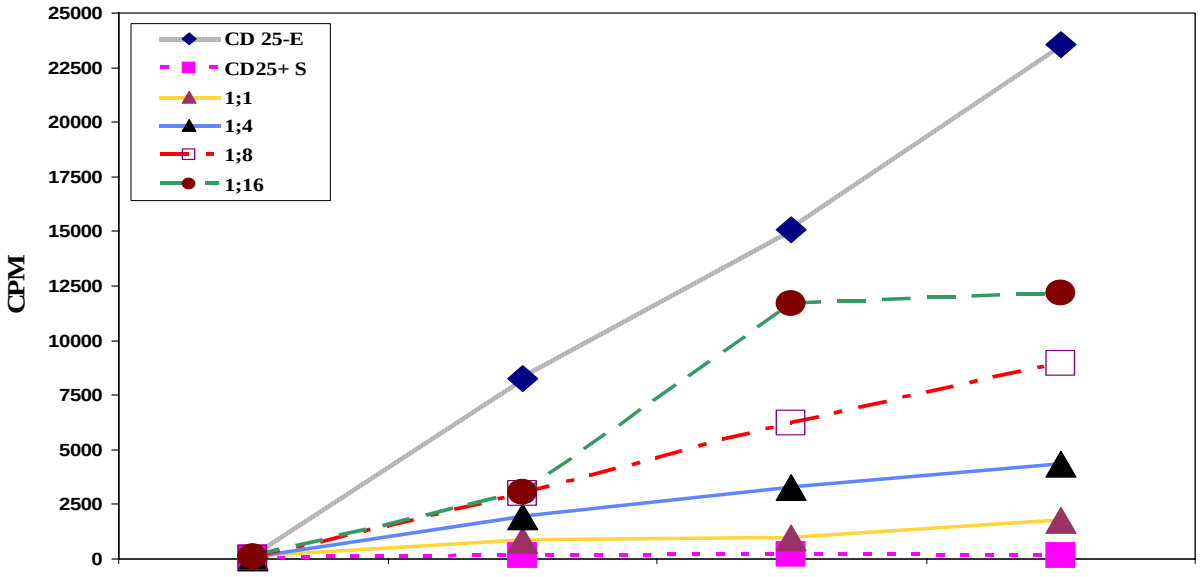


Fig. 10. Blimp-1 transduction into T cells leads to reduced viability.

4.7. Blimp-1 Transduced T Cells Share Some Features with CD4+CD25+ Tr Cells but Fail to Suppress CD4+CD25- T Cells

The proliferation of CD25- T cells induced by CD3-specific antibodies can be inhibited by adding CD4+CD25+ Tr cells to CD4+ CD25- T cells. Suppression occurs only when CD4+CD25+ Tr cells are previously activated via their T-cell receptor (TCR) (Thornton and Shevach, 2000). The main mechanism of suppression appears to be inhibition of the transcription of IL-2 in responders. Suppression could be abrogated by the addition of exogenous IL-2. The CD4+CD25+ Tr cells might act on APC to inhibit the upregulation of expression of co-stimulatory molecules which are required for activation of responders, indirectly leading to suppression of IL-2 in CD25- T cells. Clearly, cell contact between suppressors and responders is required (Takahashi, Kuniyasu et al. 1998; Thornton and Shevach 1998).

A



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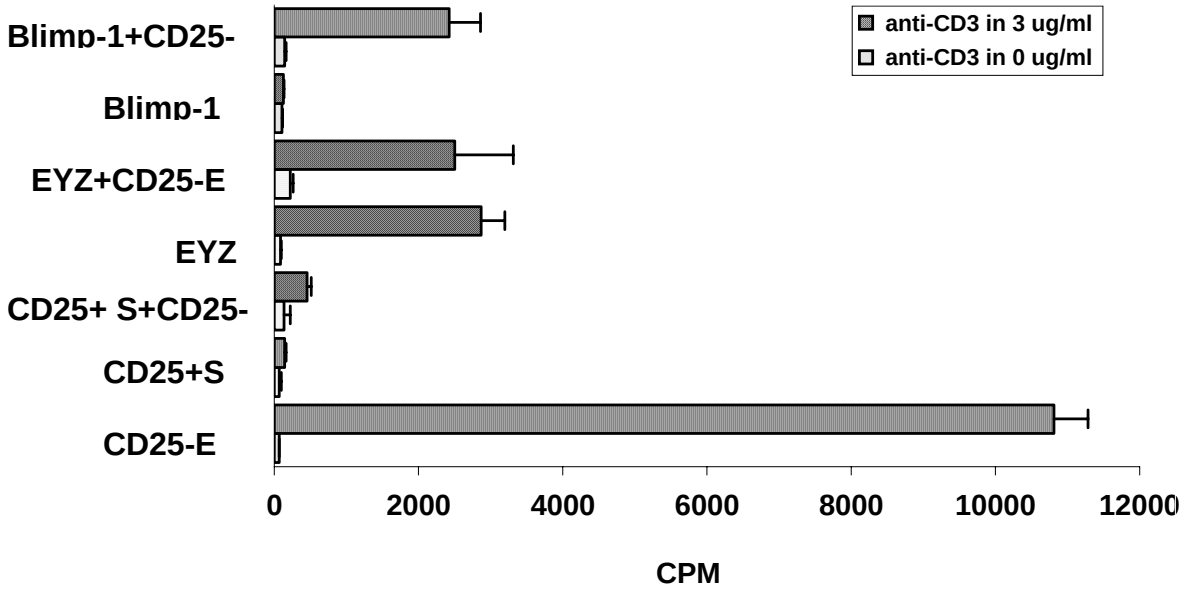


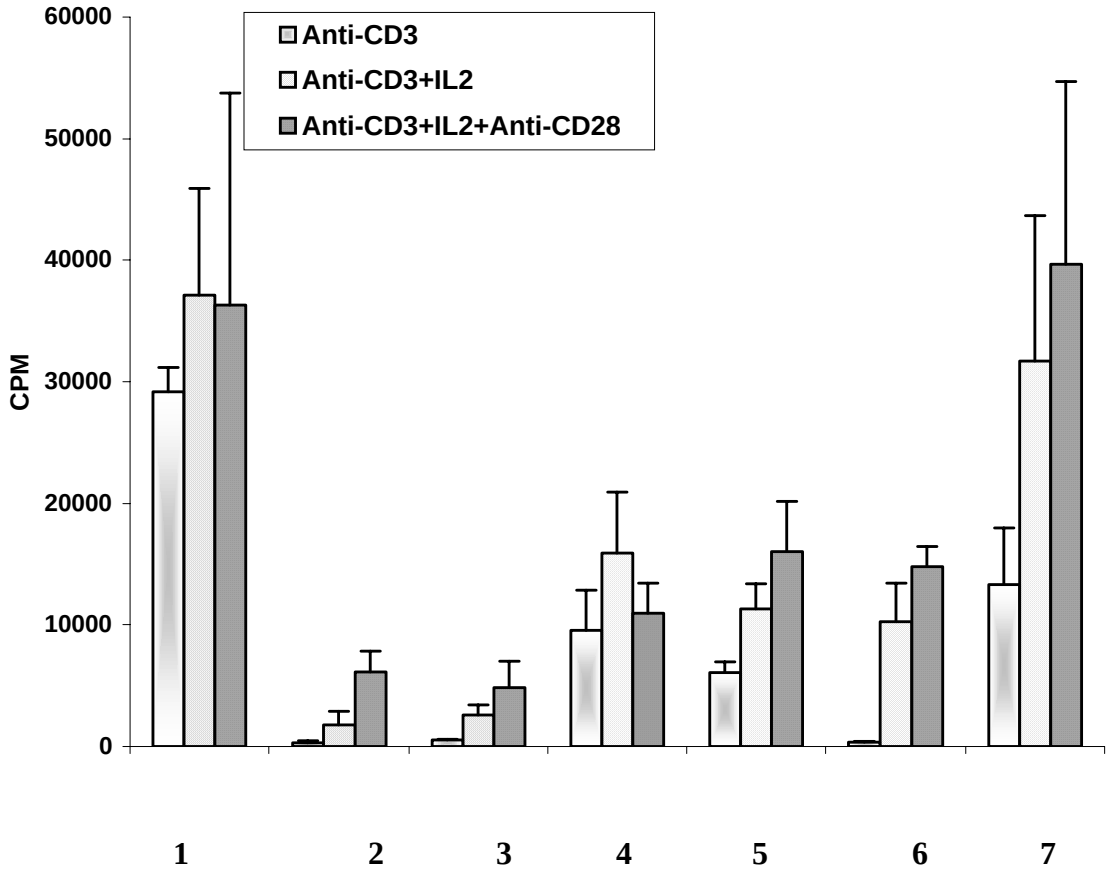
Fig.11. CD4+CD25+ Tr cells but not Blimp-1 transduced primary T cells suppress the response of CD4+CD25- T cells. A. CD4+CD25+ Tr cells suppress response of CD4+CD25- T cells at different ratios. B. Blimp-1 transduced T cells neither suppress CD4+CD25- T cells nor respond to stimulation via anti-CD3 antibody. E: ex-vivo freshly isolated; S: stimulated with anti-CD3 for three days.

We first compared the proliferative function of Blimp-1 transduced, EYZ transduced and naturally occurring CD4+CD25+ T cells with that of freshly isolated CD4+CD25- T cells. Fig 11.B showed that Blimp-1 transduced T cells did not respond to stimulation with soluble anti-CD3, comparable to CD4+CD25+ Tr cells. On the other hand, EYZ transduced T cells responded to stimulation with soluble anti-CD3, even though not quite as well as freshly isolated CD4+CD25- T cells.

We next tested whether Blimp-1 transduced T cells were capable to inhibit the proliferation of CD4+CD25- T cells in a comparison with freshly isolated CD4+CD25+ Tr cells. When the CD4+CD25+ population was cocultured with CD4+CD25- T cells, marked suppression of the response to stimulation with soluble anti-CD3 was observed. In multiple experiments of this type, significant suppression was observed at a final ratio of suppressors/responders of 1:4 and complete suppression was seen at ratio of cells with 1:1 when CD4+CD25- T cells stimulated with anti-CD3 were no longer responsive at all. Moreover, this suppression required stimulation with anti-CD3 and iDC. Most importantly, when either the Blimp-1 or EYZ transduced T cells population was cocultured with CD4+CD25- T cells, no significant difference in suppression of the response to stimulation with soluble anti-CD3 was observed. This result showed that the primary activated T cells with ectopically induced Blimp-1 did not acquire inhibitory phenotype of CD4+CD25+ Tr cells, but might be unresponsive or anergic to stimulation with anti-CD3 antibody.

4.8. Suppression of CD4+CD25+ T Cells as Well as the 'Anergic' Status of Blimp-1 Transduced Primary T Cells Are Abrogated by the Addition of Exogenous IL-2 and Anti-CD28 Antibody

As suppression mediated by CD4+CD25+ T cells can be abrogated by additional exogenous IL-2 and anti-CD28 antibody, the main mechanism of suppression appeared to be inhibition of the transcription of IL-2 in responders. Our experiments shown in Fig.12 confirm that IL-2 and anti-CD28 antibody indeed rescued the response of CD4+CD25- T cells stimulated via anti-CD3 antibody when they were cocultured with CD4+CD25+ T cells. In particular, Blimp-1 ectopically induced primary T cells also proliferated



1. CD25- E (ex-vivo); 2. CD25+S (stimulated); 3. CD25-E+CD25+S; 4. EYZ (transduced); 5. EYZ+CD25-E; 6. Blimp-1 (transduced); 7. Blimp-1+CD25-E

Fig.12. Suppression of CD4+CD25+ T cells is abrogated by the addition of exogenous IL-2 as well as anti-CD28 antibody. Hyporesponse of Blimp-1 ectopically transduced naïve T cells also can be reverted by the addition of exogenous IL-2 and anti-CD28 antibodies.

following addition of IL-2 and anti-CD28 antibody and this led to abrogation of suppression by CD4+CD25+ T cells even though they had no responses to stimulation by anti-CD3 antibody itself. This result also indicated that Blimp-1 ectopically transduced T cells might be in shortage of IL-2 which then results in a failure to respond to stimulation via anti-CD3 antibody. EYZ transduced primary T cells gave similar responses to exogenous IL-2 and anti-CD28 antibody, but the difference between EYZ and Blimp-1

transduced T cells was that only EYZ transduced T cells proliferated after stimulation with anti-CD3 antibody while Blimp-1 transduced primary T cells did not.

5. Discussion

5.1. Differentiation of Lymphocytes Mediated by Transcription Regulation

T and B cells fate is determinate by strength and timing of the signals they receive via their receptors after antigen stimulation. To maintain homeostasis of the immune systems, it has to be regulated accurately, in particular, terminal differentiation of lymphocytes. It is certain that terminal differentiation of lymphocytes involves the regulation of transcriptional programs that controls the cell cycle, their response to cytokines, effector function and susceptibility to activation-induced cell death (AICD). For instance, once signals from TCR are accumulated and integrated, naïve T cells proliferate, secret homeostatic cytokines, acquire effector function and peripheral-tissue-homing capability, develop to memory T cells, while excessive responses will be terminated by AICD (Lanzavecchia and Sallusto, 2002). Most importantly, regulatory T cells can suppress naïve T cells, to avoid self-reaction as well as overreaction of immune responses (Read et al., 1998; Thornton and Shevach, 1998). More recently, Foxp3, also called Scurfin which is a transcriptional repressor, has been shown to play an essential role in the generation and potential also the maintenance of naturally occurring CD4+25+ Tr cells. Similarly, plasma cells, the terminal differentiated B cells that are responsible for immune reaction, are also under control by transcriptional regulation, in their cases mediated by Blimp-1 (Lanzavecchia and Sallusto, 2002). Further, Blimp-1 is now known to have a much broader pattern of distribution, including the myeloid lineage and several organs during mouse embryonic development (Chang et al., 2000) (Chang et al., 2002). Interestingly, naïve T cells as well as CD4+25+ Tr cells were found to express Blimp-1 (Santner-Nanan et al., 2004). However, whether Blimp-1 acts as a terminal differentiation factor in T cells as it does in B cells or whether it plays a role in T cell development like Foxp3/Scurfin is still uncertain. The current studies were initiated to further understanding of the relationship between Blimp-1 and T cell differentiation.

5.2. Could a Blimp-1 Isoform mRNA Act as Dominant Negative in T Cells?

Blimp-1 is qualified as "master regulator' of plasma cell development, especially in down regulation of B cell proliferation, while up regulation of Ig secretion (Shaffer et al., 2002). RNA editing is a regulatory mechanism for protein expression, e.g. the use or disuse of additional splice site leading to full length or truncated mRNA (Tauson, 2004). Three major Blimp-1 mRNA isoforms are synthesized by different polyadenylation, ~5.7, 4.3 and 3.6 kb in mice, respectively. All these major mRNA isoforms are translated to the same protein. However, a minor splice variant is generated by using alternative splice site, lacking part of exon 7 (Tunyaplin et al., 2000). Of particular interest, this splice variant is found in freshly isolated CD4+CD25- and CD4+CD25+ T cells, besides the major full length mRNA of Blimp-1. More importantly, the ratio between the minor and major mRNA of Blimp-1 was almost equal in T cells, while plasma cells only showed the major form. Given that this minor splice variant can be translated into protein, this protein would lack zinc finger 2 and part of zinc finger 1 and 3, leading to inefficient DNA binding. Thus, this truncated protein may play a dominant negative role compared with full length Blimp-1 protein, particularly, in T cells. Furthermore, another question to be addressed would be whether a balance between major and minor Blimp-1 isoforms could be a key to prevent terminal T cells development. We also can speculate that Blimp-1 acts as an unattenuated transcriptional repressor efficiently in plasma cells, since there this minor mRNA is undetectable. Certainly, whether ectopic expression of minor mRNA leads to dysfunction of Blimp-1 in B or T cells differentiation need further investigations.

5.3. Functions of Blimp-1 Revealed by Ectopic Expression in Primary T Cells.

5.3.1. Blimp-1 Transduced T Cells do not Show the Characteristics of Regulatory T Cells

Surface molecules were first used in this study to characterize the phenotypes of Blimp-1 transduced T cells. Several molecules have been implicated in the suppressor mechanism

underlying the immunoregulatory function of CD4+CD25+ Tr cells, including cell surface molecules such as CTLA4, glucocorticoid-induced TNFR as well as CD25 (Shevach, 2002). We could not observe a significant difference in expression of either of the above surface markers when Blimp-1 was transduced into primary T cells activated with anti-CD3. Using a slightly different system, i.e. Ag specific stimulation of TCR transgenic naïve T cells, B. Santner-Nanan could show that CTLA4 was upregulated in unpolarized T cells by Blimp-1. Additionally, IL-10 is crucial for development of Tr. Production of IL-10 by intracellular staining also showed no increase in IL-10 following induction of Blimp-1.

To study Blimp-1 transduced T cells functionally, they were co-cultured with CD4+CD25- T cells. No significant suppression of the response to soluble anti-CD3 was observed. In addition, Blimp-1 transduced T cells did not express more Foxp3/Scurfin than T cells transduced with a control virus. These results show that the T cells ectopically expressing Blimp-1 did not acquire the inhibitory phenotype of CD4+CD25+ Tr cells. Unlike Foxp3/Scurfin, which plays a primary role in the generation and, potentially, the maintenance of naturally occurring CD4+25+ Tr cells (Khattri et al., 2003), Blimp-1 can not drive naïve T cells to develop into CD4+CD25+ Tr cells.

5.3.2. Blimp-1 Could Control Terminal Differentiation of T Cells

There are four main events during immune responses which are controlled by cytokines-initiation, clonal expansion, contraction and memory generation. Evidence has accumulated that cytokines play a fundamental role in the development and survival of T cells as well as effector functions (Schluns and Lefrancois, 2003).

Compared with EYZ transduced T cells, a dramatic decrease in IL-2 levels was detected in Blimp-1 transduced primary T cells which produced up to 20 fold less IL-2. TNF-α levels were also significantly decreased in Blimp-1 transduced primary T cells, while the difference in IFN-γ was variable, ranging from no change to 5 fold decrease in Blimp-1 transduced primary T cells. The lower production of IL-2 is consistent with the results that Blimp-1 transduced T cells were incapable of proliferation when stimulated with anti-CD3 antibody. The non-response status was abrogated by addition of IL-2 and anti-

CD28 antibody. Thus, Blimp-1 transduced T cells exhibited reduced replicative potential, but still could exert effector functions, such as production of IFN-γ. Moreover, viabilities were significantly decreased in Blimp-1 transduced T cells after 3 days of culture. Many activated T cells die via a pathway that involves members of the Bcl-2 family and is mediated by the mitochondrion (Marrack and Kappler, 2004). On the other hand, Blimp-1 transduced T cells were not found to highly express either Fas or Fas ligand (data not shown). It therefore seems likely that Blimp-1 transduced (or naturally expressing) T cells die from cytokine withdrawal, particularly of lack of IL-2.

IL-2 was originally described as a T cell growth factor, which was essential for the activation, proliferation, survival and development of T cell. The initial encounter with specific antigen in the presence of the required co-stimulatory signal triggers entry of T cells into the G1 phase of the cell cycle. Simultaneously, IL-2 also upregulates the synthesis of IL-2 receptor, particularly the α chain (Marrack and Kappler, 2004). Binding IL-2 receptor results in heterodimerization of receptor subunits, activation of JAK kinase and PI3 kinase activity which promotes proliferation of T cells. In addition to stimulating T cell activation and proliferation, IL-2 also blocks T cell apoptosis through multiple pathways, e.g. induction of Bcl-2, an anti-apoptotic factor.

It was noted that IL-2-/- and IL-2 receptor -chain (*IL-2rb*)-/- mice developed an autoimmune syndrome with absence of CD25+ T cells, respectively. (Papiernik et al., 1998) (Malek et al., 2000; Malek et al., 2002). This finding suggested that IL-2 and IL-2R are required for the differentiation and/or survival of the CD25+ T cells even if CD25+ T cells never produce IL-2.

Taking together our recent findings, especially the decline in IL-2 production and the non-proliferating status of ectopically Blimp-1 transduced T cells, the most likely scenario for the role of Blimp-1 in T cells is as follows: Blimp-1 could suppress the transcription of genes and thus lead to reduced cytokines secretion as well as an incapability of proliferation. Therefore, Blimp-1 might mark an end stage of lineage differentiation in T cells, similar to its role in B cells and monocyte.

Obviously, there are many unsolved questions. The key questions are whether Blimp-1 controls terminal differentiation of T cells in vivo and how this occurs. It is, however,

fascinating to speculate that T and B cell may share similar pathways for terminal differentiation.

6. Summary

The transcriptional repressor-Blimp-1 terminates differentiation of B lymphocytes as well as myeloid cells. Our data show that Blimp-1 is highly expressed in freshly isolated murine primary T lymphocytes, particularly its minor splice variant. Ectopic expression of Blimp-1 by retroviral transduction neither dramatically altered secretion of IFN-γ or IL-4 nor did it induce the ability to suppress as regulatory T cells. However, induction of Blimp-1 resulted in not only a significant reduction in the production of IL-2 but also an inability to proliferate as well as in the reduced viability. These results demonstrate that Blimp-1 might mark end stages of lineage differentiation in T cells.

7. Literature

Angelin-Duclos, C., Cattoretti, G., Lin, K. I., and Calame, K. (2000). Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression in vivo. J Immunol *165*, 5462-5471.

Bennett, C. L., Christie, J., Ramsdell, F., Brunkow, M. E., Ferguson, P. J., Whitesell, L., Kelly, T. E., Saulsbury, F. T., Chance, P. F., and Ochs, H. D. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet *27*, 20-21.

Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., Wilkinson, J. E., Galas, D., Ziegler, S. F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet *27*, 68-73.

Calame, K. L., Lin, K. I., and Tunyaplin, C. (2003). Regulatory mechanisms that determine the development and function of plasma cells. Annu Rev Immunol *21*, 205-230.

Carson, R., and D.Vignali (1999). Simultaneous quantitation of fifteen cytokines using a multiplexed flow cytometric assay. J Immunol Methods *227*, 41-52.

Chang, C. H., and Flavell, R. A. (1995). Class II transactivator regulates the expression of multiple genes involved in antigen presentation. J Exp Med *181*, 765-767.

Chang, D. H., Angelin-Duclos, C., and Calame, K. (2000). BLIMP-1: trigger for differentiation of myeloid lineage. Nat Immunol *1*, 169-176.

Chang, D. H., Cattoretti, G., and Calame, K. L. (2002). The dynamic expression pattern of B lymphocyte induced maturation protein-1 (Blimp-1) during mouse embryonic development. Mech Dev *117*, 305-309.

Fontenot, J. D., Gavin, M. A., and Rudensky, A. Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol *4*, 330-336.

Garrett-Sinha, L. A., Dahl, R., Rao, S., Barton, K. P., and Simon, M. C. (2001). PU.1 exhibits partial functional redundancy with Spi-B, but not with Ets-1 or Elf-1. Blood *97*, 2908-2912.

Graham, F. L., and van der Eb, A. J. (1973). Transformation of rat cells by DNA of human adenovirus 5. Virology *54*, 536-539.

Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science *299*, 1057-1061.

Janeway, C. (2001). Immunobiology: the immune system in health and disease).

Khattri, R., Cox, T., Yasayko, S. A., and Ramsdell, F. (2003). An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol *4*, 337-342.

Khattri, R., Kasprowicz, D., Cox, T., Mortrud, M., Appleby, M. W., Brunkow, M. E., Ziegler, S. F., and Ramsdell, F. (2001). The amount of scurfin protein determines peripheral T cell number and responsiveness. J Immunol *167*, 6312-6320.

Knodel, M., Kuss, A. W., Berberich, I., and Schimpl, A. (2001). Blimp-1 over-expression abrogates IL-4- and CD40-mediated suppression of terminal B cell differentiation but arrests isotype switching. Eur J Immunol *31*, 1972-1980.

Knodel, M., Kuss, A. W., Lindemann, D., Berberich, I., and Schimpl, A. (1999). Reversal of Blimp-1-mediated apoptosis by A1, a member of the Bcl-2 family. Eur J Immunol *29*, 2988-2998.

Lanzavecchia, A., and Sallusto, F. (2002). Progressive differentiation and selection of the fittest in the immune response. Nat Rev Immunol *2*, 982-987.

Lin, K. I., Angelin-Duclos, C., Kuo, T. C., and Calame, K. (2002). Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. Mol Cell Biol *22*, 4771-4780.

Malek, T. R., Porter, B. O., Codias, E. K., Scibelli, P., and Yu, A. (2000). Normal lymphoid homeostasis and lack of lethal autoimmunity in mice containing mature T cells with severely impaired IL-2 receptors. J Immunol *164*, 2905-2914.

Malek, T. R., Yu, A., Vincek, V., Scibelli, P., and Kong, L. (2002). CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. Immunity *17*, 167-178.

Marrack, P., and Kappler, J. (2004). Control of T cell viability. Annu Rev Immunol *22*, 765-787.

McHugh, R. S., Whitters, M. J., Piccirillo, C. A., Young, D. A., Shevach, E. M., Collins, M., and Byrne, M. C. (2002). CD4(+)CD25(+) immunoregulatory T cells: gene

expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. Immunity *16*, 311-323.

Messika, E. J., Lu, P. S., Sung, Y. J., Yao, T., Chi, J. T., Chien, Y. H., and Davis, M. M. (1998). Differential effect of B lymphocyte-induced maturation protein (Blimp-1) expression on cell fate during B cell development. J Exp Med *188*, 515-525.

Mikkola, I., Heavey, B., Horcher, M., and Busslinger, M. (2002). Reversion of B cell commitment upon loss of Pax5 expression. Science *297*, 110-113.

Nakamura, K., Kitani, A., Fuss, I., Pedersen, A., Harada, N., Nawata, H., and Strober, W. (2004). TGF-beta1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. J Immunol *172*, 834-842.

Nutt, S. L., Eberhard, D., Horcher, M., Rolink, A. G., and Busslinger, M. (2001). Pax5 determines the identity of B cells from the beginning to the end of B-lymphopoiesis. Int Rev Immunol *20*, 65-82.

Nutt, S. L., Heavey, B., Rolink, A. G., and Busslinger, M. (1999). Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature *401*, 556-562.

Pan, L., Sato, S., Frederick, J. P., Sun, X. H., and Zhuang, Y. (1999). Impaired immune responses and B-cell proliferation in mice lacking the Id3 gene. Mol Cell Biol *19*, 5969-5980.

Papiernik, M., de Moraes, M. L., Pontoux, C., Vasseur, F., and Penit, C. (1998). Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. Int Immunol *10*, 371-378.

Piccirillo, C. A., and Shevach, E. M. (2001). Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. J Immunol *167*, 1137-1140.

Piskurich, J. F., Lin, K. I., Lin, Y., Wang, Y., Ting, J. P., and Calame, K. (2000). BLIMP-I mediates extinction of major histocompatibility class II transactivator expression in plasma cells. Nat Immunol *1*, 526-532.

Read, S., Mauze, S., Asseman, C., Bean, A., Coffman, R., and Powrie, F. (1998). CD38+ CD45RB(low) CD4+ T cells: a population of T cells with immune regulatory activities in vitro. Eur J Immunol *28*, 3435-3447.

Rosenthal, A., and Charnock-Jones, D. S. (1992). New protocols for DNA sequencing with dye terminators. DNA Seq *3*, 61-64.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol *155*, 1151-1164.

Santner-Nanan, B., Berberich-Siebelt, and Xiao, Z. (2004). Blimp-1 is expressed in human and mouse T cell subsets and leads to loss of IL-2 production and defective proliferation. Immunology Letters. Submitted.

Schliephake, D. E., and Schimpl, A. (1996). Blimp-1 overcomes the block in IgM secretion in lipopolysaccharide/anti-mu F(ab')2-co-stimulated B lymphocytes. Eur J Immunol *26*, 268-271.

Schluns, K. S., and Lefrancois, L. (2003). Cytokine control of memory T-cell development and survival. Nat Rev Immunol *3*, 269-279.

Schubert, L. A., Jeffery, E., Zhang, Y., Ramsdell, F., and Ziegler, S. F. (2001). Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. J Biol Chem *276*, 37672-37679.

Shaffer, A. L., Lin, K. I., Kuo, T. C., Yu, X., Hurt, E. M., Rosenwald, A., Giltnane, J. M., Yang, L., Zhao, H., Calame, K., and Staudt, L. M. (2002). Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity *17*, 51-62.

Shapiro-Shelef, M., Lin, K. I., McHeyzer-Williams, L. J., Liao, J., McHeyzer-Williams, M. G., and Calame, K. (2003). Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. Immunity *19*, 607-620.

Shevach, E. M. (2002). CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol *2*, 389-400.

Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y., and Sakaguchi, S. (2002). Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol *3*, 135-142.

Sinclair, N. R. (2000). Immunoreceptor tyrosine-based inhibitory motifs on activating molecules. Crit Rev Immunol *20*, 89-102.

Steimle, V., Otten, L. A., Zufferey, M., and Mach, B. (1993). Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). Cell *75*, 135-146.

Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J., and Sakaguchi, S. (1998). Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol *10*, 1969-1980.

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W., and Sakaguchi, S. (2000). Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med *192*, 303-310.

Tauson, E. L. (2004). [RNA editing in different genetic systems]. Zh Obshch Biol *65*, 52-73.

Thornton, A. M., and Shevach, E. M. (1998). CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med *188*, 287-296.

Thornton, A. M., and Shevach, E. M. (2000). Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. J Immunol *164*, 183-190.

Tunyaplin, C., Shapiro, M. A., and Calame, K. L. (2000). Characterization of the B lymphocyte-induced maturation protein-1 (Blimp-1) gene, mRNA isoforms and basal promoter. Nucleic Acids Res *28*, 4846-4855.

Turner, C. A., Jr., Mack, D. H., and Davis, M. M. (1994). Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. Cell *77*, 297-306.

Wildin, R. S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J. L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L.*, et al.* (2001). X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet *27*, 18-20.

Yu, J., Angelin-Duclos, C., Greenwood, J., Liao, J., and Calame, K. (2000). Transcriptional repression by blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. Mol Cell Biol *20*, 2592-2603.

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