Regulation of cell-fate decisions in T lymphocyte differentiation

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Regulation of cell-fate decisions in T lymphocyte differentiation

Regulatie van de ontwikkeling van T lymfocyten

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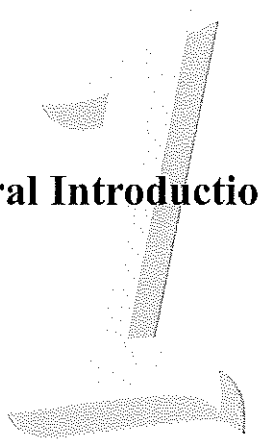
Mens is een zachte machine,
een buigzaam zuiltje met gaatjes,
propvol tengere draadjes
en slangetjes die dienen
voor niets dan tederheid
en om warmer te zijn dan lucht.
Och, hij heeft ademzucht
en hart-arbeid.

Leo Vroman
Uit: Slaapwandelen (1975)
Em. Querido te Amsterdam

The regulation of cell-fate decisions in T cell differentiation

CONTENTS

Chapter 1



General Introduction

The acquisition of protective immunity is essential for survival. Protective immunity can be divided in innate immunity and acquired immunity. These two parts of the immune system have evolved to closely interact (1, 2). Cells of the innate immune system, such as dendritic cells and macrophages, recognize potentially hazardous substances by their particular carbohydrate signature using germline-encoded receptors (3). In addition, innate immunity is thought to play a major role during the early phases of the immune response as well as in activating and regulating the acquired immune response. Acquired immunity mediates antigen-specific immune responses directed against those antigens selected by the innate immune response to be potentially noxious (1). The cells which are essential in effecting the acquired immune responses are the B and T lymphocytes. These cells specifically recognize foreign antigens using their antigen receptors, the B cell receptor (BCR) and the T cell receptor (TCR), respectively. These antigen receptors are encoded by genes which are produced by somatic gene rearrangement processes (3). The interaction between the two parts of the immune system is essential both for the maintenance of immunity (the presence of an immune response) towards infectious agents and for the maintenance of tolerance (the absence of an immune response) to the body's own tissues. Tolerance can be divided into central tolerance, which is implemented during the differentiation of T lymphocytes in the thymus, and peripheral tolerance, which is implemented in the periphery.

The acquired immune response is orchestrated by helper T (Th) lymphocytes, regulating antibody production by B lymphocytes, clonal expansion of cytotoxic T lymphocytes and intracellular killing by macrophages. Th lymphocytes differentiate in the thymus from a common pluripotent precursor. During their thymic differentiation, T cells are selected for their ability to recognize non-self antigens presented in the context of major histocompatibility (MHC) antigens. This restriction to a non-self-specific TCR repertoire is essential for the maintenance of central tolerance. Mature Th cells in the periphery are activated by the cognate interaction with antigen-presenting cells (APC), such as dendritic cells and macrophages, which have internalized (parts of) potentially infectious substances. During this interaction, helper T lymphocytes specifically recognize these antigens in the context of the MHC class II molecules presented on the surface of the APC (4). In this way, antigens which have been non-specifically internalized and degraded by the APC are specifically recognized by the T cells using their TCR. The activation state of the APC is the decisive factor in the outcome of the T cell activation in terms of induction of peripheral tolerance (no response is needed) or immunity (an immune response is required).

During the differentiation from pluripotent progenitor cells in the thymus to mature naive T cells in the circulation and eventually to activated effector cells during an immune response, T cells continuously undergo fate decisions until at some point apoptosis is induced. At many of these critical moments in T cell differentiation, the cell must be receptive to signals from the environment, which mediate regulation of the genetic program. Thereafter, the coordinated expression of cohorts of genes will alter the phenotypical and the

functional characteristics of the differentiating T cell.

The work addressed in this thesis investigates various aspects of the regulation of T cell differentiation and helper T cell-mediated immune responses. This introduction will give a brief overview of the current knowledge on T cell differentiation, activation, effector functions and memory formation.

THYMIC T CELL DIFFERENTIATION

1.1. INTRODUCTION

T lymphocytes arise from multipotential bone marrow-derived precursor cells within a specialized organ, the thymus. Extrathymic T cell differentiation has been reported, but is considered to contribute minimally to the peripheral T lymphocyte pool (5, 6). T cells are 'educated' in the thymus to recognize peptides in the context of MHC molecules and undergo two selection processes. These are known as positive selection (selection for MHC-restricted recognition of antigens) and negative selection (selection against recognition of self-peptides) (7-10). In order to avoid reactivity to self, T cells recognizing self-peptides in the context of MHC are clonally deleted (negatively selected) during T cell differentiation (11-14). Only a small proportion of all T cells are positively selected and will eventually survive to mature T lymphocytes. Generally, positive and negative selection are thought to result from the same MHC/Ag-TCR$\alpha\beta$ interaction (15, 16).

In addition to the processes of positive and negative selection, developing T cells also enter a differentiative program resulting in the commitment of the T cell into one of the two distinct lineages of MHC class I- or MHC class II-restricted T lymphocytes. MHC class I recognition will result in the formation of $CD8^+$ T lymphocytes, the cytotoxic T cells (17, 18). Recognition of MHC class II will result in the formation of $CD4^+$ T cells, the helper T cells (19, 20). This differentiative program results in the selective expression of the appropriate coreceptor gene, and is known as lineage commitment (21). The process of lineage commitment is thought to be regulated independently of positive selection (22). Lineage commitment ensures the functional competence of mature T cells: $CD4^+$ T cells recognize MHCII/peptide complexes presented on APC whereas $CD8^+$ T cells recognize MHCI/peptide complexes presented on all cells of the body (23). Fully matured $CD4^+$ and $CD8^+$ T cells will enter the periphery, and, as recent thymic emigrants, they contribute to the naive peripheral T cell population.

1.2. STAGES OF T CELL DIFFERENTIATION AND SELECTION PROCESSES IN THE THYMUS

T cells differentiating in the thymus are phenotypically distinguished by the expression of CD4 and CD8 coreceptors. T cells are either double negative (DN), double positive (DP) or single positive (SP) for these two cell-surface antigens (figure 1; adapted from 16).

Upon entry into the thymus, the bone marrow-derived progenitor cell is at the DN stage. The DN population is generally subdivided into four distinct subsets, defined by differential expression of the alpha chain of the interleukin 2 receptor (IL-2Rα; CD25) and phagocyte glycoprotein-1 (PGP-1; CD44) (24). At the most immature stage, the precursor cells express CD44, but not CD25. These so-called thymic lymphoid progenitor (TLP) cells maintain the capacity to differentiate into all lymphoid lineages: B lymphocytes, T lymphocytes, NK cells and lymphoid dendritic cells (25). Commitment to the T cell lineage is accompanied by the induction of CD25 expression, and the immature T cells enter the pro-T cell stage

($CD25^+CD44^+$). Pro-T cells are cycling cells, which are dependent on IL-7 for their proliferation. At the pro-T cell stage, *TCRβ* gene rearrangements have not been initiated yet, but rearrangements at the *TCRγ* locus can be detected (26). This implies that pro-T cells initiate expression of *recombination-activating* genes (*Rag*). RAG proteins (RAG-1 and RAG-2) independently mediate somatic rearrangement of *TCR* and *BCR* genes by the induction of double-strand DNA breaks at specific sites in the *TCR* and *BCR* locus (27, 28). During the transition to the next stage of T cell maturation, the early pre-T cell stage, the cells lose CD44 expression and shut down proliDferation (29). During this early pre-T cell stage ($CD25^+CD44^-$), *TCRβ* genes begin to be rearranged. The rearranged *TCRβ* is expressed at the cell surface complexed to pre-Tα as the pre-TCR-complex. Only those cells which produce a functional TCRβ protein will downregulate CD25 expression and proceed to the next phase of T cell maturation. These late pre-T cells are proliferating cells which have a $CD25^-CD44^-$ phenotype (30-32). This selection process is referred to as β-selection (16). Cells which have undergone β-selection will then initiate expression of CD4 and CD8 coreceptors genes.

At the $CD4^+CD8^+$ DP stage, T cells start rearranging their *TCRα* genes. Upon the recognition of Ag/MHC complexes by the newly formed TCRαβ receptor -the event initiating positive selection-, T cells will terminate *RAG* expression, which results in the abrogation of *TCRα* gene rearrangement. In addition, the T cells increase expression of TCRαβ on their cell surface and start to express the activation marker CD69. The process of positive selection serves to select for a peripheral T cell pool with an MHC-restricted TCR repertoire. Double-positive T cells which fail to receive the TCRαβ-mediated signal within 3-4 days will enter the apoptotic pathway (16). Positively selected cells will downregulate coreceptor

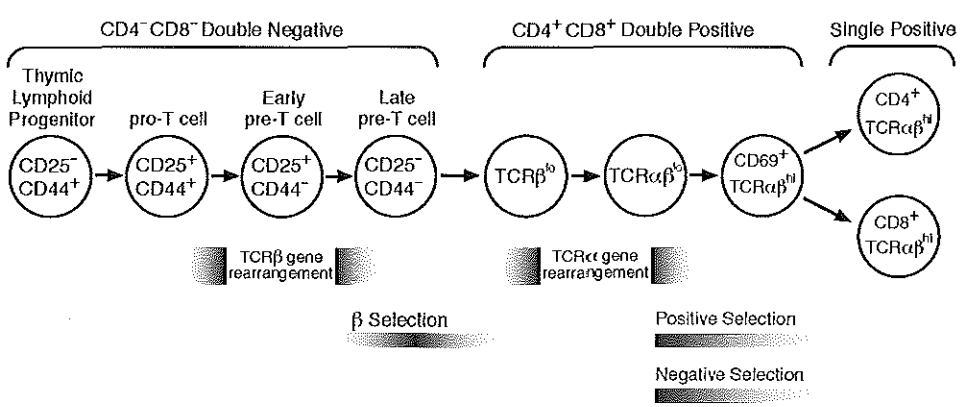


FIGURE 1

Stages of T cell differentiation in the thymus.

Several stages of T cell differentiation can be distinguished in the thymus based on the expression of cell-surface markers. Based on the expression of CD4 and CD8 coreceptors, three major stages of differentiation can be characterized: CD4/CD8 double negative, CD4/CD8 double positive and CD4 or CD8 single positive. CD4/CD8 double negative T cells can be further divided in 4 developmental stages based on the expression of CD25 and CD44.

expression, thereby developing into the $CD4^{lo}CD8^{lo}$ TCR^{int} $CD69^{+}$ cells (33). Subsequently, CD4 coreceptor expression is induced again, giving rise to $CD4^{+}CD8^{lo}$ $TCR\alpha\beta^{int/hi}$ $CD69^{+}$ cells. This subpopulation still contains precursors for both CD4 and CD8 SP cells. At this point, T cells differentiate into either CD4 SP or CD8 SP cells. In both lineages, maturing cells will eventually terminate CD69 expression and initiate high expression of CD62L (L-selectin), a cell surface receptor which is functionally involved in homing of the mature T cells to the lymph nodes. Mature thymocytes exit the thymus as CD4 or CD8 SP $CD69^{-}$ $CD62L^{hi}$ cells.

T cells which are specific for self peptides are negatively selected during thymic differentiation (34-36). Negative selection functionally restricts the peripheral TCR repertoire to the recognition of non-self peptides presented in the context of MHC. As such, negative selection is responsible for maintaining central tolerance. Negative selection can occur at any stage of T cell development from the DP $CD69^{+}$ stage onward. The mechanisms responsible for positive and negative selection have not yet been revealed into great molecular detail.

Two major models have been proposed to explain the processes of positive and negative selection. As both positive and negative selection are mediated by interactions of the TCRαβ receptor complex with MHC/Ag complexes, the nature of the antigenic peptide is usually considered to be of critical importance for the outcome of the thymic selection processes (16). The first model, the *qualitative/peptide model*, proposes that it is solely the nature of the antigenic peptide and the quality of the TCRαβ derived signal it induces, which determines the outcome of the selection process: low affinity peptides will induce positive selection, whereas high affinity peptides will induce negative selection (37-40). In contrast, the *avidity/quantitative model* proposes that positive selection is the result of low avidity thymocyte interactions, whereas high avidity interactions will result in negative selection (38, 41, 42). This model therefore allows for an effect of costimulatory receptors on the outcome of the selection process. Most importantly, however, in this model multiple TCRαβ-MHC/Ag interactions are integrated into one cumulative signal, which determines the cell fate, thereby encompassing not only peptide affinity but also peptide abundance. This integrated signal thus allows for a 'signalling gradient' with functional thresholds, ranging from low avidity interactions resulting in apoptosis ('death by neglect'), to a certain avidity-threshold which needs to be surpassed to ensure positive selection, to a high-avidity threshold, which, when surpassed, will induce negative selection by clonal deletion (16).

1.3. LINEAGE COMMITMENT DURING T CELL DIFFERENTIATION

In addition to positive selection, the recognition of Ag/MHC complexes at the DP stage of T cell differentiation also initiates the process of lineage commitment. Lineage commitment results in T cell differentiation into CD4 or CD8 SP cells (21). Studies on TCRαβ transgenic and MHC class I and II-deficient mice have shown that the MHC-specificity of the TCR dictates commitment of the developing T cells into the CD4 or CD8 lineages (17, 18, 20). T cells expressing an MHCI-restricted TCRαβ complex will maintain expression of the

CD8 coreceptor, and differentiate into CD8 single positive cells. Alternatively, T cells expressing an MHCII-restricted TCRαβ complex will maintain CD4 expression, committing to the CD4 lineage. Lineage commitment of DP cells into single-positive cells expressing a CD4/CD8 coreceptor with an MHC-specificity matching that of their TCRαβ complex ensures the immunological competence of the mature CD4 and CD8 positive T cells (23). Several mechanisms have been proposed to be responsible for lineage commitment (figure 2; adapted from 43). The *instructional model* postulates that the simultaneous co-engagement of TCRαβ and either of the coreceptors instructs the T cell to selectively downregulate expression of the other coreceptor gene, resulting in the commitment to the appropriate lineage (44). In contrast, the *stochastic/selection model* for lineage commitment postulates that the T cell randomly terminates expression of either coreceptor gene, resulting in 'intermediate' phenotypes ($CD4^+CD8^{lo}$ and $CD4^{lo}CD8^+$) which are then selected for the expression of the appropriate coreceptor (with matching MHC-specificity) (45). Finally, the *asymmetric commitment model* postulates that CD4 and CD8 lineage commitment have fundamentally different requirements. According to this model, CD8-lineage commitment requires lineage-instructional signals, whereas CD4 lineage commitment occurs in the absence of these signals (46, 47).

Recently, an attractive mechanism for lineage commitment has been proposed (figure 3; 48). According to this *coreceptor reversal model*, all DP T cells downregulate CD8 expression upon MHC/Ag recognition by the newly formed TCRαβ complexes. Downmodulation of CD8 coreceptor expression will result in a decreased TCR-mediated signal for MHCI-restricted TCRαβ-expressing cells. For cells expressing MHCII-restricted TCRαβ complexes, however, loss of cell surface expression of CD8 will not affect the strength of the TCRαβ-derived signal. In the latter case, the $CD4^+CD8^{lo}$ T cells will continue to shut down CD8 expression, resulting in the differentiation into MHCII-restricted CD4 SP thymocytes. Alternatively, loss of TCRαβ-mediated signal transduction results in an enhanced sensitivity of the developing T cell to IL-7-mediated signal transduction, allowing for the delivery of a differentiative signal by IL-7, which then results in coreceptor reversal: upon IL-7-induced signal transduction the $CD4^+CD8^{lo}$ T cells expressing MHCI-restricted TCRαβ complexes will terminate expression of CD4 and induce re-expression of CD8, eventually resulting in the differentiation into CD8 SP cells.

In a sense, the *coreceptor reversal model* combines the *instructional model* with the *asymmetric commitment model*, recognizing the $CD4^+CD8^{lo}$ stage of differentiation as the moment of selection/instruction rather than the DP stage. T cells at the $CD4^+CD8^{lo}$ stage are instructed (by TCR-mediated signals) to become CD4 SP when they carry an MHCII-restricted TCRαβ, but are instructed (by IL-7R-mediated signals in the absence of TCR-mediated signals) to become CD8 SP instead when they carry an MHCI-restricted TCRαβ. Therefore, the processes of CD4 and CD8 lineage commitment have fundamentally different mechanisms. This novel concept might reflect a broader mechanism for appropriate cell fate decisions of bipotential cells.

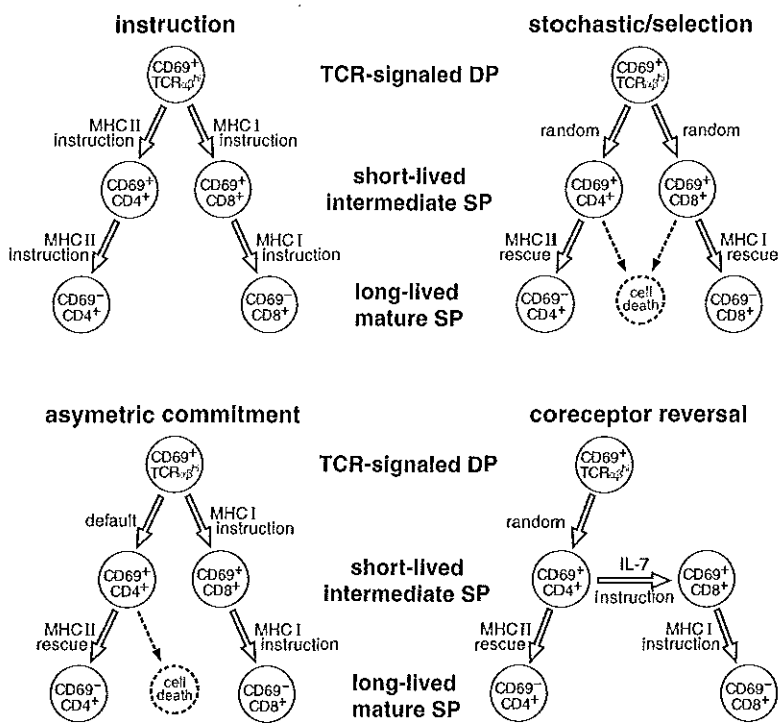


FIGURE 2

Models for CD4/CD8 lineage commitment.

Four models have been proposed for the process of lineage commitment to $CD4^+$ or $CD8^+$ T cells by positively selected DP T cells. The *instructional model* assumes that MHC-delivered signals will instruct the T cell to down-modulate expression of the inappropriate coreceptor gene. The *stochastic or selection model* assumes that signaled DP T cells randomly shut down expression of one of the two coreceptor genes. The cells which have shut down-expression of the irrelevant coreceptor gene, will be selected by MHC-dependent signals. The *asymmetric commitment model* assumes that CD4 lineage commitment is the default pathway, whereas CD8 lineage commitment requires MHCI-delivered instructional signals. The *coreceptor reversal model* assumes that all positively selected DP T cells will initially downregulate expression of CD8. At the subsequent $CD4^+CD8^{lo}CD69^+$ stage, the T cells commit to the appropriate lineage: MHCII-restricted T cells will continue to downmodulate *CD8* coreceptor gene expression and develop into CD4 SP cells. MHCI-restricted T cells will revert coreceptor gene expression by re-expression of the *CD8* gene and downmodulation of *CD4* gene expression. This coreceptor reversal is dependent on IL-7-delivered instructional signals.

1.4. REGULATION OF T CELL DIFFERENTIATION

T cells mature in close interaction with their thymic microenvironment (49). Commitment to the T cell lineage occurs at the pro-T cell stage, before the onset of *TCR* gene rearrangement (25). Pro-T cells are dependent on IL-7 for their proliferation. At the next stage, expression of *Rag* genes induces *TCR* gene rearrangement. Expression of *Rag* genes and somatic rearrangement of antigen-receptor genes is specific for cells of the lymphoid lineage. Although the thymic lymphoid progenitor retains the capacity to develop into the B cell

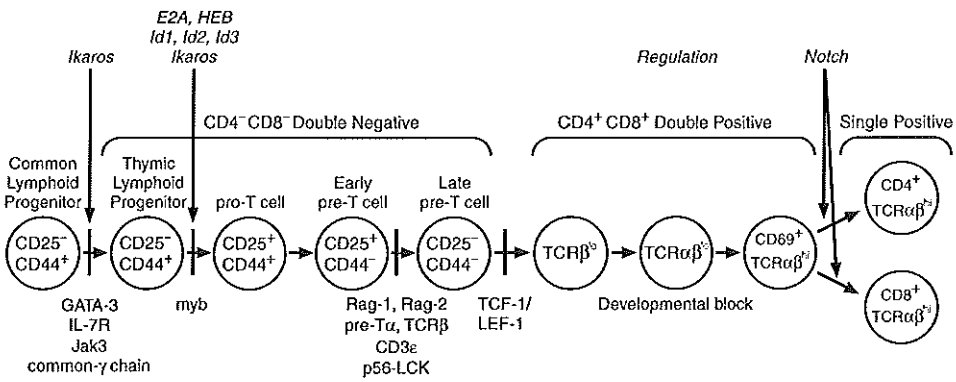


FIGURE 3

Regulation of T cell differentiation in the thymus.

Regulation of T cell thymic T cell differentiation by several families of proteins. Bold annotations indicate a block in the corresponding phase of T cell differentiation in the absence of the respective gene. Annotations in italics indicate a regulatory effect of the protein at the indicated stage of T cell differentiation. (see text for details).

lineage, the unique microenvironment of the thymus supports the differentiation into the T cell lineage (49). It has been shown that the unique three-dimensional architecture of the thymus plays an important role in allowing for efficient differentiation of progenitor cells into the T cell lineage (50). Stepwise reciprocal interactions between the T cells and the thymic stroma are of critical importance for the acquisition and maintenance of this unique thymic architecture (51).

T cell differentiation in the thymus is subject to stringent regulation. Cellular differentiation is usually accompanied by global changes in gene expression, resulting in the acquisition of a gene expression profile characteristic for a particular cell type or stage of differentiation or development (ontogeny). Transcriptional regulation of eukaryotic gene expression occurs at multiple levels (52). The most direct regulation of transcriptional activity is implemented by control elements which are part of the core promotor, the regulatory promotor or potential enhancer sites, interacting with DNA-binding proteins (called transcription factors) which directly influence the ability of the RNA polymerase II complex to initiate transcription (53). Transcription factors are proteins which have a DNA-binding domain as well as a protein-protein interaction domain. An additional important regulatory mechanism acts at the level of accessibility for transcription factors and the RNA polymerase II complex to the DNA. This level of regulation is mediated by more specialized elements like silencers and locus control regions (LCR), as well as by the degree of DNA methylation (53). Transcriptionally silent genes are often found in regions of 'condensed' chromatin. Induction of transcriptional activity at these loci is often associated with chromatin remodeling, resulting in decondensation of the chromatin. Chromatin remodeling sustains extensive changes in chromatin structure, such as increased histone acytelation of nucleosomal DNA and decreased DNA methylation on cytosine-guanine (CpG) dinucleotides (53-55). These

changes in chromatin structure also result in enhanced accessibility for transcription factors, which can be experimentally monitored by the analysis of DNase hypersensitive sites (52).

Cell-fate decisions of pluripotent progenitor cells are characterized both by the expression of lineage-associated transcription factors and by changes in the balance of these transcription factors (56). A number of transcription factors have been implicated in the regulation of various steps of T cell differentiation (figures 4 en 5; 25, 56-59). The fetal outgrowth of the earliest thymic progenitors is dependent on the transcription factors Ikaros, PU.1 and GATA-3. Whereas *PU.1*$^{-/-}$ and *Ikaros*$^{-/-}$ TCR$\alpha\beta^{+}$ T cells do develop after birth, *GATA-3*$^{-/-}$ embryonic stem (ES) cells fail to contribute to the T cell lineage (60-62). The transition from the thymic lymphoid progenitor stage to the pre-T cell stage ($CD44^{+}CD25^{-}$ to $CD44^{+}CD25^{+}$) has been shown to be critically dependent on the transcription factor c-myb (63). The transition from the late pre-T cell stage to the DP stage is dependent on presence of either lymphoid enhancer factor-1 (LEF1) or T cell factor-1 (TCF1), two related members of the high-mobility group (HMG) family of DNA-binding proteins (64, 65). Two other HMG family members, HMG-1 and HMG-2, have been shown to be involved in VDJ recombination by promoting RAG-mediated dsDNA cleavage at 23-mer recombination signal sequence (RSS) sites (66). The HMG factors are assumed to constitute downstream components of the evolutionary conserved Wingless/Wnt signaling cascade (59). In addition, helix-loop-helix (HLH) proteins have also been implicated in the regulation of thymic T cell differentiation.

Helix-loop-helix proteins

Two families of HLH proteins have been shown to regulate various stages of T cell differentiation. The E-box family of basic HLH DNA-binding proteins recognize conserved DNA sequences known as E-box elements, which are implicated in the regulation of cell-type specific gene transcription (67). The Id family of HLH proteins, lacking the basic DNA-binding domain, form non-DNA-binding heterodimeric complexes with E-box proteins (68). In this way, Id proteins have been shown to act in a dominant negative fashion on the function of the E-box family members. The members of the E-box family of DNA-binding basic HLH proteins HEB, E2A and E2-2 have been shown to be involved in T cell differentiation (69). Analysis of thymocyte populations in *E2A*$^{-/-}$ mice revealed a marked reduction of overall thymocyte numbers, especially at the DN and the DP stage, accompanied by increased SP cell numbers, especially of $CD8^{+}$ SP cells. Numbers of thymic lymphoid progenitor cells were within normal ranges, whereas strongly decreased numbers of pre-T cells were observed, consistent with a partial block of the commitment of the TLP to the T cell lineage (70). The *E2A* encoded protein E47 was shown to regulate the maturation of DP thymocytes (71).

Targeted deletion of the *HEB* gene resulted in a partial block in T cell differentiation at the transition from the late pre-T cell stage to the DP stage (69, 72). A more severe phenotype than that of either *HEB*$^{-/-}$ of *E2A*$^{-/-}$ animals was observed in mice homozygous for a *HEB* allele encoding a dominant-negative HEB protein, indicating a role for HEB-E2A heterodimers in T cell differentiation. In these mice, $\alpha\beta$ T cell differentiation was blocked at the pre-T cell stage (73). The Id protein family member Id3 has been shown to play multiple roles

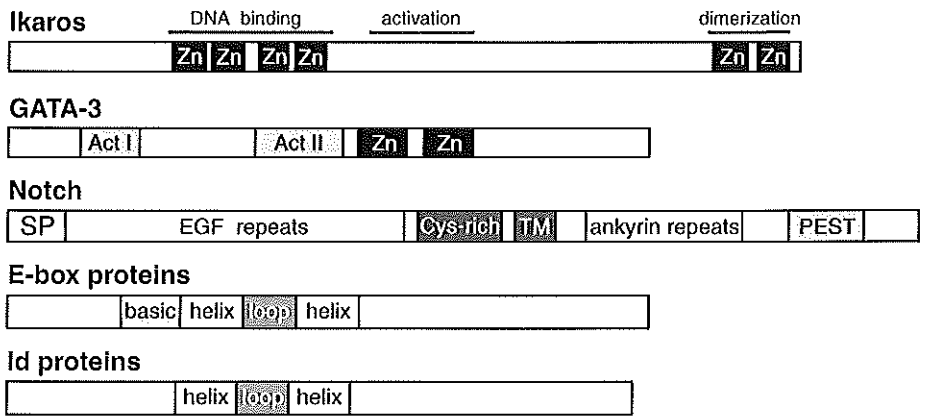


FIGURE 4
Proteins involved in the regulation of thymic T cell differentiation.
Several families of proteins are involved in the regulation of T cell differentiation in the thymus. Proteins are schematically depicted with relevant protein domains. (Zn, Zinc-finger domain; Act, activation domain; SP, signal peptide; Cys-rich, Cystein rich domain; TM, transmembrane domain; PEST, PEST-sequence).

in T cell differentiation (74, 75). Depending on the maturation stage under scrutiny, overexpression of *Id3* inhibited commitment to the T cell lineage or to the αβ-TCR lineage in fetal thymic organ cultures (FTOC), resulting in strongly increased numbers of NK cells and TCRγδ T cells, respectively (74, 76). Detailed analysis of T cell differentiation in *Id3*$^{-/-}$ mice revealed a block in its role in the regulation of positive and negative selection at the DP stage (75). As these data are consistent with the observed phenotype in *HEB*$^{-/-}$ and *E2A*$^{-/-}$ mice, the effect of *Id3* on T cell differentiation can be attributed to its dominant-negative role on bHLH protein function. Overexpression of *Id1* in a transgenic approach resulted in a massive induction of apoptosis during thymic T cell differentiation as well as a strong increase of the number of DN T cells (77).

Ikaros

The transcription factor Ikaros, a member of the Kruppel family of Zinc finger DNA-binding proteins, was shown to control various essential steps in T cell differentiation (78). The *Ikaros* gene encodes a family of proteins by means of alternative splicing (79-81). Mice homozygous for a targeted deletion of the *Ikaros* gene exons encoding the C-terminal Zinc-finger/DNA-binding domain (DN$^{-/-}$ mice) have a complete lack of lymphoid cells, including all precursor stages (82). This C-terminally truncated Ikaros protein binds Ikaros family members but inhibits DNA-binding of the heterodimer, and therefore exerts a dominant-negative effect on Ikaros function. In heterozygous mice (DN$^{+/-}$) this dominant-negative activity of the truncated Ikaros protein results in thymocytes with augmented proliferation. These mice rapidly develop lymphoproliferative disorders and die of T cell lymphomas (83).

Mice with a targeted deletion of exon 7 of the *Ikaros* gene, coding for the N-terminal

Zinc finger domain of Ikaros proteins (*Ikaros-C$^{-/-}$*), resulting in the expression of a true null mutation of the *Ikaros* gene, have a complete block in B cell differentiation from the earliest pro-B cell stages onwards. T cell development was absent at all fetal stages in *Ikaros-C$^{-/-}$* mice, but thymocyte precursors are detected starting a few days after birth. Detailed analysis of the various T cell populations in adult *Ikaros-C$^{-/-}$* mice revealed normal numbers of thymocytes at 1 month of age. However, CD4$^+$ SP TCR$\alpha\beta$ thymocyte numbers were strongly enhanced. In addition, oligoclonal and monoclonal thymocyte populations predominate in the thymuses of older *Ikaros-C$^{-/-}$* mice. Peripheral TRC$\alpha\beta$ T cell numbers are restored in adult animals, but NK cells and certain types of TCR$\gamma\delta$ T cells are absent (84). A more detailed analysis of mice homozygous for the Ikaros null mutation (*Ikaros-C$^{-/-}$*) revealed decreased numbers of CD44$^+$ DN and CD44$^-$CD25$^+$ DN thymocytes, but normal numbers of CD44$^-$ CD25$^-$ DN thymocytes. In addition, the *Ikaros-C$^{-/-}$* null mutation could rescue the block in T cell development of *RAG-1$^{-/-}$* mice, resulting in development of DP and even CD4 SP T cells in the thymus of double knockout mice (78). Taken together, these data show that Ikaros is involved in the regulation of TCR-signalling thresholds during β-selection (pre-TCR) and during positive selection and lineage commitment (TCR$\alpha\beta$).

A possible mechanism for Ikaros function was revealed in microscopy studies of B and T lymphocytes (85-89). Using confocal microscopy and immuno-fluorescence in situ hybridization (immuno-FISH), it was shown that Ikaros proteins associate with transcriptionally silent genes in heterochromatin foci in interphase nuclei, comprising clusters of centromeric DNA (87). The association of Ikaros with heterochromatin was independently observed by another group as well, using immunogold electron microscopy (89). Interaction of a gene with centromeric heterochromatin can mediate transcriptional repression, indicating that Ikaros may function as a regulator of transcriptional repression (87, 90). Protein complexes containing Ikaros proteins purified from a thymoma cell line were shown to contain an additional T-cell specific Ikaros-family member, designated Helios. Helios/Ikaros complexes were shown to associate selectively with centromeric clusters in T cell nuclei (88). Centromeric clustering of Ikaros with transcriptionally silent genes was shown to occur in cells preparing for cell cycle in lymphocytes differentiating along the B- or the T-cell lineage. Mature B- and T-lymphocytes only displayed the centromeric heterochromatin foci when they were induced to enter cell cycle, but not when they were allowed to maintain in a resting state (86). These observations implicate Ikaros proteins in differentiation-induced transcriptional silencing of genes by repositioning of these genes to centromeric heterochromatin loci (86).

Notch

The Notch family of cell surface receptors regulate developmental cell-fate decisions and affect the processes of proliferation, differentiation and programmed cell death (91, 92). In general, Notch receptors regulate cell-fate decisions by mediating either signals derived from identical, equipotent cells (lateral specification/inhibition), signals derived from a different cell-type (inductive signaling) or signals derived from the cell itself (cell-autonomous

signaling) (91, 93). Notch-1, -2 and -3 and their ligand Jagged-2 are all expressed in the thymus in variable fractions of all subpopulations of immature T cells. Thymic stroma also expresses the Notch-ligands Jagged-1 and -2, indicating a multitude of potential interactions putatively regulating T cell differentiation via the Notch pathway (94).

Notch family members have been shown to play a role in regulating cell-fate decisions at T cell lineage commitment, at αβ- versus γδ-T cell lineage commitment and at CD4 versus CD8 lineage commitment (95). An essential role for *Notch1* in the regulation of precursor cell commitment to the T cell lineage was shown in mice in which inactivation of the *Notch1* gene could be induced using the Cre/loxP system (96). In the absence of Notch1, T cell differentiation was arrested at the TLP ($CD25^-CD44^+$ DN) stage. These mice displayed B cell differentiation in the thymus, indicating that the thymic lymphoid progenitor cells might have adapted an alternative cell fate in the absence of *Notch1*. This notion was further substantiated by the observation that retroviral transduction of an activated form of *Notch1* into bone marrow precursor cells resulted in T cell differentiation in the bone marrow in the absence of B cell differentiation (97). Using mice which were heterozygous for a targeted deletion of one of the two *Notch1* alleles, it was shown that $Notch1^{+/+}$ and $Notch1^{+/-}$ cells did not contribute equally to the TCRαβ and TCRγδ lineages, whereas they did contribute equally to the B cell lineage. As the overrepresentation of $Notch1^{+/+}$ cells was more pronounced in the TCRαβ lineage than in the TCRγδ lineage, it was concluded that Notch apparently regulates the αβ- versus γδ-T cell lineage commitment (98). The effect of Notch1 on αβ-lineage commitment was only observed in the presence of $Notch1^{+/+}$ cells, indicating that the Notch-mediated regulation might be the result of lateral inhibition (95). However, mice homozygous for a targeted deletion of *Jagged-2*, a ligand for Notch which is highly expressed in the thymus, had a specific defect in γδ-T cell development, indicating that Notch also plays a role in γδ-lineage commitment (99). Together, these data suggest a complicated regulation of T cell commitment into the αβ and γδ lineages by Notch family members. At the DP stage of T cell development, Notch1 has been implicated in the regulation of thymocyte maturation independently of TCR/MHC interactions by preventing apoptosis due to death by neglect or negative selection (91, 100-102). In addition, an activated form of Notch1 has been shown to induce an increased differentiation of DP T cells into CD8 SP T cells in transgenic mice (103).

GATA-3

The transcription factor GATA-3 belongs to a family of proteins which bind to DNA at GATA-consensus sites through a highly conserved C_4 Zinc finger domain (104, 105). The GATA family of DNA binding proteins contains 6 mammalian members which can be divided into two subfamilies based on expression pattern and genomic structure. GATA-1, -2 and -3 are all expressed in hematopoietic cells, and display a similar genomic organization. GATA-4, -5 and -6 are expressed in developing cardiac structures, in the gut, the urogenital system and in the brain, and share two independent transactivation domains in the N-terminal region, which are conserved between GATA-4, -5 and -6 but not between the other GATA-family members (106-110). All GATA-family proteins contain a DNA binding domain

of two C4 Zinc fingers. The C-terminal Zinc finger is essential and sufficient for DNA binding to the GATA recognition sequence (T/A)GATA(A/G) (111-113). In addition, the N-terminal Zinc finger and the region placed in between the two Zinc fingers has been shown to play a role in protein-protein interactions. The N-terminal Zinc finger does not display independent DNA binding, but does stabilize the GATA-1-DNA interaction (113-116). Recently, the interaction between the two Zinc fingers of GATA-1 has been shown to modify the GATA-1/DNA interaction, resulting in stabilization or disruption of the interaction, or in modification of the specificity of the interaction (117).

GATA-proteins regulate transcriptional checkpoints during mammalian differentiation (58). GATA-1, GATA-2 and GATA-3 play different roles in hematopoiesis. *GATA-1* is expressed in erythroid, eosinophilic, megakaryocytic and mast cells (113, 118-120), and is essential for terminal differentiation of erythrocytes and megakaryocytes (104, 121). *GATA-1*$^{-/-}$ animals die at an early embryonic stage, due to severe anemia. Erythroid development of *GATA-1*$^{-/-}$ ES cells is arrested at an early proerythroblast stage (104, 122, 123). GATA-2 is involved in the regulation of the hematopoietic progenitor cell proliferation. Targeted deletion of the *GATA-2* gene results in early embryonic death associated with severe anemia. *GATA-2*$^{-/-}$ ES cells make no contribution to any hematopoietic lineage in chimeric mice (124, 125). GATA-3 was first identified as a transcription factor binding of the *TCRα* enhancer (126). GATA-3 binding sites have also been found in the *TCRβ* and *TCRδ* enhancers, as well as in the *CD8α* and the *IL-5* promotor regions (127-130). In addition to the expression of *GATA-3* in hematopoietic cells, *GATA-3* is also expressed during embryonic development in kidney and the central nervous system (131).

The two Zinc finger domains and the region in between these two Zinc fingers are highly conserved between GATA-1 and GATA-3 (132, 133). In addition to the conserved Zinc finger domains, the large N-terminal and C-terminal regions of the GATA-3 protein were shown to be essential for efficient transcription of a reporter gene placed under transcriptional control of the minimal *c-fos* promotor and four GATA-binding sites from the *TCRδ* enhancer (133). Mice homozygous for a targeted deletion of the *GATA-3* gene die at an early fetal stage (day 11). *GATA-3*$^{-/-}$ mice display massive internal bleeding, severe deformations of the brain and spinal cord and growth retardation (134). A more recent study revealed that an attenuated induction of tyrosine hydroxilase and dopamine β-hydroxilase in the sympathetic nervous system leading to noradrenaline deficiency in *GATA-3*$^{-/-}$ animals is a major cause of early fetal death (135). Pharmacological rescue of *GATA-3*$^{-/-}$ fetuses beyond day 11 by feeding catechol intermediates to pregnant females carrying *GATA-3*$^{-/-}$ fetuses, revealed the occurrence of various abnormalities in the absence of *GATA-3* later in fetal development, such as renal hypoplasia and aberrant development of cephalic neural crest-derived structures, implicating a role for GATA-3 in the differentiation of multiple cell lineages. These pharmacologically rescued *GATA-3*$^{-/-}$ fetuses also displayed severe thymic hypoplasia at fetal day 16.5 (135). *Rag-2*$^{-/-}$ complementation analysis revealed contribution of *GATA-3*$^{-/-}$ embryonic stem (ES) cells to all hematopoietic lineages except the T lymphocyte lineage (61). Therefore, it was concluded that development of *GATA-3*$^{-/-}$ T lymphocytes was blocked before or at the DN

progenitor stage (61). In addition, targeting *GATA-3* mRNA by an antisense approach in FTOC inhibited generation of thymocytes from fetal liver progenitors but not from fetal thymic cells (136). Taken together, these data implicate a crucial role for GATA-3 in early T cell development.

PERIPHERAL T CELL DIFFERENTIATION

1.5. T CELL ACTIVATION AND DIFFERENTIATION

During the differentiation of T cells in the thymus, T cells are selected for their capacity to recognize non-self peptides presented in the context of MHC molecules. Recent thymic emigrants contribute to the peripheral T cell pool as circulating naïve, resting T lymphocytes. These naïve T cells will become activated upon TCR-dependent recognition of a peptide presented in the context of MHC on the surface of an APC. Such peptides are derived from extracellular antigens which have been internalized by these cells, either aspecifically (in the case of dendritic cells and macrophages) or specifically (in the case of B lymphocytes). TCR-induced signaling pathways eventually result in phenotypical changes in the activated T cell which are characteristic for the acquisition of an effector cell phenotype, such as (1) cytoskeletal changes, resulting in an altered structure, mobility and size of the T cell, (2) changes in chromatin structure (histone acetylation and CpG demethylation) allowing the activated T cell to induce transcription of large numbers of genes, and (3) changes in the gene expression profile, endorsing new effector functions onto the activated T cell (137-140).

Although recognition of an MHC/peptide complex by the TCR will initiate the activation of a naïve T cell, a second (costimulatory) signal is required for a naïve T cell to become fully activated (141). TCR-mediated T cell activation will be further discussed in section 1.6. In the absence of costimulatory signals, the T cell will be functionally disabled by transition into an unresponsive state defined as anergy (142, 143). This so-called two-signal model for T cell activation plays an essential role in the maintenance of peripheral tolerance (144-146). B7 family members (CD80 and CD86) expressed on the cell surface of APC deliver costimulation by interaction with the T cell surface receptor CD28 (147). As the various types of APC express different levels of costimulatory molecules on their surface, presentation of antigenic peptides by the various types of APC will result in functionally different outcomes of the activation of naïve T cells. Only activated dendritic cells (the so-called professional APC) express sufficient levels of costimulatory molecules (CD80 and CD86) to fully activate a naïve T cell and induce differentiation of an activated T cell into an effector cell. Other types of APC, such as macrophages and B lymphocytes, will functionally disable a naïve T cell by the induction of anergy. Most APC can functionally activate antigen-experienced T cells, as these have far less stringent costimulatory requirements (148).

Eventually, fully activated naïve Th cells will acquire an effector phenotype after 4-5 days, and will very rapidly induce effector functions upon renewed recognition of the appropriate MHC/Ag complexes. $CD4^+$ T cells regulate Th cell-dependent immune responses both

by the production of soluble mediators (cytokines and chemokines) and by the cognate interaction with B cells and macrophages. Cytokines are pleiotropic proteins which induce a multitude of responses in various cell types (see table 1&2). Chemokines are chemotactic molecules which are involved in the regulation of leukocyte migration (149). The capacity to provide help to B cells in proliferation and antibody production is an important effector function of $CD4^+$ T cells, in which the production of cytokines such as IL-2, IL-4 and IL-6 as well as CD40/CD40L-dependent cognate B cell-interactions play critical roles (150). In addition, $CD4^+$ T cells mediate delayed-type hypersensitivity (DTH) responses by the production of cytokines such as IFNγ, lymphotoxin, GM-CSF and TNFα and by cognate interactions with

TABLE 1: The effects of various cytokines on B- and T-lymphocytes and other hematopoietic cell types.

Cytokine	Effect on B lymphoctye	Effect on T lymphocytes	Effect on other cells
IL-2	Growth, J chain synthesis	Growth, Survival	NK cells: Growth
IL-3	-	-	Hematopoietic progenitors: growth
IL-4	Growth, activation, *IgH* isotype switching (γ_1, ε), MHCII φ	Growth, survival, type-2 differentiation	Macrophages: inhibits activation
IL-5	Differentiation, IgH isotype switching	-	Eosinophils: Growth, differentiation
IL-6	Growth, Differentiation, Ig production	Induces IL-4 production in $CD4^+$ T cells	
IL-10	Anti-proliferative, MHCII φ	Anti-proliferative	Inhibits macrophage cytokine production; co-stimulates mast cell growth
IL-12		Type-1 differentiation, induces IFNγ production	NK cell activation, IFNγ production
IL-13	Differentiation, IgH isotype switching	-	
IFNγ	Differentiation, *IgH* isotype switching (γ_{2A})	Anti-proliferative	Macrophage and NK cell activation, induction of MHCI/II

macrophages through membrane-bound forms of TNF (151, 152). $CD8^+$ T cells mediate cytotoxicity against cells infected with virus or intracellular bacteria and against tumor cells, both by secretion of the content of cytotoxic granules (mainly perforin and granzymes) and by cognate interaction with the target cell (153).

Upon antigen-specific activation, the naïve T cell enters a program of cellular differen-

TABLE 2: A short overview of the producing cell types of the most relevant cytokines and overview of Jak and Stat molecules involved in signal transduction of each individual cytokine.

Cytokine	Producers	Stats	Jaks
IL-2	activated T cells	Stat5a, Stat5b, Stat3	Jak1, Jak3
IL-3	T cells	Stat5a, Stat5b	Jak1, Jak2
IL-4	activated Th2 cells, $NK1.1^+$ T cells	Stat6	Jak1, Jak3
IL-5	activated Th2 cells	Stat5a, Stat5b	Jak1, Jak2
IL-6	activated Th2 cells	Stat3	Jak1, Jak2, Tyk2
IL-10	activated Th2 cells	Stat3	Jak1, Tyk2
IL-12	Dendritic cells	Stat3, Stat4	Jak2, Tyk2
IL-13	activated Th2 cells	Stat6	Jak1,Jak3
IFNγ	activated Th1 cells, $CD8^+$ T cells, NK cells	Stat1	Jak1, Jak2

tiation. The nature of the antigen, the strength of the TCR-mediated signal, the nature of the costimulatory signal provided by the APC and the cytokines present in the microenvironment of the T cell direct the differentiation into one of various types of effector T cells. The two most extensively characterized T cell effector phenotypes are the type-1 and type-2 T cells. This distinction can be made amongst both $CD4^+$ and $CD8^+$ T cells (154). The two types of helper T cell and their differentiation from the naïve Th cell will be discussed in sections 1.7 and 1.8. In a later phase of the immune response, when the antigen which initiated the immune response has been cleared from the body, effector T cells will enter their final genetic program in a physiological process called activation-induced cell death (AICD), leading to the induction of apoptosis (155). A small number of antigen-experienced cells will remain in the circulation as resting cells, which are responsible for the maintenance of immunological memory.

Traditionally, immunological memory is defined functionally as the faster and stronger response of an animal following a renewed exposure to an antigen it has already encountered in the past (156). These enhanced responses could in part be explained by a higher frequency of antigen-experienced cells, or alternatively by qualitatively different functional properties of the memory cells (157-159). The functional differences between naïve and memory Th cells have only recently been extensively characterized (160). Using transfer studies of TCR-transgenic Th cells into T cell-deficient hosts, it was shown that memory Th cells display

enhanced kinetics of cytokine production and cell division as well as enhanced production of cytokines and an enhanced proliferation to suboptimal Ag-specific stimulation, as compared to naïve Th cells with the same clonotypic specificity (160). This study confirms the results from previous studies performed on polyclonal T cell populations which already suggested these functional differences between memory and naïve cells (148, 161-163). A complicating factor in the study of memory T cells has always been the use of memory T cell-specific markers to phenotypically distinguish these cells from the majority of resting naïve and activated effector cells (156). Generally, naïve murine T cells are characterized as $CD62L^{hi}$, $CD45RB^{hi}$ and $CD44^{lo}$, whereas memory T cells have the opposite expression of these cell surface markers: $CD62L^{lo}$, $CD45RB^{lo}$ and $CD44^{hi}$. Recently activated T cells, however, express the same pattern of cell surface markers as memory cells, but can be distinguished by the expression of the activation markers CD25 and CD69. So, resting memory cells are contained within the $CD25^{-}$, $CD69^{-}$, $CD44^{hi}$, $CD45RB^{lo}$, $CD62L^{lo}$ cell population (156).

1.6. SIGNAL TRANSDUCTION BY THE TCRαβ-COMPLEX

Recognition of MHC/Ag complexes on the surface of an APC initiates a signaling cascade mediated by the TCR complex. The TCR complex consists of the antigen-specific TCR-α and -β chains which are non-covalently associated with the invariant CD3-γ,-δ,-ε and TCR-ζ chains (figure 5; 164). The clonotypic α and β chains of the TCR complex have only minimal intracellular portions and cannot perform signal transduction. The signal transducing components of the TCR complex, the CD3-γ, -δ, -ε and TCR-ζ chains, are members of a family of immunoreceptor signal transduction subunits which contain immunoreceptor tyrosine-based activation motifs (ITAM) (138, 139). None of the components of the TCR complex has endogenous enzymatic activity. Signal transduction by the TCR is performed by non-receptor protein tyrosine kinases (PTK; figure 6, 7), which are recruited into the TCR complex upon activation (165). Recognition of MHCII/Ag complexes on the APC will bring Lck, which is associated to the CD4 coreceptor, into the TCR complex (figure 6). Lck, a Src-family PTK, will then phosphorylate tyrosine residues in the ITAM motifs of the CD3-γ, -δ, -ε (one ITAM motif in each chain) and TCR-ζ chains (three ITAM motifs per chain). Phosphorylated ITAM motifs serve as docking sites for the Src-homology 2 (SH2) domain of the ZAP-70 PTK (165). Importantly, the recruitment and activation of ZAP-70 and the Src families of kinases result in a cascade of downstream signaling events (figure 8) (137).

One major pathway inducing TCR-dependent T cell activation is the phospholipase C-γ1 (PLCγ1) pathway. Phosphorylation of the adaptor protein LAT (linker of activated T cells) attracts PLCγ1 to the cytoplasmic membrane, leading to activation of protein kinase C (PKC) and consequently in increases in intracellular calcium concentrations. As a result, the calcium/calmodulin-dependent phosphatase calcineurin is activated, which in turn dephosphorylates the transcription factor NF-AT (nuclear factor of activated T cells). Consequently, NF-AT is translocated to the nucleus and its affinity for DNA is increased (166, 167). It has been show that Tec-family protein-tyrosine kinases Itk and Rlk play an essential role in

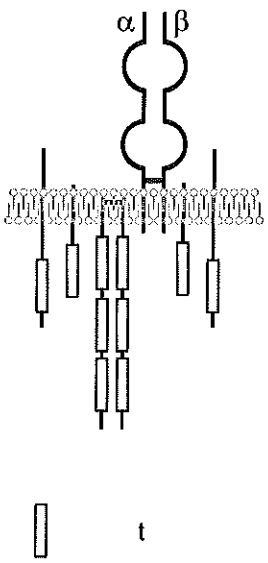


FIGURE 5
Structure of the T cell receptor complex.
The T cell receptor contains of the TCRα and TCRβ chain, which are the major extracellular components of the receptor complex and recognize the MHC/Ag complex on the antigen-presenting cell. In addition, the CD3γ, CD3δ, CD3ε and TCRζ chains comprise the intracellular signaling portion of the TCR complex. These receptor subunits contain immunoreceptor tyrosine activation motifs (ITAM), which are essential for the initiation of signal transduction by the TCR.

PLCγ1 activation upon TCR-mediated signaling. T cells from mice with a targeted deletion of the *Itk* gene displayed altered TCR-signaling, resulting in impaired proliferation and IL-2 production upon TCR-crosslinking as well as defects in thymic T cell differentiation and thymocyte selection (168-170). Enforced expression of *Rlk* throughout T cell differentiation resulted in the development of hyperresponsive T cells, characterized by augmented PLCγ1 activation. The expression of the *Rlk* transgene could partially restore the defects observed in $Itk^{-/-}$ mice, suggesting a functional redundancy between these two Tec family members (171). Mice with a targeted deletion of the *Rlk* gene displayed only mild defects in TCR signaling. T cells from mice with a targeted deletion of both the *Itk* and the *Rlk* gene, however, were shown to be unable to perform inositol triphosphate (IP_3) production, calcium immobilization and mitogen-activated protein (MAP)-kinase activation upon TCR-signaling (172). These data indicate a critical role for the combined function of Itk and Rlk in PLCγ1 activation. The mechanism of Itk-dependent PLCγ1 activation involves the association of Itk with phosphorylated linker for activation of T cells (LAT) (173, 174). In contrast, Rlk has been shown to associate with and phosphorylate the adaptor molecule SLP-76, also resulting in PLCγ1 activation (175). Both Itk and Rlk associate with the recently identified T cell-specific adaptor molecule RIBP, which was shown to be essential for TCR-induced IL-2 production and proliferation (176).

Lck-dependent activation of ZAP-70 also results in the activation of $p21^{RAS}$. The adaptor molecules LAT and SLP-76 are essential for $p21^{RAS}$ activation by ZAP-70 (177, 178). $p21^{RAS}$ plays an essential role in the induction *IL-2* gene transcription by the activation of mitogen-activated kinases (179), leading to c-Fos activation, as well as by activation of the JNK pathway, resulting in c-Jun activation. Jun/Fos heterodimerization will induce their translocation into the nucleus as transcription factor AP-1, which acts in concert with NF-AT.

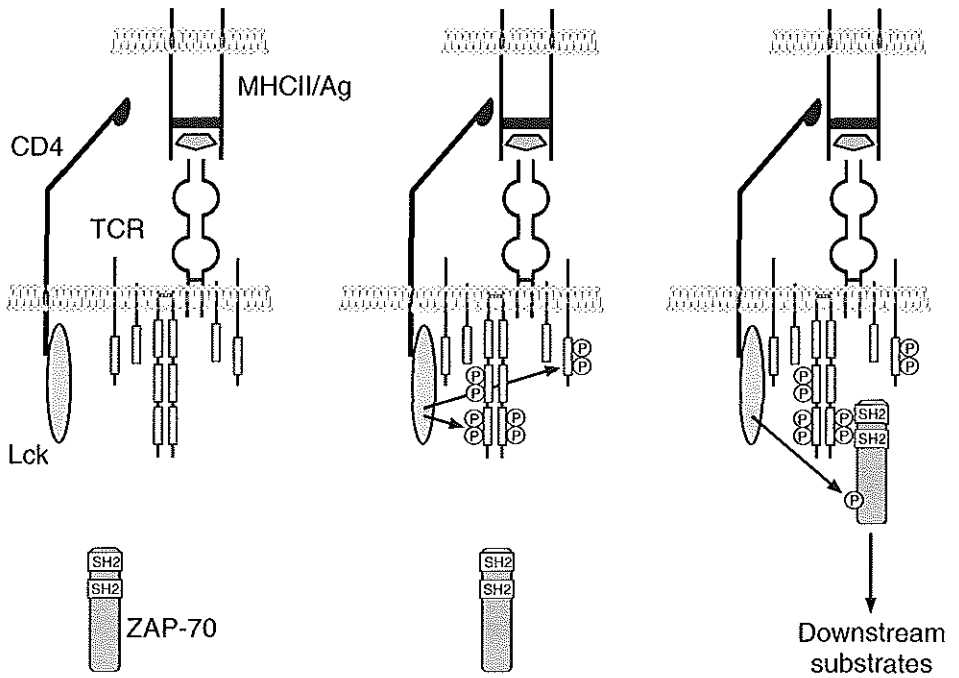


FIGURE 6
Proximal signalling events in TCR-mediated signal transduction.
Upon recognition of the MHCII/Ag complex on the antigen-presenting cell, the CD4 coreceptor molecule will be brought into close proximity of the TCR complex. Lck, a Syk-family tyrosine kinase which is constitutively associated with the CD4 molecule will phosphorylate critical tyrosine residues in the ITAM motifs of the TCRζ and CD3 chains. Phosphorylated ITAM motifs serve as docking sites for adapter proteins such as ZAP-70, which will then be phosphorylated by Lck. Activated ZAP-70 will initiate a number of downstream signaling pathways.

Amongst the gene-products that are induced by activated NF-AT are a number of cytokines, such as IL-2, IL-4, IFNγ, GM-CSF and TNFα as well as various cell surface receptors, including CD40L and FASL, all of which are phenotypical characteristics of effector T cells. In addition to the induction of gene transcription, TCR-signaling also induces changes in the organization of the cytoskeleton (180).

An additional downstream pathway is induced by the activation of phosphatidyl-inositol 3-kinase (PI3-K), resulting in the increased inositol lipid turnover and production of phosphatidyl inositol 3,4,5-tri-phosphate (PIP_3) (181). Two adaptor molecules have been suggested to recruit PI3-K to the cell membrane upon TCR-signaling: LAT and T cell receptor-interacting molecule (TRIM) (182). PI3-K regulates the Tec family member Itk, which in turn regulates PLCγ1 (183). In addition, PI3-K regulates guanosine exchange factors (GEF) for Rho family GTPases such as Rac, Rho and Cdc42 (180). The best characterized lymphocytic Rho family GEF is Vav-1, which is recruited to the plasma membrane by interaction with PIP_3

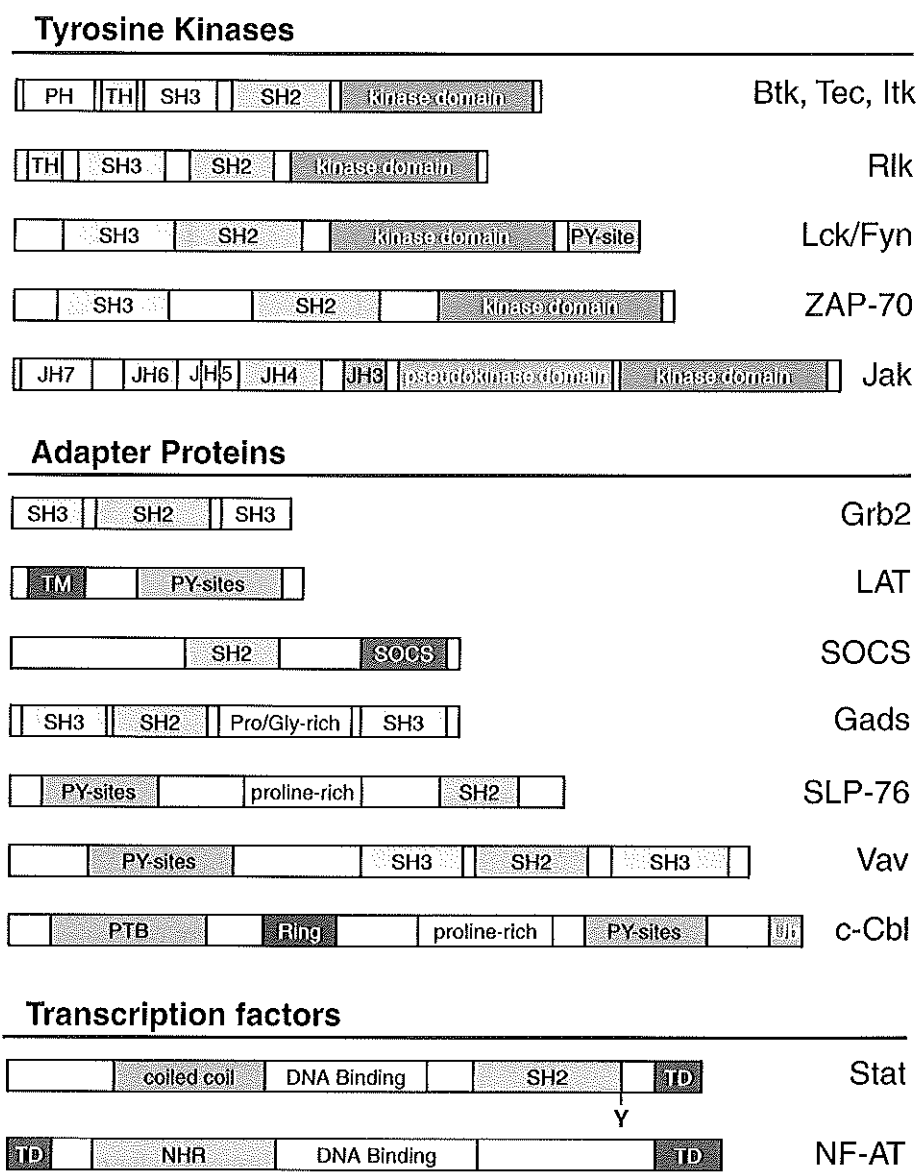


FIGURE 7

Proteins involved in signal transduction in T cells.

Numerous proteins have been implicated in TCR and cytokine-receptor signal transduction pathways. Here, a selection of the most relevant proteins have been depicted schematically with relevant protein domains (PH, pleckstrin homology; SH, Src-homology; TH, Tec-homology; PY, phosphotyrosine; JH, Jak-homology; TM, trans-membrane region; SOCS, SOCS-box domain; Pro/Gly-rich, proline/glycine rich domain; Ring, Ring-finger domain; Ub, Ubiquitination domain; TD, transactivation domain; NHR, NF-AT homology region).

(180). Tyrosine kinase activity at the membrane will than activate the nucleotide exchange factor Vav-1. Subsequently, Rho GTPases will initiate various signaling pathways, amongst which the regulation of the dynamic organization of the actin cytoskeleton (184, 185).

1.7. HELPER T CELL SUBSETS

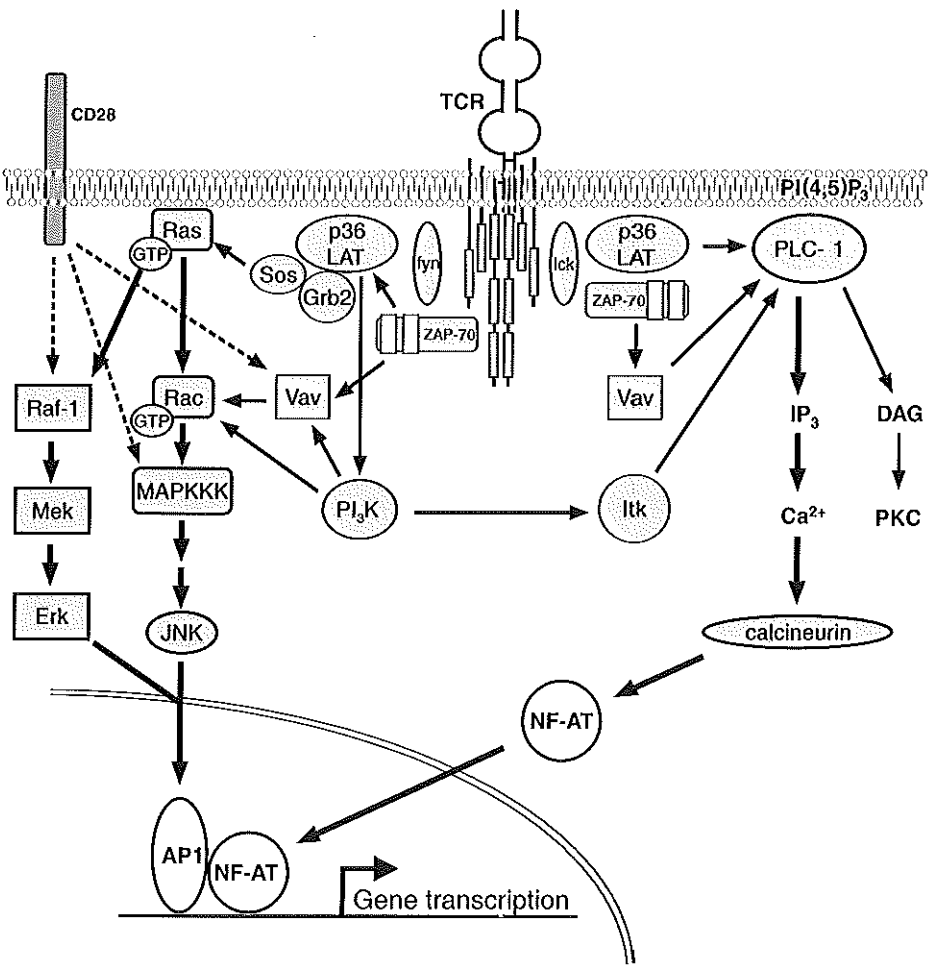


FIGURE 8

Downstream pathways in TCR-induced signal transduction.

Lck phosphorylation of adapter proteins induces activation of several downstream signaling pathways. Some relevant signaling pathways are schematically depicted here: Raf/Mek/Erk and MAP-K/JNK pathway leading to formation of the transcription factor AP-1 (Jun/Fos heterodimer); PLCγ1 activation inducing the PKC pathway and the Ca^{2+}/calcineurin pathway, leading to NF-AT activation. Gene transcription is induced by cooperate transcriptional activity of AP-1 and NF-AT.

Since their original description in the 1980s, functionally distinct effector Th subsets have been distinguished phenotypically on the basis of the profile of cytokines they produce after activation (Table 3) (186, 187). Th1 cells are characterized by the production of IL-2, interferon-γ (IFNγ) and tumor necrosis factor-β (TNFβ), whereas Th2 cells typically produce IL-4, IL-5, IL-9, IL-10 and IL-13. An intermediate effector phenotype (Th0) can also be detected, and is characterized by the concomitant production of IL-2, IFNγ, and IL-4. In addition to their cytokine production profiles, Th cell subsets can also be distinguished on the basis of their expression pattern of cytokine and chemokine receptors as well as some other cell surface markers (149, 188, 189). Th1 cells have lost cell surface expression of IFNγR2, whereas the expression of IL-12Rβ2 and IL-18R is absent on Th2 cells (190-192). In addition, Th1 cells are characterized by the expression of P-selectin glycoprotein ligand-1 (PSGL-1), whereas Th2 cells specifically express T1/ST2, a member of the IL-1R family of proteins (193, 194).

The expression patterns of chemokine receptors on human helper T cell subsets have been characterized in great detail. The level of expression of some of these receptors (CCR1, CCR2, CCR3, CCR5) is determined both by effector phenotype and activation state of the cell (189). From the studies on Th cells, it can be concluded that CCR5 (195) and CXCR3 (196) are preferentially expressed by Th1 cells whereas expression of CCR3 (197), CCR4 (195, 197) and CCR8 (198) is specific for human Th2 cells. Th0 effector cells have a mixed chemokine receptor expression profile (189). The chemokine expression patterns for murine helper T cell subsets have been studied in far less detail. It has been shown, however, that murine naïve Th cells and Th1 cells express CCR7, which is absent on murine Th2 cells, resulting in a different localization of these cells in the spleen (199).

The two Th cell subsets mediate distinct effector functions *in vivo*. Th1 cells are involved in immune responses against intracellular pathogens and mediate immune responses such as DTH and in some cases direct cytotoxicity. Th2 cells are important in the defense against extracellular pathogens and mediate humoral immune responses (200-202). Activated Th2 effector cells deliver essential signals to B cells (cytokine- and CD40L-mediated), both for the induction of B cell differentiation into immunoglobulin (Ig)-producing plasma cells as well as for the induction of Ig isotype switching to the IgG1 and IgE isotypes (203).

TABLE 3: Phenotypical characteristics of Th1 and Th2 effector cells.

Specific phenotype	Th1	Th2
Cytokine profile	GM-CSF, IL-2, IL-3, IFNγ, LT, TNFα	GM-CSF, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13
Cytokine receptors	IL12Rβ2, IL18R	IFNγR2
Other membrane receptors	PSGL-1	T1/ST2
Chemokine receptors	CCR5, CCR7, CXCR3	CCR3, CCR4, CCR8
Elicited by	Intracellular pathogens	Extracellular pathogens

Cytokines produced by Th2 cells also activate mast cells and eosinophils (200-202).

The ability of CD4+ T cells to differentiate into Th2 or Th1 cells is critical in effecting a susceptible or resistant phenotype to *Leishmania major* infection in mice, reflecting a possible *in vivo* relevance of polarized Th cell responses (204-206). In addition to the regulation of the type of immune response which is induced, effector Th cells also regulate the activity of the opposite subset. The production of IFNγ by Th1 effector cells inhibits Th2 cell differentiation and proliferation (207, 208), whereas the Th1 cells themselves have lost responsiveness to IFNγ at the receptor level (192, 209). The production of cytokines by Th1-differentiated cells is inhibited indirectly by the production of IL-10 by Th2 effector cells, which acts on the APC compartment (210).

Over the last few years, it has become clear that Th cells can differentiate into various effector phenotypes other than the 'classical' Th1 and Th2 phenotypes. These alternative Th subsets are characterized by the expression of cytokines which are involved in the negative regulation of immune responses. The Th3 subset of CD4+ T cells is characterized by the production of high levels of TGFβ as well as IL-4 (211). Functionally, Th3 cells are implicated in regulating mucosal immune responses and can play a role in preventing autoimmune responses. In addition, a CD4+ T cell subset specifically regulating Th1 responses has been described (212). This effector phenotype, designated Tr1 (regulatory T cell-1), is characterized by the production of high levels of IL-10 in the absence of appreciable amounts of IL-4.

1.8. REGULATION OF HELPER T CELL DIFFERENTIATION

The differentiation of uncommitted naïve Th cells into Th1 or Th2 effector cells is a tightly regulated process. Recently, considerable progress has been made in understanding the underlying mechanisms governing this program of cellular differentiation (188, 213-215). In particular, the regulation of Th2 differentiation has been revealed into great detail (191, 216-226). A new model for Th cell differentiation can be extracted from these studies. This model is depicted in figure 10 for Th1 differentiation and in figure 11 for Th2 differentiation.

In order to enter the genetic program leading to Th cell differentiation, the resting naïve Th cell needs to receive concomittant activating (TCR-delivered) and differentiation-inducing (cytokine receptor-delivered) signals (226). Upon activation by TCRαβ mediated signals (in the presence of costimulation), the naïve Th cell will enter the cell cycle and start expressing low levels of *IL-2* (217, 226). Expression of other cytokine genes upon activation requires entry of the activated naïve Th cell into the S phase of the first cell cycle, underscoring the requirement for *de novo* DNA synthesis of cytokine gene expression (217, 226). Full differentiation into Th1 or Th2 effector cells, characterized by high-level cytokine production requires several cell divisions, taking generally up to 4 days (213).

Cytokine-induced signaling pathways are essential in driving the differentiation of activated naïve CD4+ T cells from the common precursor into either phenotype (187, 201, 202, 227, 228). IL-12 induces the differentiation of naïve Th cells into the Th1 effector phenotype (229-232) by activating the transcription factor Stat4 (for signal transducer and activator of

transcription-4) (225, 233, 234). IL-4 directs $CD4^+$ T cell differentiation towards the Th2 effector phenotype (235-238), which is mediated by Stat6 activation (239-241). Therefore, the activation of Stat4 versus Stat6 is critical in inducing Th cell differentiation in response to cytokines. Stat-independent induction of Th cell differentiation, however, has also been reported (224, 242-244). Nevertheless, in the majority of Th cell-mediated immune responses, Th cell differentiation seems to be dependent on cytokine-mediated activation of Stat-factors.

Over time, Th cell phenotypes become stabilized (245). This is in part due to loss of sensitivity to cytokine-induced differentiative signals (188, 246). Whereas Th1 cells have been shown to have a loss of sensitivity to IL-4R-mediated signal transduction (247), Th2 cells actively downregulate the IL-12Rβ2 subunit, resulting in a complete loss of IL-12-induced signal transduction (191, 225). In addition to a loss-of-sensitivity to cytokine-mediated differentiative signals, Th cells acquire fixed and heritable chromatin structures at relevant genetic loci, such as those containing cytokine genes (213, 248). This epigenetic regulation of effector gene expression contributes to Th cell phenotype stabilization.

In addition to chromatin changes at relevant genetic loci, transcription factors play a central role both in the regulation of Th cell differentiation and in the cell type-specific expression of (cytokine) genes (188, 213, 215, 246). Recently, it has become clear that transcription factors play a dual role in the regulation of Th cell differentiation by actively inducing differentiation into the one lineage while actively repressing differentiation into the opposite lineage (188, 214, 215). The recently identified transcription factor T-box expressed in T cells (T-bet), was shown to be expressed in Th1 cells but not in Th2 cells (249). Expression of T-bet, however, is not confined to the T cell lineage, and is also correlated with expression of IFNγ in B cells and NK cells. During Th cell differentiation, expression of T-bet was shown to induce IFNγ production and actively repress IL-4 and especially IL-5 production. In Th2 clones, ectopic expression of T-bet resulted in suppression of IL-4 and IL-5 expression, but not in induction of IFNγ expression (249).

Conversely, expression of the transcription factor GATA-3 has been shown to be upregulated during Th2 differentiation and downregulated during Th1 differentiation (250, 251). GATA-3 expression in uncommitted helper T cells results in repression of IFNγ-production and induction of Th2 differentiation independently of Stat6 (224). In addition, ectopic expression of GATA-3 in committed Th1 cells induced Th2 cytokine expression and chromatin remodeling at the *IL-4* locus reminiscent of Th2-committed cells, whereas repression of IFNγ-production by GATA-3 was inconsistently observed (221). Both GATA-3 and T-bet are induced by TCR-mediated Th cell activation (249, 250). In addition, Stat4 induces T-bet and represses GATA-3, whereas Stat6 induces GATA-3 (191, 223, 249). The effect of Stat6 on T-bet has not yet been analyzed (249). In conclusion, the induction of T-bet versus GATA-3 might be decisive in the differentiation of naïve Th cells into Th1 or Th2 phenotypes (188, 215).

1.9. SIGNAL TRANSDUCTION BY THE IL-4 RECEPTOR

IL-4-induced signaling pathways are key regulators for driving Th2 differentiation. The IL-4 receptor (IL-4R) complex is responsible for transmitting the IL-4-delivered signals across the cell membrane (252). The IL-4R complex contains the IL-4Rα chain which binds IL-4 with high affinity, and the common gamma chain (γc), which is also used by the IL-2 and IL-7 receptor complexes (253). Non-hematopoietic cells have been described to use IL-13Rα rather than γc to form a functional IL-4R complex (254).

Signal transduction by the IL-4R complex is dependent on activation of Janus kinase (Jak)-family members of receptor associated tyrosine kinases (255). Jak-1 and Jak-3 are constitutively associated with IL-4Rα and γc chain, respectively (figure 9; 256, 257). The IL-4Rα chain is 785 amino acids long, containing a 553 amino acid cytoplasmic region (258), which contains five highly conserved tyrosine residues. Binding of IL-4 to IL-4Rα induces heterodimerization of the IL-4Rα chain with the γc chain, resulting in tyrosine phosphoryla-

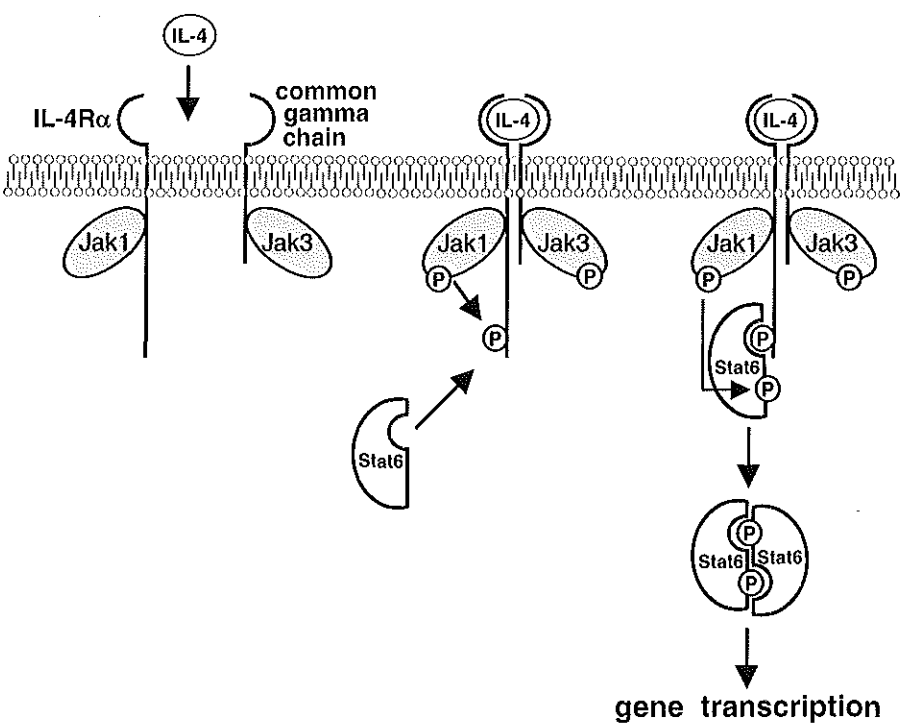


FIGURE 9

Signal transduction by the IL-4 receptor complex.

Binding of IL-4 to the extracellular part of the IL-4Rα chain induces heterodimerization with the common gamma chain. As a result, Jak kinases, which are constitutively associated to the intracellular part of the receptor chains, will phosphorylate critical tyrosine residues in the cytoplasmic tail of the cytokine receptor. These phosphorylated tyrosine residues serve as docking sites for multiple signaling molecules. In the case of IL-4, Jak1 phosphorylates tyrosine residues in the IL-4Rα chain, which induced Stat6 docking to these phosphotyosine residues. Phosphorylated Stat6 homodimerizes and translocates to the nucleus to initiate gene transcription of IL-4-responsive genes.

tion of Jak-1 and Jak-3 and consequently in rapid tyrosine phosphorylation of the IL-4Rα chain itself (252, 259). These phosphorylated tyrosine residues then serve as docking sites for downstream signaling proteins which recognize these residues with their Src-homology-2 (SH2) or phosphotyrosine-binding (PTB) domains (252). Downstream signal transduction pathways include the insulin receptor substrate (IRS)-1/2 pathway and the activation of Stat factors. Activation of IRS-1/2 by IL-4R engagement induces cellular proliferation in response to IL-4 (260, 261).

Activation of Stat factors by activated Jak family members is responsible for the rapid induction of gene transcription by cytokines (262, 263). Stat-6 is the primary Stat protein family member activated in response to IL-4, and is responsible for the induced or enhanced transcription of IL-4-responsive genes (239, 241, 264, 265). Stat-6 binds the phosphorylated tyrosine residues in the IL-4Rα chain through a highly conserved SH-2 domain. Activated Jak kinases then activate Stat-6 by phosphorylation of a C-terminal tyrosine residue (266). Phosphorylated Stat-6 homodimerizes and translocates into the nucleus to activate gene transcription (263, 267). Activated and dimerized Stat factors recognize the semi-palindromic DNA sequence TTC(N)3/4GAA known as IFNγ-activated sequence (GAS) motif (268).

1.10. REGULATION OF IL-4R-MEDIATED SIGNAL TRANSDUCTION

Signal transduction of the IL-4R complex can be modified by multiple mechanisms. A more generalized regulatory mechanism in tyrosine-phosphorylation-dependent signal transduction is dephosphorylation of signaling intermediates by phosphotyrosine phosphatases (PTP) such as SH-2 containing posphatases SHP-1 and SHP-2 or the SH-2-containing inositol-5-phosphatase (SHIP) (269). A modulatory role for these PTP in IL-4-induced signal transduction has been suggested by studies using phosphatase inhibitors (270).

Another regulatory mechanism in IL-4R mediated signal transduction involves direct inhibition of Jak-1 activity by suppressor of cytokine signalling-1 (SOCS-1) (271). SOCS-1 inhibition of Jak activity is mediated through interaction of a conserved SH-2 domain in SOCS-1 with a critical tyrosine residue in the activation loop of Jak proteins, resulting in diminished Stat activation (272). This effect of SOCS-1 on Jak-1-mediated Stat6 activation could be due to shielding of the critical phosphotyrosine in Jak-1, or alternatively to targeting of Jak-1 for proteosomal breakdown. As SOCS-1 is an immediate-early gene induced by IL-4-mediated Stat-6 activation, this regulatory pathway resembles a classical negative feedback loop on IL-4-mediated signal transduction (273).

SOCS-1 is a member of a family of SH-2-containing proteins which exert a specific negative regulation of the Jak/Stat pathway (273-276). The regulatory activities of this family of proteins were first recognized in SOCS-1, which was characterized as an inhibitor of IL-6-induced Jak/Stat-mediated signal transduction by three groups independently (277-279). These groups called the protein SOCS-1, Stat-induced Stat-inhibitor-1 (SSI-1) and JAK-binding protein (JAB), respectively. The molecule was characterized to be a family member of the already cloned cytokine-induced Scr-homology-2 containing (CIS)-1 protein (280). The SOCS-family of adaptor proteins is characterized by a central SH-2 domain and a C-ter-

minal conserved region called the SOCS-box domain (figure 7). The CIS/SOCS family of proteins contains 8 members (CIS-1 and SOCS1-SOCS7) (281). The mechanisms of inhibition of Stat activation differ between family members (276). CIS-1 has been shown to directly bind to phosphorylated residues on the IL-3Rβ and erythropoietin (EPO) receptor chain, and plays a role in the inhibition of IL-3- and EPO-induced Stat activation (280). SOCS-1 and SOCS-3 have been shown to specifically associate with phosphorylated Jak proteins using their central SH-2 domains and N-terminal flanking sequences (282, 283). SOCS proteins have been implicated in proteosomal breakdown of their targets, as it was shown that the SOCS-box interacted with elongin-B/C (284). Mice with a targeted deletion of *SOCS-1* were reported to succumb to a complex disease associated with IFNγ hyperresponsiveness (285, 286). *SOCS-2*-targeted mice displayed marked gigantism, associated with deregulated growth hormone and insulin-like growth factor signaling (287). Targeted deletion of *SOCS-3* resulted in early embryonic death associated with marked erythrocytosis, whereas enforced expression of *SOCS-3* results in a specific suppression of fetal erythropoiesis. These data implicate SOCS-3 in the negative regulation of fetal liver hematopoiesis (288).

1.11. REGULATION OF Th1 DIFFERENTIATION AND GENE EXPRESSION

Th1 differentiation of activated naïve Th cells depends critically on IL-12-induced activation of Stat4 (225, 229-234). The mechanism of Stat4-induced Th1 differentiation, however, remains unclear (188, 215). Stat4 has been shown to downregulate expression of GATA-3 and is suggested to induce expression of T-bet (188, 223, 249). Taken together, these activities would favor differentiation of Th cells into an IFNγ-producing phenotype, as T-bet has been shown to strongly induce IFNγ production, whereas GATA-3 downregulates IFNγ production (221, 223, 249). However, *IL-2* gene expression, which is maintained in differentiated Th1 but not in differentiated Th2 cells, cannot be dependent on T-bet, as this transcription factor exerts a negative regulatory effect on *IL-2* transcription (249). The activity of T-bet might be reflected in the lower levels of IL-2 expressed by differentiated Th1 cells as compared to naïve activated Th cells (188). Still, additional factors must be responsible for the Th2-specific down-regulation of IL-2.

As discussed earlier, expression of the Th1-specific *IFNγ* gene is in part regulated by chromatin structure at the *IFNγ* locus (216). Several intracellular signalling pathways contribute to *IFNγ* gene transcription (figure 10) (215). Activation-inducible transcription factors implicated in *IFNγ* gene expression are NF-κB, NF-AT and ATF-2 (215). IL-18-mediated signal transduction, resulting in NF-κB activation, has been reported to strongly enhance IFNγ production (289). Two NF-AT binding sites were initially characterized, one of which was shown to also bind NF-κB (290, 291). In addition, two NF-AT sites have been identified by DNA footprinting analyses (292). Finally, the MAP kinase pathway appears to contribute to IFNγ production (293, 294). Additional transcription factors putatively regulating *IFNγ* gene expression are jun, CREB and Oct-1, which together with ATF-2 bind a 25 bp proximal promotor region (295). Regulatory elements in the *IFNγ* promotor region interacting with the newly described Th1-specific transcription factor T-bet, which has been shown to induce

potent IFNγ production, have not yet been described (249).

Regulation of *IL-2* gene expression seems to be directly controlled by the 300 bp proximal promotor region, which has been shown to confer tissue specificity and inducibility in transient transfection studies *in vitro* (296). The proximal 300 bp promotor region contains multiple composite NF-AT/AP-1 binding sites as well as an NF-κB site, two Oct sites and the

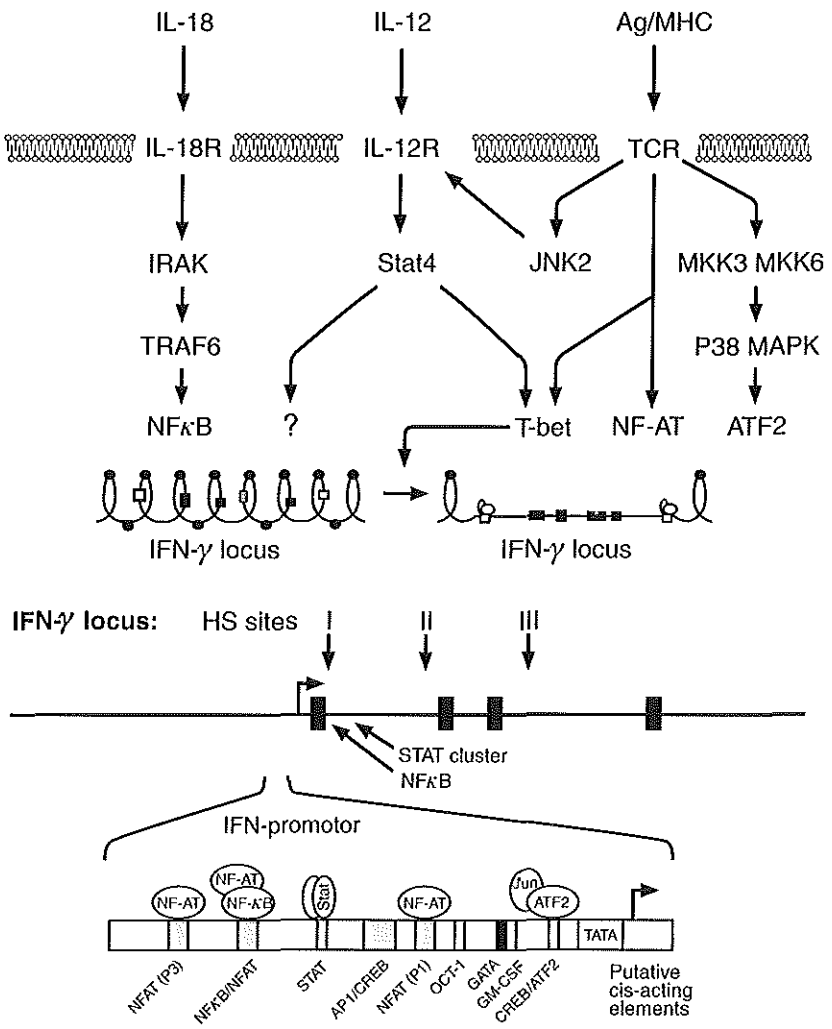


FIGURE 10
Regulation of Th1 differentiation and *IFNγ* gene transcription.
A schematical representation signaling pathways inducing transcription factors involved in Th1 differentiation and IFNγ gene transcription as well as a schematical outline of the IFNγ proximal promotor region (see text for details).

CD28RE, all of which are essential for optimal *IL-2* expression (297). This proximal promotor region, however, was insufficient for *in vivo* high level *IL-2* expression in a transgenic mouse model, which instead required a more extended 600 bp *IL-2* promotor region (298). The additional 300 bp upstream of the proximal promotor region probably contain regulatory elements required for chromatin remodeling at the *IL-2* locus (297, 299). In addition, post-translational regulation implemented at the level of *IL-2* mRNA stability is mediated by CD28-delivered signals, and reflects the biochemical equivalent of costimulation (300).

1.12. REGULATION OF Th2 DIFFERENTIATION AND GENE EXPRESSION

Th2 differentiation of activated naïve Th cells is most efficiently induced by IL-4-dependent Stat6 activation (235-241), although Stat6-independent Th2 differentiation has been observed (224, 243, 244). In one of these cases, the Th2 differentiation was dependent on GATA-3, suggesting an autoregulatory mechanism to be responsible for the Stat6-independent GATA-3 activity (224). The other reports have not addressed the issue of the GATA-3 dependence of the Stat6-independent Th2 differentiation (243, 244). It seems unlikely that Stat6 directly induces *IL-4* gene transcription (301). An inducible activated form of Stat6 was shown to induce GATA-3 and c-Maf in Th1 cells, resulting in IL-4 production (220). Ectopic expression of *GATA-3* in polarized Th1 cells induced Th2 cytokine expression and chromatin remodeling at the *IL-4* locus reminiscent of Th2-committed cells (221). So, activation of GATA-3 by Stat6 seems to be the key event in Th2 differentiation (figure 11).

Transcription of the *IL-4* gene is regulated by accessibility of chromatin at the *IL-4* locus as described before, as well as by Th2 cell-specific and activation-inducible transcription factors (213). Studies in transgenic mice initially showed that a 3 kb region containing the *IL-4* promotor could transfer tissue-specific expression of *IL-4* (302). The elements responsible for preferential expression in Th2 cells were then demonstrated to be located in the –741-bp to +60-bp region of the *IL-4* promotor (303). A more detailed analysis of the *IL-4* promotor region revealed the presence of five NF-AT sites, two of which are composite NF-AT/AP-1 sites which were shown to be critical for *in vitro* induction of *IL-4* gene transcription (302, 304-308). NF-AT family members are differentially expressed between Th1 and Th2 cells. NF-ATc1 was shown to be a direct transcriptional activator of the *IL-4* gene (309-311). The two other lymphoid family members, NF-ATc2 and NF-ATc3, have redundant roles in negatively controlling Th2 differentiation and IL-4 production, most likely by the induction of unidentified NF-AT target genes which play a critical role in negatively regulating Th2 responses (188).

The Th2 cell-type specific transcription factor c-Maf has been shown to play an important role in IL-4 production (218, 219, 312). In mice with a targeted deletion of the *c-Maf* gene, IL-4 production was severely impaired, whereas transgenic expression of *c-Maf* resulted in an IL-4-dependent increased Th2 differentiation and effector function (218, 219). The AP-1 family member c-Maf is a basic region/leucine zipper factor and binds a consensus sequence (MARE for c-Maf responsive element) in the *IL-4* proximal promotor region (312). Expression of *c-Maf* is induced by TCR-mediated Th2 cell activation. Finally, the role of

GATA-3 in direct transactivation of the *IL-4* promotor seems to be minimal (188). Transcription of the *IL-5* gene, however, is directly transactivated by GATA-3, which binds a double GATA-consensus site at positions -70 to -59 in the proximal *IL-5* promotor (128, 251, 313, 314).

The coordinate expression of the *IL-4*, *IL-5* and *IL-13* genes has recently been found to

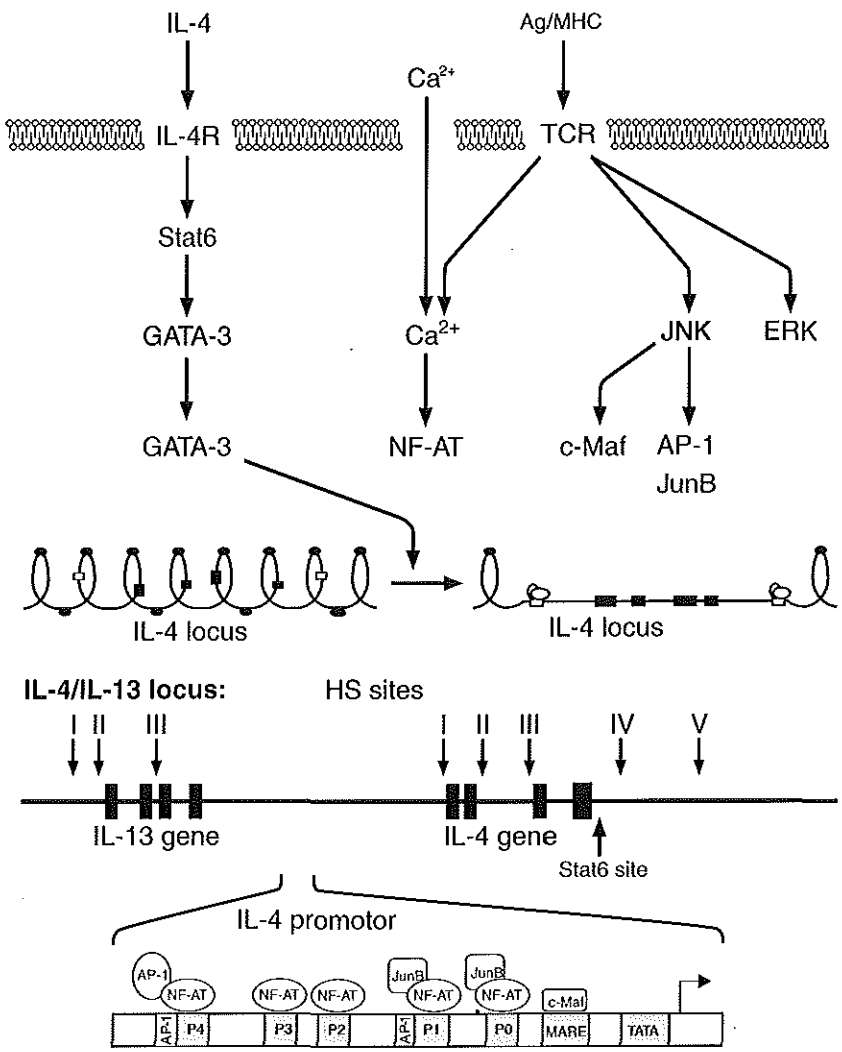


FIGURE 11

Regulation of Th2 differentiation and *IL-4* gene transcription.

A schematical representation signaling pathways inducing transcription factors involved in Th2 differentiation and *IL-4* gene transcription, as well as a schematical outline of the *IL-4* proximal promotor region (see text for details).

be regulated by a conserved (84% homology between human and mouse) non-coding sequence located in between the *IL-4* and *IL-13* loci. This 402 bp sequence appears to be involved in the long-range regulation of gene transcription by modulating chromatin structure, and therefore functions as an LCR (222). It has been observed that this sequence contains putative GATA-3 binding sites (315). Therefore, the *IL-4*, *IL-5* and *IL-13* proximal promotor regions might only play a limited role in the regulation of cell-type specific expression of these Th2 cytokine genes.

AIM OF THIS THESIS

T cell differentiation is tightly regulated. Most of the regulatory processes involved in the differentiation of the various types of mature T cells, have not been elucidated into great detail. The aim of this thesis is to investigate the regulation of cell-fate decisions by T lymphocytes at various stages of their differentiation.

We analyze regulation of Th cell differentiation at various levels, including regulation at the cellular level by production of cytokines IL-4 and IL-10, intracellular regulation of the signals elicited by the TCR and the IL-4 receptor and the regulation by the transcription factor GATA-3, which was shown to be essential and sufficient for Th2 differentiation. As GATA-3 has been shown to be critical for the development of the T cell lineage, we also analyze the role of this transcription factor during thymic differentiation of T cells into detail.

In Chapter 1 a general introduction on T cell differentiation in the thymus and in the periphery is provided. In Chapters 2, 3 and 4 the role of GATA-3 in T cell differentiation is analyzed. To analyze transcription of the *GATA-3* gene during T cell development, we generated a strain of mice in which a *lacZ* reporter gene was inserted into the *GATA-3* locus. In Chapter 2, the levels of *GATA-3* gene transcription at the subsequent stages of T cell differentiation are analyzed. In addition, we evaluated the contribution of *LacZ*-tagged *GATA-3*-deficient ES cells to the T cell lineage in chimeric mice.

To be able to investigate the role of GATA-3 in thymic as well as in peripheral T cell differentiation *in vivo*, we generated transgenic mice in which *GATA-3* expression was modified. In contrast to the normal, modulated expression, i.e. low levels during phases of *TCRα* and *TCRβ* gene rearrangement, in CD8 SP cells and in Th1 cells, the *CD2* LCR provided expression throughout T cell differentiation. In Chapter 3, we describe the effect of dysregulation of *GATA-3* expression on T cell differentiation in the thymus, and in Chapter 4 the effect of the enforced expression of *GATA-3* on the differentiation of Th1 and Th2 cells and on Th memory cell formation.

Chapter 5 describes the analysis of *in vivo* differentiation of SJA/9 $CD4^+$ T cells into effector phenotypes. These mice have a specific Th2-defect, characterized by low IgE responses, which can be attributed to a failure of SJA/9 $CD4^+$ T cells to produce sufficient

IL-4. In Chapter 6, an attempt is made to analyze the role of SOCS-3 in T cell activation and differentiation. The SOCS family of proteins has been implicated in the regulation of cytokine receptor signaling, indicating a potential role in Th1 and Th2 differentiation. In Chapter 7, the results obtained in this thesis project are discussed in the context of the current knowledge of T cell differentiation.

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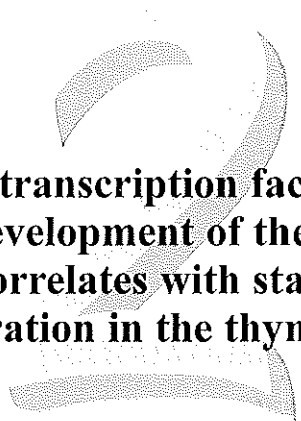
Chapter 2



Expression of the transcription factor *GATA-3* is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus

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ABSTRACT

GATA-3 is a zinc-finger transcription factor which is essential for both early T cell development and differentiation of Th2 cells. To quantify *GATA-3* expression during T cell differentiation *in vivo* in the mouse, the *GATA-3* gene was targeted by insertion of a *lacZ* reporter by homologous recombination in ES cells. Although we could detect *GATA-3*$^+$ cells throughout T cell differentiation in the thymus, the proportions of *GATA-3*$^+$ cells varied considerably between the distinct stages of differentiation. The two periods of T cell receptor (*TCR*)α and *TCR*β gene recombination, which occur in quiescent or slowly dividing cells, were associated with low proportions of *GATA-3*$^+$ cells. Conversely, the stage of rapidly proliferating cells, which insulates these two waves of *TCR* gene rearrangement, was characterised by a large proportion of *GATA-3*$^+$ cells. In addition, we generated chimeric mice by injection of *GATA-3*-deficient, *lacZ*-expressing embryonic stem cells into wild-type blastocysts. In this *in vivo* competition analysis, no contribution of *GATA-3*-deficient cells to the T cell lineage was detected, not even in the earliest CD44$^+$CD25$^-$ double negative (CD4$^-$CD8$^-$) cell stage in the thymus. These results parallel data implicating other GATA family members as key regulators of proliferation and survival of early hematopoietic cells. We therefore propose that GATA-3 is required for the expansion of T cell progenitors, and for the control of subsequent proliferation steps, which alternate periods of *TCR* gene recombination in the thymus.

INTRODUCTION

T-cell differentiation proceeds in the thymus through a highly regulated program in which precursor cells from fetal liver or bone marrow expand and progress through a series of distinct maturational stages (1,2). On the basis of CD4 and CD8 expression, thymocytes can be subdivided into four main populations, the most immature of which are the CD4$^-$CD8$^-$ double negative (DN) cells. A detailed assessment of differentiation stages within the DN compartment, which represent only 2-5% of adult thymocytes, is based upon *TCR* loci configuration and expression of the surface markers CD44 (phagocyte glycoprotein-1) and CD25 (IL-2R α-chain) (2-3). The most immature DN population has the CD44$^+$CD25$^-$ phenotype, and the transition to the next stage is marked by the induction of CD25 (4-7). During the next step, the CD44$^+$CD25$^+$ DN cells decrease in size and *TCR*β gene rearrangements are initiated and completed. Only those cells that produce a functional TCRβ chain are then selected for further maturation, enter the cell cycle and expand rapidly to generate CD44$^-$CD25$^-$ cells (1,2.4). These cells upregulate CD4 and CD8 expression and concurrently initiate *TCR*α gene rearrangement. Subsequently, CD4$^+$CD8$^+$ double positive (DP) cells are positively selected on the basis of TCR$\alpha\beta$ expression and differentiate further into either CD4$^+$ or CD8$^+$ single positive (SP) mature thymocytes (1).

Several transcription factors have been shown to be essential for the differentiation of

the T cell lineage (5,6). One of these is *GATA-3*, which belongs to a family of transcription factors that bind to a *GATA* consensus motif through a highly conserved C4 zinc finger domain (7-9). *GATA-3* is abundantly expressed in the developing central nervous system, adrenal gland and kidney, but within the haematopoietic system expression appears to be confined to T cells (10-12). Mice with targeted null mutations of the *GATA-3* gene die on embryonic day 12, displaying anaemia and central nervous system defects (10). *GATA-3* was shown to be expressed in the most immature subset of day 12 fetal thymus cells, which have the $CD44^+CD25^-$ DN phenotype, and was shown to be essential in early T cell development, using anti-sense *GATA-3* oligonucleotides in fetal thymic organ cultures (13). Moreover, *RAG-2*$^{-/-}$ complementation experiments *in vivo* demonstrated that the differentiation of *GATA-3*$^{-/-}$ T cells is blocked at or before the $CD4^-CD8^-$ DN stage of thymocyte development (14). In addition to this very early requirement, *GATA-3* is selectively expressed in mature Th2 cells (15,16). *GATA-3* binding sites are present in the promoter regions of all Th2 cytokine genes, as well as in regulatory regions of the *CD8α*, *TCRα*, *TCRβ* and *TCRδ* genes (5 and references therein).

It is currently not known at which maturational stages *GATA-3* exerts its critical function. We have targeted the *GATA-3* gene by inserting a *lacZ* reporter by homologous recombination in ES cells. The presence of the *lacZ* reporter gene, placed under direct *GATA-3* transcriptional and translational control, enabled us to quantify the *GATA-3* expression profile during T cell differentiation *in vivo*, revealing an association between *GATA-3* expression and steps of cellular proliferation. We have also generated chimeric mice by injecting *lacZ*-expressing embryonic stem (ES) cells into wild-type blastocysts, and followed these cells during T cell differentiation.

MATERIALS AND METHODS

GENERATION OF HETEROZYGOUS AND CHIMERIC MICE

The generation of the targeting vectors, containing the *E.* coli *nlslacZ* and the *taulacZ- hygromycin* fusion genes has been described (Van Doorninck et al., submitted). Briefly, the genomic murine *GATA-3* gene was PCR-modified by introducing an *NcoI* restriction site at the start codon. Subsequently, a *BspHI-XhoI* fragment with a *nlslacZ* cassette (19) was inserted in-frame into the *NcoI* and *ClaI* sites of the genomic GATA-3 fragment, deleting most of the second exon. To generate the *nlslacZ* targeting construct, the modified fragment was ligated to a subclone, containing the PGK-hygromycin resistance gene cassette (kindly provided by H. te Riele), and to the downstream 4-kb *EcoRI-SacI* genomic fragment, containing the thymidine kinase gene from the previously described *GATA-3*^{neo} targeting construct (10). The *GATA-3 taulacZ* vector was derived from the *nlslacZ* targeting construct by exchanging the *nls* by a *tau* fragment (Van Doorninck et al., submitted). E14 ES cells were cultured and transfected as described (19). Homologous recombinants were screened by Southern blot analysis and chimeric mice were generated by injection of homologous recom-

binant ES cells into C57BL/6 blastocysts. To obtain the heterozygous *GATA-3*$^{+/nlslacZ}$ and *GATA-3*$^{+/taulacZ}$ for the *lacZ* expression studies, wild type E14 ES cells were transfected. Chimeras were crossed with C57BL/6 wild type mice to yield *GATA-3*$^{+/nlslacZ}$ mice, *GATA-3*$^{+/taulacZ}$ mice, as well as *GATA-3*$^{+/+}$ mice, which served as controls. For the generation of the chimeric mice (figure 4) *GATA-3*$^{+/lacZ}$ and *GATA-3*$^{neo/lacZ}$ ES cells were obtained by transfection of *GATA-3*$^{+/neo}$ ES cells (10), with a targeted deletion within the *GATA-3* gene on one allele (figure 1).

FLOW CYTOMETRIC ANALYSES

Preparation of single-cell suspensions, determination of β-Galactosidase activity using FDG and subsequent incubations with mAb have been described previously (19). For three-colour flow cytometry 0.5-1x10^5 events were scored using a FACScan analyzer (Becton Dickinson, Sunnyvale, CA). For four-colour analysis, 1x10^5 - 2x10^6 events were scored using a FACSCalibur dual laser instrument (BD). The following mAb were purchased from Pharmingen (San Diego, CA): PE-conjugated anti-CD4 (L3T4), anti-CD25 and anti-CD11b/Mac-1, Cy-Chrome conjugated anti-CD8 (Ly-2), anti-CD44 and anti-B220 (RA3-6B2), biotinylated anti-CD4, anti-CD8 and anti-IgM. Secondary Ab used were PE-conjugated (Caltag Laboratories, CA) or allophycocyanin (APC)-conjugated streptavidin (Pharmingen). FDG and To-Pro3 were from Molecular Probes Europe BV (Leiden, The Netherlands).

Th1 AND Th2 CELL CULTURES

Single cell suspensions from spleen were enriched for T cells by incubation with mAb to MHC class II, CD16/32, heat stable antigen, and Gr-1 and subsequent treatment with rabbit C. The resulting cell suspensions were incubated with biotinylated anti-CD40, anti-B220 and anti-CD8, and subsequently with streptavidin-conjugated microbeads. Using a MACS column separation, cell populations were obtained that were ~90% CD4. Cells were cultured in the presence of 10 mg/ml anti-CD28 and 50 U/ml IL-2 in plates pre-coated with 50 mg/ml anti-CD3. Priming for Th1 responses was with 50 ng/ml IL-12, 100 U/ml IFNγ and 10 μg/ml anti-IL-4 and for Th2 responses with 50 μg/ml IL-4 and 10 μg/ml anti-IFNγ. After 4 days of culture, cells were transferred to new 96-wells plates, coated with anti-CD3, and at day 7 *lacZ* expression was determined as described above.

RESULTS

WITHIN THE HEMATOPOIETIC SYSTEM *GATA-3* EXPRESSION IS RESTRICTED TO THE T-CELL LINEAGE

To follow the expression pattern of *GATA-3*, mice were generated in which a *lacZ* gene was introduced at the ATG translation start, either fused a nuclear localisation signal sequence (*nlslacZ*), or to the *tau* gene (*taulacZ*), resulting in cytoplasmic *lacZ* localisation (figure 1). The expression of *GATA-3*-directed *lacZ* was analyzed in heterozygous *GATA-3*$^{+/nlslacZ}$

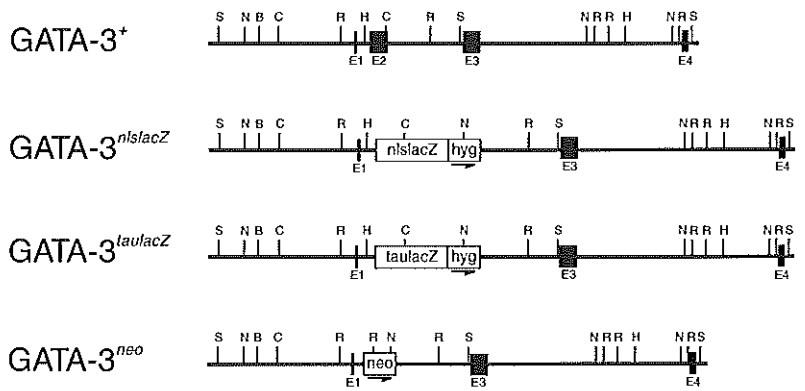

FIGURE 1

Schematic representation of the wild-type and target *GATA-3* alleles.

The horizontal line represents *GATA-3* genomic DNA. The four exons (E1 to E4) of the *GATA-3* gene are depicted by boxes. The arrows indicate the orientation of the hygromycin (hyg) and the neomycin (neo) resistance genes. The restriction enzyme sites shown include B, BamHI; C, ClaI; H, HindIII; R, EcoRI; N, NcoI and S, SacI.

and *GATA-3*$^{+/taulacZ}$ mice, by using fluorescein-di-β-D-galactopyranoside (FDG) as a substrate in flow cytometric analyses. Single cell suspensions from thymus, spleen, mesenteric LN and peritoneal cavity of 5 week-old mice were evaluated for *lacZ* activity in conjunction with surface expression of B cell- or T cell-specific surface markers. The analyses described below were performed on *GATA-3*$^{+/nlslacZ}$ mice. In addition, we independently investigated *GATA-3*$^{+/taulacZ}$ mice, in which comparable proportions of *lacZ*$^+$ cells were identified in the individual stages of T cell development.

In spleen and mesenteric LN, expression of *GATA-3* was found to be restricted to CD4$^+$ T cells, which manifested proportions of *lacZ* expressing cells of 22-25%, while *lacZ* activity was very low in CD8$^+$ T cells, and absent in B cells (figure 2). No *lacZ* activity was detected in the Mac-1^{hi} myeloid or Mac-1^{lo} NK cell populations in the spleen and peritoneal cavity (data not shown).

To verify that the *lacZ* activity of the targeted *GATA-3*^{nlslacZ} allele reflected the actual expression pattern of the *GATA-3* gene, we investigated purified splenic CD4$^+$ T cells from heterozygous *GATA-3*$^{+/nlslacZ}$ mice after *in vitro* culture using priming conditions for either Th1 or Th2 cells. During the differentiation to Th2 cells, the proportion of *lacZ*$^+$ cells increased from 6-12% in freshly isolated splenic CD4$^+$ cells to ~40% in activated CD4$^+$ T cell blasts at day 7, whereas after an analogous Th1 priming, *lacZ* expression decreased to undetectable levels. These results are consistent with the reported specific expression of *GATA-3* in Th2 cells (15,16).

EXPRESSION OF *GATA-3* DURING EARLY T CELL DIFFERENTIATION

In the thymus, a significant proportion of the CD4$^-$CD8$^-$ DN early cell fraction (28%±13) expressed *lacZ* (figure 2B). To investigate the *GATA-3* expression profile in this fraction in

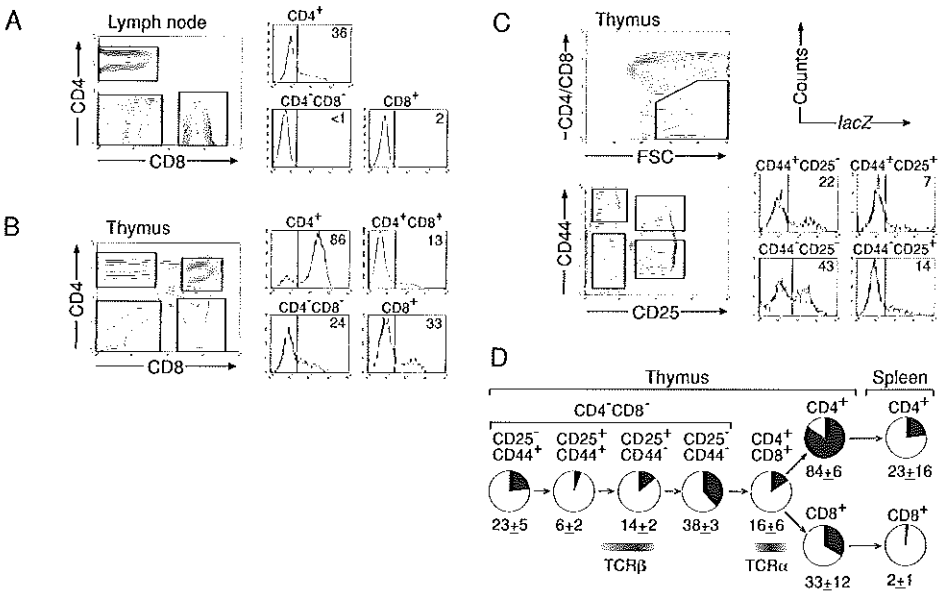

FIGURE 2

***GATA-3* gene expression during T cell differentiation.**

Three-colour flow cytometric analyses of *GATA-3*$^{+/nlslacZ}$ cells from mesenteric LN (A) and thymus (B). Data are shown as 5% probability CD4/CD8 contour plots (left). The indicated populations were gated and analysed for *lacZ* expression. The results are displayed as histograms with the percentages of *lacZ*$^+$ cells indicated (right). The CD4$^-$ CD8$^-$ cell population in LN consisted for >98% of B cells.

(C) Four-colour flow cytometric analyses of *GATA-3*$^{+/nlslacZ}$ cells from thymus. CD4$^-$CD8$^-$ DN cells with relatively high forward scatter were analysed for CD25 and CD44 expression, as displayed in 5% probability contour plots (left). The gated four populations were analysed for *lacZ* expression; the percentage of *lacZ*$^+$ cells is indicated in the histograms (right).

(D) Summary of *GATA-3* gene expression during T cell differentiation. In the indicated stages, the proportions of *lacZ*$^+$ cells are given as mean ± SD of 6-13 mice examined.

more detail, we performed four-colour flow cytometry. The DN cell populations with high forward scatter characteristics were gated, and subdivided into four populations, based on differential expression of CD44 and CD25 (figure 2C and 2D). *LacZ* was expressed in 23±5% of the most immature population of CD44$^+$CD25$^-$ pro-T cells. The size of this *lacZ*$^+$ fraction decreased to 6%±2 after transition to the next differentiation stage of CD44$^+$CD25$^+$ DN pro-T cells. In the next stage of CD44$^-$CD25$^+$ DN pre-T cells 14%±2 of the cells were *lacZ*$^+$. This population mainly contains non-dividing small cells, where *TCRβ* rearrangements are initiated and completed (1,17,18), but 8-10% are large-sized cycling cells in which the appearance of a functional TCRβ chain has triggered rapid cell division (1,18). These β-selected cells subsequently downregulate CD25 to give rise to the CD25$^-$CD44$^-$ DN subpopulation that entirely consists of large proliferating cells. This subpopulation manifested a high proportion of *lacZ* expressing cells (38±3%, figure 2C and 2D). When they progress to the next matura-

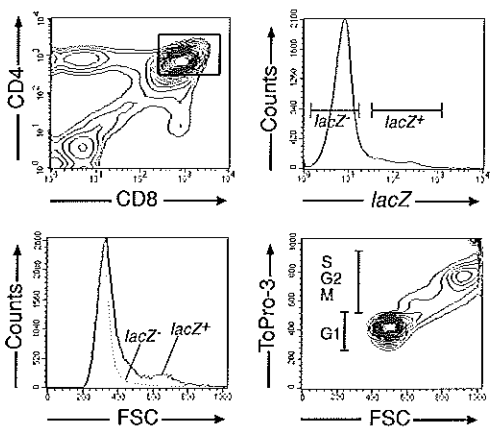

FIGURE 3
Correlation between *GATA-3* expression and cell size. (D) Three-colour flow cytometric analyses of GATA-3$^{+/nlslacZ}$ cells from thymus. A 5% probability CD4/CD8 contour plots is shown (top left). The indicated CD4$^+$CD8$^+$ DP thymocyte population was gated and analysed for *lacZ* expression (top right). The FSC of the *lacZ*$^+$ and *lacZ*$^+$ DP cell populations are displayed as histogram overlays (bottom left). The mean FSC was 380 for the *lacZ*$^+$ and 327 for the *lacZ*$^-$ cell population. For cell cycle analyses, thymocyte cell suspensions were labelled with anti-CD4, anti-CD8 and To-Pro3. CD4$^+$CD8$^+$ DP thymocytes were gated and analysed for FSC and DNA-content. The 5% probability contour plot displayed (bottom right) shows that cycling cells (S, G2 and M phase) have larger FSC values.

tion stage of smaller, non-dividing CD4$^+$CD8$^+$ DP cells, in which the *TCRα* gene rearrangements occur (1,17), *GATA-3* expression decreased: 16%±6 of the cells were *lacZ*$^+$. At the transition to the SP stages, the proportion of *GATA-3* expressing cells increased, particularly in the CD4$^+$ cells (to 84%±6) but also in the CD8$^+$ cells (to 33±12; figure 2).

In summary, we found that the two waves *TCR* gene rearrangements were associated with low proportions of *GATA-3* expressing cells, insulated by the CD25$^-$CD44$^+$ DN cell stage, which contained a large proportion of *GATA-3* positive cells (figure 2D).

CORRELATION BETWEEN *GATA-3* EXPRESSION AND CELL SIZE

The observation that the proportions of *GATA-3*$^+$ cells are highest in developmental stages, in which cellular proliferation occurs, suggested coupling of *GATA-3* expression to the cell cycle. As cycling thymocytes are larger in size (figure 3; 17), we analysed the relation between *GATA-3* transcription and the cell cycle by *lacZ* expression and forward scatter (FSC) values, respectively.

We could not detect any significant differences in FSC values between *lacZ*$^+$ and *lacZ*$^-$ cells in the mature CD4$^+$ or CD8$^+$ cell populations in thymus. In contrast, within the CD4$^+$CD8$^+$ cell population, we found that the *lacZ*$^+$ population contained relatively more large cells (mean FSC=371±16, n=5), as compared to the *lacZ*$^-$ population (mean FSC= 332±5) (figure 3). Within the four subsets of CD4$^-$CD8$^-$ DN cells, a similar correlation was only found for the CD44$^-$CD25$^+$ DN subset: mean FSC=663±19 for the *lacZ*$^+$ population and mean FSC=583±8 for the *lacZ*$^-$ population. These results indicate that the correlation between expression of *GATA-3* and cell size is only present during the phases of *TCRβ* rearrangement (in CD44$^+$CD25$^-$ DN cells) and *TCRα* rearrangement (in CD4$^+$CD8$^+$ cells).

GATA-3 IS ALREADY REQUIRED AT THE EARLIEST T CELL PROGENITOR STAGE IN THE THYMUS

To investigated whether *GATA-3* deficient cells are able to contribute to the most immature subpopulation of CD44$^+$CD25$^-$ DN thymocytes, we compared the contribution of het-

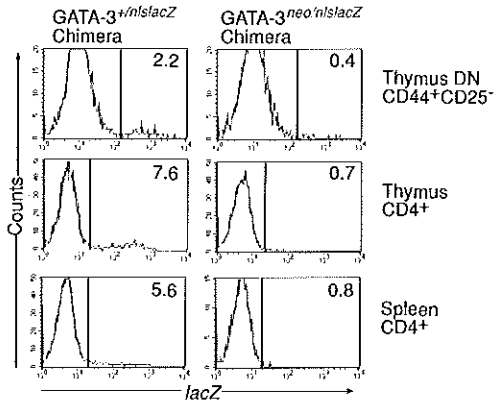

FIGURE 4

Absence of *GATA-3*$^{neo/nlslacZ}$ cells in the T cell lineage in chimeric mice.

Three- or four- colour flow cytometric analyses of thymus or spleen cells. The indicated cell populations were gated and analysed for *lacZ* expression. The results are displayed as histograms with the percentages of *lacZ*$^+$ cells indicated.

Cells were stained with FDG for lacZ activity, biotinylated anti-CD4 and anti-CD8 and streptavidin-APC, anti-CD25-PE and anti-CD44-CyChrome (top) or with FDG, anti-CD4-PE and anti-CD8-CyChrome (middle, bottom). The data shown are representative for two *GATA-3*$^{+/nlslacZ}$ and five *GATA-3*$^{neo/nlslacZ}$ chimeric mice analysed.

erozygous *GATA-3*$^{+/nlslacZ}$ and *GATA-3* deficient *GATA-3*$^{neo/nlslacZ}$ ES cells to the T cell lineage in chimeric mice generated by injection into wild-type blastocysts. These two different types of ES cells were obtained by electroporation of *GATA-3*$^{+/neo}$ ES cells, which have a targeted deletion within the *GATA-3* gene on one allele (figure 1; 10), dependent on the allele where the homologous recombination with the *GATA-3/lacZ-Hygro* targeting vector had occurred. Because we could follow the ES-cell derived thymocytes and T cells by their *lacZ* expression, this system provides an *in vivo* competition assay (19) to detect the proliferative ability of *GATA-3* targeted cells.

Both types of ES cells contributed significantly (50-70%) to heart, brain, kidney and lungs, as assessed by glucose phosphate isomerase isozymes and Southern blot densito-metric analyses (data not shown). In these assays, only the *GATA-3*$^{+/nlslacZ}$ ES cells contributed to the thymus. Consistent with these findings, *lacZ*$^+$ cells were present in the CD4$^+$ population in spleen and thymus of the two *GATA-3*$^{+/nlslacZ}$ chimeras analysed (figure 4). Also within the CD4$^-$CD8$^-$ DN compartment, low but detectable numbers of *lacZ* expressing cells were present in the *GATA-3*$^{+/nlslacZ}$ chimeric mice. When we analysed the most immature thymocyte population of CD25$^-$CD44$^+$ DN cells, the *GATA-3*$^{+/nlslacZ}$ chimeras contained ~2% *lacZ*$^+$ cells (figure 4).

In contrast, no contribution of the *GATA-3*$^{neo/nlslacZ}$ ES cells to the T cell population in the spleen or thymus was found in the five chimeras analysed (figure 4). Also within the CD44$^+$CD25$^-$ DN subpopulation of *GATA-3*$^{+/nlslacZ}$ chimeras, a distinguishable population of *lacZ* expressing cells could not be identified. The fractions of cells (<0.5%) that were present as a shoulder of the histogram, were also found in non-chimeric wild-type control mice, and largely consisted of Mac-1$^+$ cells with high background autofluorescence levels (data not shown). Likewise, *lacZ*$^+$ cells could not be detected in any of the three other DN subsets, defined by CD44 and CD25. In summary, these results showed that *GATA-3*-deficient cells did not contribute to the T cell lineage, not even to the earliest CD44$^+$CD25$^-$ DN cell stage in the thymus.

DISCUSSION

The insertion of a *lacZ* reporter cassette enabled us to perform a quantitative assessment of *in vivo GATA-3* expression in each successive step of T cell differentiation. The two thymocyte populations that largely consist of slowly dividing cells that are in the process of rearranging their *TCRβ* and *TCRα* loci, contained the lowest proportions of *GATA-3* expressing cells. Moreover, these two cell populations were the only ones in which we observed a correlation between *GATA-3* expression and cell size (figure 3). The two waves of *TCR* gene rearrangements were insulated by a developmental phase of large cycling cells with a high proportion of *GATA-3*$^+$ cells. As the CD44$^-$CD25$^+$ DN population consists of two subsets, representing resting cells prior to TCRβ selection and cycling cells that have passed TCRβ selection (18), our findings suggests that *GATA-3* expression is upregulated in those cells that have passed TCRβ selection.

The apparent coupling of *GATA-3* expression to the cell cycle would parallel findings for other GATA family members. Overexpression of *GATA-1* was shown to overcome terminal erythroid differentiation and to result in a higher level of cell division, suggesting that *GATA-1* is a master regulator of the choice between proliferation and differentiation, by acting on the cell-cycle apparatus in a dose-responsive manner (20). The activation of an oestrogen-inducible *GATA-2* protein promoted proliferation and inhibited the terminal differentiation of chicken primary erythroblasts (21). Analysis of *GATA-2*$^{-/-}$ ES cells and hematopoietic cells from yolk sac *in vitro* showed that *GATA-2* is required for the expansion of multipotential hematopoietic progenitors, but dispensable for the terminal differentiation of erythroid cells and macrophages (9,22). Over-expression of the cardiac *GATA-4* protein in P19 cells increased the number of cardiomyocytes following differentiation (23). As over-expression of *GATA-6* in *Xenopus* blocked cardiac differentiation and increased the number of cells in the myocardium, it was concluded that *GATA-6* may act to maintain heart cells in the precursor state (24). The *GATA-3* expression pattern, which we have identified in this report, would allow a role for *GATA-3* in the regulation of early T cell development towards cell division and differentiation via the cell-cycle machinery, thereby establishing the inverse relationship between *TCR* gene segment recombination and cellular proliferation.

Our findings in the *GATA-3*$^{neo/nlslacZ}$ chimeras demonstrated that the absence of *GATA-3* abolished the development of the most immature T-lineage cell precursors in the thymus, *i.e.* the CD44$^+$CD25$^-$ DN cells. GATA-3 activity could be required for the proliferation or survival of cells within this immature fraction in the thymus, or alternatively for the development of T-lineage restricted progenitors in fetal liver or bone marrow, which seed the thymus (2). The latter would be supported by the observation that *GATA-3* antisense oligonucleotides strongly inhibited the generation of T cells from fetal liver progenitors in fetal thymic organ cultures, while antisense oligonucleotides only had a limited suppressive effect on T cell development from fetal thymus progenitors (13).

In summary, our results show that *GATA-3* is required for the development of the earliest T cell progenitors and point at a role for *GATA-3* in the control of proliferation steps,

which alternate the stages of differentiation in which *TCRβ* and *TCRα* gene rearrangements occur. The observed association of *GATA-3* expression and cellular proliferation in early T cell precursors, makes it attractive to investigate T cell development in transgenic mice with manipulated expression windows or expression levels of the *GATA-3* transcription factor.

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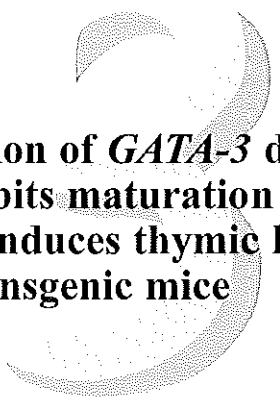
Chapter 3



Enforced expression of *GATA-3* during T cell differentiation inhibits maturation of CD8 single positive cells and induces thymic lymphoma in transgenic mice

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ABSTRACT

The zinc-finger transcription factor GATA-3 is of critical importance for development of the T cell lineage and for commitment of Th2 cells. To study the role of GATA-3 in T cell differentiation, we analyzed and modified *GATA-3* expression *in vivo*. In mice carrying a targeted insertion of a *lacZ* reporter on one allele, we found that *GATA-3* transcription in $CD4^+CD8^+$ double positive (DP) thymocytes correlated with the onset of positive selection events, i.e. TCRαβ upregulation and CD69 expression. *LacZ* expression remained high (~80 % of cells) during maturation of CD4 single positive (SP) cells in the thymus, whereas the fraction of *lacZ* expressing cells decreased to <20% in CD8 SP cells. We modified this pattern by ectopic *GATA-3* expression driven by the *CD2* locus control region, which provides transcription of *GATA-3* throughout T cell differentiation. In two independent *CD2-GATA-3* transgenic lines, ~50% of the mice developed thymic lymphomas before the age of 9 months. These lymphoblastoid tumors were DP $CD69^+$ $CD3^+$ cells that appeared to differentiate along the CD4 lineage. In tumor-free *CD2-GATA-3* transgenic mice the total numbers of CD8 SP cells in the thymus were in the normal ranges, but their maturation was hampered, as indicated by increased cell death of CD8 SP cells and a selective deficiency of mature $CD69^{lo}HSA^{lo}$ CD8 SP cells. In the spleen and lymph nodes the numbers of $CD8^+$ T cells were significantly reduced. These findings indicate that GATA-3 supports differentiation of the CD4 lineage and inhibits the maturation of CD8 SP cells in the thymus.

INTRODUCTION

In the thymus early $CD4^-CD8^-$ double negative (DN) precursors develop into mature CD4 or CD8 single positive (SP) T cells following a tightly regulated program of cellular differentiation (1-4). The DN population is generally subdivided into four distinct stages of differentiation, defined by differential expression of the surface makers IL-2 receptor α chain CD25 and phagocyte glycoprotein-1 CD44 (5). Precursor T cells rearrange their *TCRβ* genes during the $CD25^+CD44^-$ DN stage, and only those cells that produce a functional TCRβ protein proceed via a proliferative phase to the $CD25^-CD44^-$ DN stage (3, 6, 7). These cells rapidly upregulate CD4 and CD8, and start to rearrange their *TCRα* genes.

After successful *TCRα* gene rearrangement, TCRαβ-bearing immature cells are selected for MHC recognition during the process of positive selection (8-10). Concomitantly, DP T cells will undergo lineage commitment to ensure the correlation of the TCR specificity for MHC class I with the CD8 lineage and for MHC class II with the CD4 lineage, respectively (2, 11, 12). In addition, potential self-reactive T lymphocytes are eliminated by selection against self-recognition within the MHC context (13).

T cell development is regulated by a large number of transcription factors (14, 15). One of the transcription factors critically involved in T cell development is GATA-3, which was originally identified in the T cell lineage as a protein that binds to the *TCRα* gene enhancer

(16). GATA-3 is a member of a family of transcription factors that bind a GATA-consensus motif through a highly conserved C_4 zinc-finger binding domain (17). Mice with a targeted deletion of *GATA-3* die between embryonic day 11 and 12, displaying massive internal bleeding and central nervous defects (18). *GATA-3* expression is abundant in the developing central nervous system, adrenal gland and kidney. Within the hematopoietic system *GATA-3* expression is largely confined to T lymphocytes (18-21). In mature T helper cells, GATA-3 has been shown to be essential for Th2 differentiation (22, 23), and has been implicated in the regulation of locus accessibility of the *IL-4*, *IL-5* and *IL-13* genes by chromatin remodeling (24-26).

The *GATA-3* gene is already expressed in the earliest CD25$^-$CD44$^+$ DN progenitors in day 12 fetal thymus (27). Antisense *GATA-3* oligonucleotides inhibited T cell development from fetal liver precursors in fetal thymic organ cultures, indicating the critical importance of GATA-3 for T cell differentiation (27). Moreover, *RAG-2*$^{-/-}$ complementation experiments *in vivo* demonstrated that the development of *GATA-3*$^{-/-}$ embryonic stem (ES) cell-derived T cell precursors is arrested at or before the DN stage (28). In such *GATA-3*$^{-/-}$*/RAG-2*$^{-/-}$ chimeric mice, the *GATA-3*-deficient ES cells contributed significantly to nonhaematopoietic tissues and to the erythroid, myeloid and B cell lineages. In chimeric mice generated by injection of *GATA-3* deficient *lacZ*-expressing ES cells in wild-type blastocysts, we previously showed that *GATA-3*$^{-/-}$ ES cells did not contribute to the T cell lineage, not even to the earliest subset of CD25$^-$CD44$^+$ DN thymic DN progenitors (21).

As *GATA-3*$^{-/-}$ cells display a block before the earliest T cell progenitor, few data are available on the role of GATA-3 during T cell development in the thymus. Using mice with an insertion of a *lacZ* reporter in the *GATA-3* gene on one allele (*GATA-3*$^{+/nlslacZ}$), we examined the proportion of *GATA-3* expressing cells as a function of T cell development (21). We found significant *GATA-3* expression at the earliest DN stage in the thymus. The two waves of *TCRβ* and *TCRα* gene recombination were associated with low proportions of *lacZ*$^+$ cells. The stage of rapidly proliferating CD44$^-$CD25$^-$ DN cells, which demarcates these two periods of *TCR* rearrangement, was characterized by a large proportion of *lacZ* expressing cells. The proportion of *lacZ*$^+$ cells increased again as DP cells progressed into CD4 or CD8 SP cells. The presence of significant proportions of *lacZ*$^+$ cells within the CD8 SP T cell subpopulation in the thymus was in strong contrast with the almost complete absence of *lacZ* expression in mature CD8$^+$ T cells in the periphery (21).

The differential regulation of *GATA-3* gene expression in the CD4 versus the CD8 lineage prompted us to investigate its expression during positive selection and CD4/CD8 lineage commitment in the thymus in more detail. We analyzed the *GATA-3*$^{+/nlslacZ}$ mice, using additional markers for the maturation stages of DP and SP cells, including CD3, TCRαβ, heat stable antigen (HSA), L-selectin (CD62L) and particularly CD69, which is typically induced by TCR signaling and therefore marks cells that are in the process of positive selection (29-33). Furthermore, we investigated the functional role of GATA-3 during T cell development *in vivo* by the generation of transgenic mice with enforced *GATA-3* expression driven by the human *CD2* locus control region (LCR), which provides expression of the *GATA-3* transgene

throughout T cell differentiation (34).

MATERIALS AND METHODS

MICE

The *GATA-3*$^{+/nlslacZ}$ mice in which one *GATA-3* allele was replaced by a *lacZ* reporter have been described previously (21). For the generation of the *CD2-GATA-3* construct, the translation initiation site was mutated (ATG to GTG) in a murine *GATA-3* cDNA clone and 3 HA epitope tags were added together with a new ATG and Kozak's consensus sequence. Subsequently, the ~ 2 kb *mGATA-3* was cloned into a human *CD2* mini-gene Bluescript SK vector, with ~5 kb of *CD2* 5'promoter sequence and ~ 5.5 kb 3' CD2 flanking sequences (35). The latter contained the 3' untranslated sequence and poly(A) addition site of the *CD2* gene, as well as the LCR, which was shown to confer T-cell specific, copy-dependent, integration site independent expression in transgenic mice (34). A 13.2-kb linear fragment was injected into pronuclei of FVBXFVB fertilized oocytes at a concentration of ~2 ng/μl. Founder mice were identified by genomic Southern blotting and crossed onto an FVB background. To determine the genotype of the subsequent generations, tail DNA was analyzed by Southern blotting of either *Eco*RI /*Xba*I double digests hybridized to a 2 kb *Hin*dIII *CD2* LCR probe (36), or *Eco*RI digests hybridized to a 800 kb partial *GATA-3* cDNA probe (20).

FLOW CYTOMETRIC ANALYSES

The preparation of single-cell suspensions, determination of β-galactosidase activity using fluorescein-di-β-D-galactopyranoside (FDG), mAb incubations and three or four color cytometry have been described previously (37). The following mAb were purchased from Pharmingen (San Diego, CA): FITC-conjugated anti-CD3ε and anti-TCRαβ, PE-conjugated anti-CD4 (L3T4), anti-CD24/heat stable antigen (HSA), anti-CD25 (clone 3C7), anti-CD62L and anti-CD69, CyChrome-conjugated anti-CD4, anti-CD8 and anti-CD44, biotinylated anti-CD4 and anti-CD8, APC-labeled anti-CD3ε and anti-CD4. Secondary antibodies used were PE-, TriColor, or APC-conjugated streptavidin (Caltag Laboratories, Burlingame, CA). FDG and To-Pro3 were from Molecular Probes Europe BV (Leiden, The Netherlands). FITC-labeled Annexin V was from Nexins Research BV, Hoeven, The Netherlands.

For intracellular detection of GATA-3 protein, cells were fixed and permeabilized using paraformaldehyde and saponin, as described (38) and subsequently incubated with the Hg-3-31 anti-GATA-3 MoAb (Santa Cruz, Dan Diego, CA) and FITC-labeled anti-mouse IgG1 (Pharmingen) as a second step.

RESULTS

GATA-3 EXPRESSION IS STRONGLY INDUCED DURING POSITIVE SELECTION OF DOUBLE-POSITIVE T CELLS

We have previously quantified the *GATA-3* gene expression profile during thymic T cell differentiation *in vivo*, by placing a *lacZ* reporter gene, containing a nuclear localization signal, under direct *GATA-3* transcriptional control. In these *GATA-3*$^{+/nlslacZ}$ mice *GATA-3* directed *lacZ* expression was analyzed by flow cytometry using fluorescein-di-β-D-galacto-pyranoside (FDG) as a β-galactosidase substrate, and differential expression of *GATA-3* in DP (~16% *lacZ*$^{+}$ cells), CD4 SP (~84%) and CD8 SP (~33%) cells was found (21). Since it has been shown that double positive T cells differentiate into mature single positive T cells via a series of phenotypically distinct subpopulations, reflecting the multistage process of positive selection and CD4/CD8 lineage commitment (17-22), we investigated *GATA-3* gene expression in these subpopulations in more detail.

Upon MHC/TCRαβ interaction in DP cells, the surface expression of the CD69 marker is upregulated (29-32), followed by a down-regulation of the CD4/CD8 coreceptor surface expression (2). Therefore, we analyzed *lacZ* activity in conjunction with surface expression of CD4, CD8 and CD69 (figure 1). The majority of DP cells (~85%) did not express CD69 on the cell surface, and in this CD69^{-} DP population lacZ was expressed in ~19 % of cells. By contrast, within the CD69^{+} DP subpopulation, *lacZ* was expressed in ~54% of the cells. After subsequent downregulation of coreceptor expression and transition into the CD4^{lo}CD8^{lo} subpopulation, ~86% of the CD69^{+} cells expressed *lacZ* (figure 1). The CD4^{lo}CD8^{lo} cells have been shown to subsequently enhance CD4 expression (2), thereby developing into the CD4^{+}CD8^{lo} subset, which still contains precursors for both CD4 and CD8 single positive T cells (2, 39-41). *LacZ* expression was present in ~86% of these CD4^{+}CD8^{lo} CD69^{+} cells (figure 1). In addition, we found that in the DP, CD4^{lo}CD8^{lo} and CD4^{+}CD8^{lo} subpopulations, *lacZ* expression correlated with the expression levels of TCRαβ or CD3 on the cell surface (Shown for TCRαβ in figure 1). These results indicated that the induction of *GATA-3* transcription coincides with CD3 and TCRαβ upregulation and CD69 expression in DP cells.

GATA-3 GENE EXPRESSION IS DOWNREGULATED AFTER COMMITMENT TO THE CD8 LINEAGE

Bipotential CD4^{+}CD8^{lo} CD69^{+} T cells differentiate into either CD4 or CD8 SP cells by shutting down expression of the reciprocal coreceptor gene (2, 39-41). Final maturation of SP thymocytes is accompanied by downregulation of CD69 and HSA on the cell surface and induction of high-level expression of CD62L and CD44 (33, 42, 43)

As shown in figure 1, *lacZ* expression was present in ~87% of the cells committed to the CD4 lineage (CD4^{+}CD8^{-}CD69^{+}TCRαβ^{hi} cells) and in ~78% of the more mature CD4^{+} cells with a CD69^{-}HSA^{lo} surface profile. By contrast, during the maturation of CD8 lineage cells, ~40% of the CD69^{+}HSA^{+} and only ~16% of the mature CD69^{-}HSA^{lo} CD8^{+}TCRαβ^{hi} cells

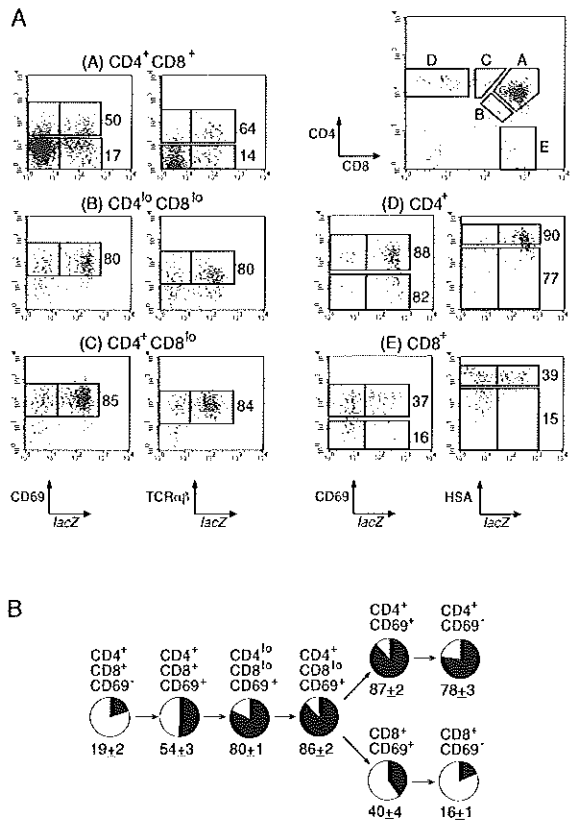

FIGURE 1

Analysis of *lacZ* expression in thymocytes from *GATA-3*$^{+/nlslacZ}$ mice by four-color flow cytometry.

Thymus cell suspensions were loaded with the β-galactosidase substrate FDG and subsequently stained with anti-CD4, anti-CD8, in combination with anti-CD69, anti-TCRαβ or anti-HSA antibodies. Cells were analyzed for the expression of CD4 and CD8, the indicated subpopulations A-E were gated, and analyzed for *lacZ* expression and the fourth surface marker (CD69, TCRαβ or HSA). The numbers indicate the percentage of *lacZ* expressing cells in the subpopulations analyzed. All samples are lymphocyte gated by forward and sideward scatter.

Overview of the proportions of *lacZ* expressing cells in the indicated thymocyte subpopulations in *GATA-3*$^{+/nlslacZ}$ mice. The numbers are mean values ± SD (n=3). The background percentages of β-galactosidase positive cells, as determined in wild-type control mice, was <1% in all subpopulations.

expressed *lacZ*. Likewise, *lacZ* expression was found to be significantly downregulated in mature CD44$^+$ and CD62L$^+$ CD8$^+$ cells (data not shown). The intensities of the fluorescence signals show that the *lacZ* expression levels per cell increased slightly during the maturation process of CD4 SP cells, whereas CD8 SP cells displayed lower and more heterogeneous *lacZ* expression levels (figure 1A).

When the T cells leave the thymus, the proportions of *GATA-3*$^+$ cells decreased to ~20% of the CD4$^+$ and to <1% of the CD8$^+$ T cell populations in the spleen and lymph nodes. (21). For the CD4$^+$ lineage cells in the spleen, we did not observe a clear correlation between *GATA-3* and the expression of the HSA, CD69, CD44, CD62L or CD25 surface markers, which are instrumental to specify subpopulations of naïve, activated or memory T cells (44, 45).

As summarized in figure 1B, the proportions of *GATA-3* expressing cells were low in CD3-TCRαβ-CD69- DP cells (~19%), and increased at the onset of positive selection events, characterized by upregulation of CD3 and TCRαβ surface expression and induction of CD69. The proportions increased to ~86% at the stage of the last uncommitted subset of CD4$^+$CD8^{lo}CD69$^+$cells, and remained high for the most mature thymic CD69-CD4$^+$ subpop-

ulation. By contrast, commitment to the CD8 lineage was associated with downregulation of GATA-3 expression, resulting in <20% GATA-3$^+$ cells within the mature population of CD69$^-$ CD8$^+$ cells.

TRANSGENIC EXPRESSION OF *GATA-3* DRIVEN BY THE HUMAN *CD2* LOCUS CONTROL REGION

In order to modify *GATA-3* expression *in vivo*, transgenic mice were generated in which the murine *GATA-3* gene, 5' tagged with 3 HA epitopes, was expressed under the control of the human *CD2* LCR (35). Two independent *CD2-GATA-3* transgenic lines were established. No differences were found between the two lines in any of the performed analyses. The offspring did not manifest developmental defects or any increased susceptibilities to infectious disease or malignancies for over 9 months of age, with the exception of the observed thymic lymphomas discussed below.

Expression of the *CD2-GATA-3* transgene was evaluated by comparing total GATA-3 levels in transgenic and non-transgenic littermates in intracellular flow cytometry experiments, using a mouse monoclonal antiserum specific for GATA-3. In the wild-type animals, the GATA-3 levels were low in DP cells, increased during positive selection in CD4^{lo}CD8^{lo} cells, and were high in CD4 SP cells (figure 2), consistent with our findings in the *GATA-3*$^{+/nlslacZ}$ mice (figure 1).

In both transgenic lines, expression of the *CD2-GATA-3* transgene resulted in elevated levels of GATA-3 protein in all stages of T cell development in the thymus, however there were major differences between the individual thymocyte subpopulations (figure 2). Due to the presence of the *CD2-GATA-3* transgene, total GATA-3 protein levels were uniformly high in DP, CD4^{lo}CD8^{lo}, CD4$^+$CD8^{lo}, and CD4$^+$ cells. In CD8$^+$ cells in the thymus and peripheral T cells, GATA-3 levels in the *CD2-GATA-3* transgenic were close to those observed in

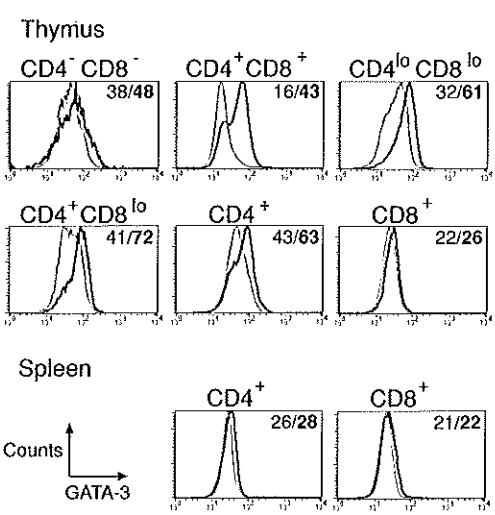

FIGURE 2

Expression of GATA-3 in thymus and spleen in wild-type and *CD2-GATA-3* transgenic mice.

Cell suspensions were stained for surface CD3, CD4, and CD8 expression, and subsequently for intracellular GATA-3 protein. The indicated T cell subpopulations were gated and analyzed for GATA-3 expression. The results are displayed as histograms of *CD2-GATA-3* transgenic mice (*bold lines*), together wit those of non-transgenic control mice (*thin lines*). CD4$^-$CD8$^-$ populations were gated on CD3$^-$ cells. CD4^{lo}CD8^{lo} cells and CD4$^+$CD8^{lo} were gated on CD3$^+$ cells. The numbers indicate the mean fluorescence intensities in nontransgenic (*normal type*) and *CD2-GATA-3* transgenic mice (*bold type*). Data shown are representative of 6 mice examined within each group.

wild-type littermates. As CD2 surface expression in the individual T cell subpopulations was comparable (data not shown), the observed modulated *GATA-3* expression profile in the transgenic mice suggested the presence of post-translational regulation or an autoregulation mechanism by which GATA-3 protein levels regulate transcription of the endogenous *GATA-3* gene (26, 46, 47).

CD2-GATA-3 TRANSGENIC MICE HAVE DECREASED CD8$^+$ T CELL NUMBERS IN THE PERIPHERY

To analyze the effect of the *CD2-GATA-3* transgene on T cell development, we examined the T cell populations in thymus, spleen and mesenteric lymph nodes from 2-3 month-old *CD2-GATA-3* transgenic mice and non-transgenic littermates by flow cytometry (figure 3). In the *CD2-GATA-3* mice the sizes of the main thymocyte subpopulations, as determined by CD4/CD8 analysis, were within the normal ranges, indicating that the enforced *GATA-3* expression did not dramatically impede thymocyte development (figure 3). No significant differences were detected between *CD2-GATA-3* transgenic mice and normal littermates within the DN subpopulations, as defined by differential CD44 and CD25 expression (data not shown). However, the *CD2-GATA-3* transgenic mice had fewer CD8$^+$ T cells (~50% of control) in spleen and lymph nodes, when compared with non-transgenic littermates (Shown for spleen in figure 3). The residual CD8$^+$ T cells present exhibited a lower CD8 and a higher CD3 expression on the cell surface. The numbers of CD4$^+$ T cells in the periphery were comparable in the two groups of mice.

In conclusion, these results indicated that forced expression of *GATA-3* did not result in any adverse effects on CD4$^+$ T cell development in 2-3 month-old *CD2-GATA-3* transgenic mice. By contrast, an inhibition of the maturation of CD8 single positive cells was observed, occurring either in the thymus or shortly after leaving the thymus.

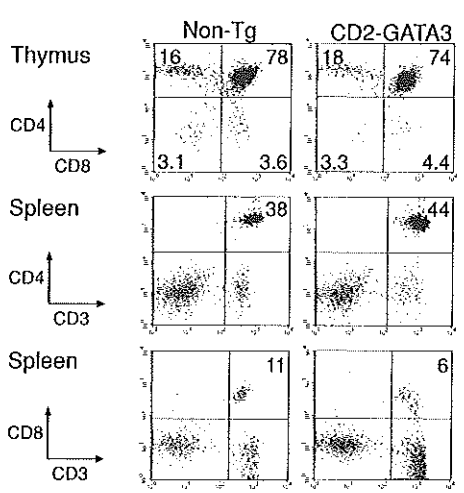

FIGURE 3
Phenotype of *CD2-GATA-3* transgenic mice.
T cell development in the presence of the *CD2-GATA-3* transgene results in a reduced pool of peripheral CD8$^+$ cells. Flow cytometric analyses of the thymus and spleen of 2-month-old wild-type and *CD2-GATA-3* mice. Single-cell suspensions were stained with anti-CD3, anti-CD4 and anti-CD8 antibodies. Results are displayed as dot plots of lymphocyte gate cells; percentages of total cells within the indicated quadrants are given. Data shown are representative of over 20 mice examined within each group.

CD2-GATA-3 TRANSGENIC MICE DEVELOP THYMIC LYMPHOMAS

When the *CD2-GATA-3* transgenic mice were followed up to 9 months of age, approximately 50% (26 out of 51) developed thymic lymphomas. Typically, these lymphomas were noticed as mice displayed respiratory distress at the age of 6-8 months, but in three cases such animals were observed at ~3 months of age. Tumor frequencies in the two independent transgenic lines were similar, while tumors were not seen in non-transgenic littermates. A Kapplan-Meyer survival curve is plotted in figure 4D.

Flow cytometric analyses demonstrated that the tumors consisted of $CD3^+$ $CD4^+$ lymphoblasts showing variable levels of CD8 coexpression, suggesting differentiation of DP tumor cells along the CD4 lineage. Several animals with a thymic lymphoma exhibited enlargement of spleen or lymph nodes. Lymphoma cells were also found to be present in the spleen, liver and lymph nodes, indicating that the thymic lymphomas metastasized to the periphery. Analysis of *TCRβ* gene rearrangements using a *Jβ1*- and a *Jβ2*-specific probe revealed that the lymphomas were monoclonal populations. The lymphoma cells displayed unique gene rearrangements in the *TCRβ* locus, indicating that the oncogenic event has taken place in a single T cell precursor (not shown). Figure 4A shows an example of a thymic lymphoma (DP and $CD4^+$ cells), with different metastases in lymph node (DP and $CD4^+$ cells) and spleen ($CD4^+$ cells only). Immunohistochemical examination of tumor tissue sections confirmed that the tumors mainly consisted of lymphoblasts, supported by a network of MHC class II negative fibroblasts, while characteristic structures of epithelial cells expressing cortical or medullar cell markers were absent (data not shown).

Among *CD2-GATA-3* transgenic mice that did not exhibit outward signs of illness, nor manifested a macroscopically visible thymic tumor at ~3 months of age, we found evidence for early stages of tumor development in 6 out of 32 cases (~ 19%). In flow cytometric analyses of thymus cell suspensions the DP or $CD4^+CD8^{lo}$ subsets contained atypical fractions of $CD3^+$ lymphoblastoid cells with high FSC characteristics, suggestive of tumor growth. In the example shown in figure 4BC, the lymphoblastoid cells had a $CD3^+$ $CD4^+CD8^{lo}$ phenotype and expressed very high levels of GATA-3 protein.

GATA-3 ENHANCES TCRαβ UPREGULATION DURING POSITIVE SELECTION

As we observed a correlation between *GATA-3* expression and TCRαβ or CD3 surface levels in *GATA-3*$^{+/nlslacZ}$ mice, we investigated these parameters in the thymocyte subpopulations of the *CD2-GATA-3* mice (figure 5A). The expression of CD69 in the DP, $CD4^{lo}CD8^{lo}$ and $CD4^+CD8^{lo}$ subpopulations was similar in *CD2-GATA-3* and wild-type mice. By contrast, the proportions of TCRαβ^{hi} or $CD3^{hi}$ cells were significantly increased in *CD2-GATA-3* mice, particularly in $CD69^+CD4^{lo}CD8^{lo}$ subpopulation (Shown for TCRαβ expression in figure 5A). In the more mature fractions ($CD4^{lo}CD8^+$ and SP cells), the expression of CD3 and TCRαβ were comparable between transgenic animals and wild-type littermates.

During the positive selection process CD3/TCRαβ^{lo} DP cells develop into CD3/TCRαβ^{hi} SP cells, which is accompanied by an increase in the average cell size (See

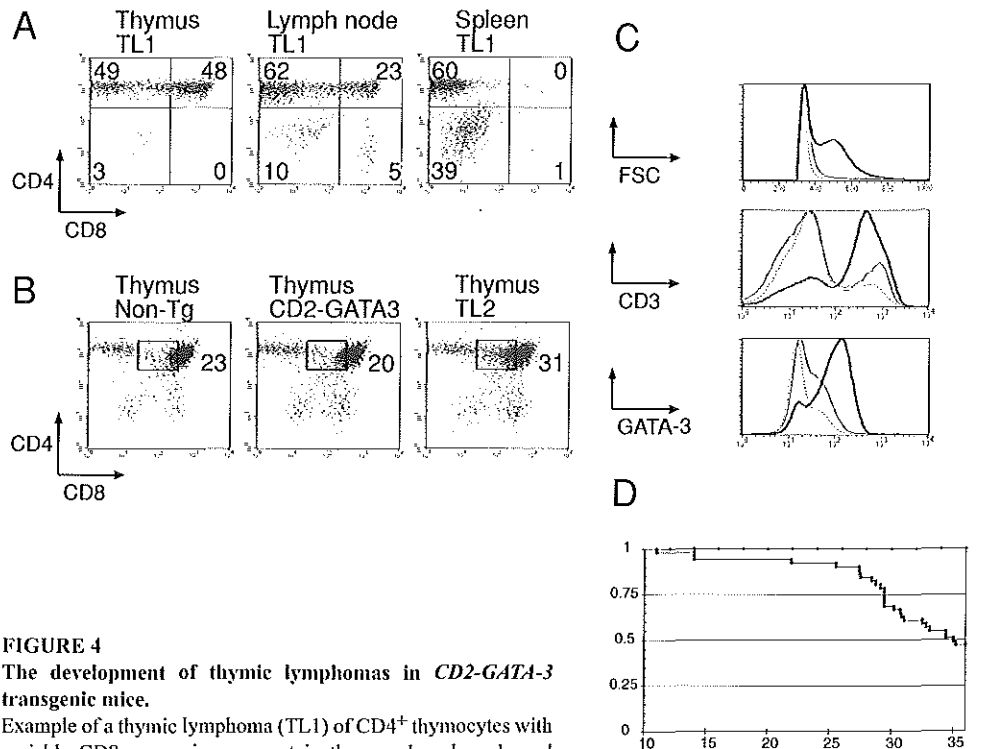

FIGURE 4

The development of thymic lymphomas in *CD2-GATA-3* transgenic mice.

Example of a thymic lymphoma (TL1) of $CD4^+$ thymocytes with variable CD8 expression, present in thymus, lymph node and spleen. The percentages of gated cells within the indicated CD4/CD8 quadrants are shown.

Identification of an atypical GATA-$3^{hi}CD3^+CD4^+CD8^{lo}$ lymphoblastoid cell population in the thymus of a *CD2-GATA-3* transgenic mouse, indicative for a thymic lymphoma (TL2). Non-transgenic and tumor-free *CD2-GATA-3* transgenic mice are shown as controls. Results are displayed as dot plots for CD4 and CD8. The given percentages of the gated $CD4^+CD8^{lo}$ populations are of all thymocytes.

Analysis of FSC, CD3 and GATA-3 expression in the gated thymocyte subpopulation shown in (B). The results are displayed as histograms of the *CD2-GATA-3* transgenic TL2 mouse (*bold lines*), together with those of a non-transgenic (dashed lines) and a tumor-free *CD2-GATA-3* transgenic mouse (*thin lines*). Cell suspensions were stained for CD3, CD4, and CD8, and subsequently for intracellular GATA-3. All samples are lymphocyte/lymphoblast gated by forward and sideward scatter.

(D) A total of 42 wild-type animals and 51 *CD2-GATA-3* transgenic animals were followed for 9 months. Survival of the animals is plotted in a Kapplan-Meyer curve as fraction of the total number of animals.

figure 5B, thin lines). However, in the *CD2-GATA-3* transgenic mice cells, the cells within the DP subpopulations had increased average forward scatter values, closer to those of normal SP cells (figure 5B). This finding suggested that in *CD2-GATA-3* transgenic mice DP cells already have some morphological characteristics of SP cells. Additional explanations for the increased cell size of DN cells would include an enhanced activation status of DP cells in the presence of high levels of GATA-3, or alternatively an effect of GATA-3 on cell cycle progression in DP T cells. However, we did not find evidence for either of these two. We failed to detect activated cells with high Th2 cytokine production in immunohistochemical analy-

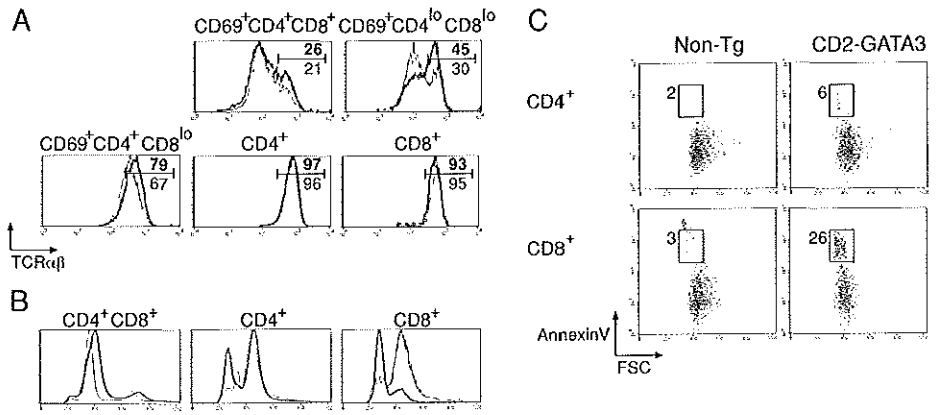

FIGURE 5

The *CD2-GATA-3* transgene enhances TCRαβ upregulation during positive selection and induces cell death in CD8 SP T cells in the thymus.

Cell suspensions were stained for CD4, CD8 and CD69 expression, together with either CD3 or TCRαβ. The indicated T cell subpopulations (see also figure 1A) were gated and analyzed for TCRαβ expression. The results are displayed as histograms of *CD2-GATA-3* transgenic mice (*bold lines*), together with those of non-transgenic control mice (*thin lines*). The percentages shown are the fractions of the $CD69^+CD4^{lo}D8^{lo}$ cells that are in the indicated $TCR\alpha\beta^{hi}$ gate in wild-type mice (below marker) and *CD2-GATA-3* transgenic mice (*above marker, bold type*).

The effects of the *CD2-GATA-3* transgene on the cell sizes of thymocyte subpopulations. Cell suspensions were stained for CD4 and CD8. The indicated T cell subpopulations were gated and analyzed for FSC; the results are displayed as histograms of *CD2-GATA-3* transgenic mice (*bold lines*), together with those of non-transgenic control mice (*thin lines*).

Thymus cell suspensions were stained for CD4, CD8 and Annexin V. The CD4 and the CD8 SP T cell subpopulations were gated and analyzed for FSC and Annexin V. The numbers indicate the percentage of gated CD4 or CD8 SP thymocytes that are Annexin V positive in the indicated mice.

ses of the thymuses of *CD2-GATA-3* mice. Flow cytometric analyses of the thymic subpopulations, using anti-CD4, anti-CD8 and To-Pro3, did not reveal differences in the cell cycle between *CD2-GATA-3* transgenic animals and their wild-type littermates (data not shown). Also, the total size of the DP compartment in the thymus was similar in wild-type and *CD2-GATA-3* transgenic animals (figure 3), arguing against increased proliferation of DP cells.

Taken together, the *CD2-GATA-3* transgenic animals manifested an increased cell size of DP cells and an accelerated upregulation of the expression of TCRαβ and CD3 on the cell surface, suggesting that enforced *GATA-3* expression accelerated T cell maturation during the phase of positive selection.

GATA-3 INHIBITS MATURATION OF CD8 SINGLE POSITIVE T CELLS

To investigate whether increased numbers of cells that had committed to the CD8 lineage died in the thymus in *CD2-GATA-3* transgenic mice, we analyzed the thymic SP compartment in more detail. We found that many cells in the CD8 SP subpopulation and to a lesser extent also in the CD4 SP subpopulation had low FSC characteristics (figure 5B), sug-

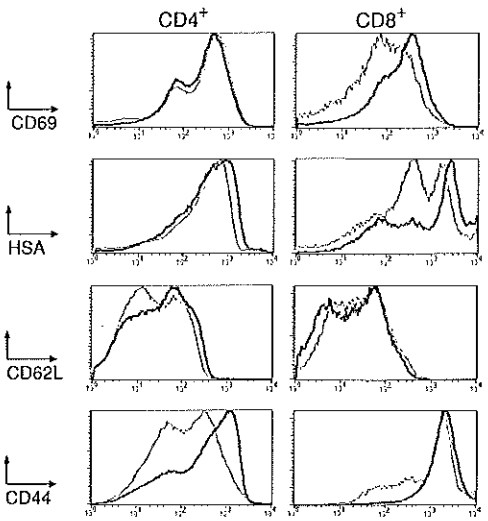

FIGURE 6
Enforced expression of *GATA-3* inhibits the maturation of CD69^{lo}HSA^{lo} CD8 SP cells in the thymus.
In four-color flow cytometry experiments, thymus cell suspensions were stained for CD4 and CD8 expression, together with anti-HSA and anti-CD69 or with anti-CD62L and anti-CD44. The CD4 and CD8 SP T cells were gated and analyzed for the expression of the indicated markers. The results are displayed as histograms of *CD2-GATA-3* transgenic mice (*bold lines*), together with those of non-transgenic control mice (*thin lines*).

gesting that a major fraction of these cells were apoptotic. Indeed, the numbers of annexin V positive mature CD8 SP T cells, which had small forward scatter values, were significantly increased in *CD2-GATA-3* mice, as compared to wild-type littermates (figure 5C). In the CD4 SP T cell subpopulation only a minor increase in the fraction of annexin V positive cells was observed, from ~2% in nontransgenic to ~6% in *CD2-GATA-3* transgenic mice (figure 5C).

It has been reported that final maturation of single positive T cells is accompanied by a downregulation of CD69 and HSA expression (33, 43). The enforced GATA-3 expression appeared to inhibit the final maturation of CD8$^+$ cells, as a selective deficiency of CD69^{lo} HSA^{lo} cells was observed, when *CD2-GATA-3* transgenic and wild-type littermates were compared (figure 6). For CD4$^+$ cells the enforced *GATA-3* expression only mildly affected the final thymic maturation steps. In addition, in the *CD2-GATA-3* transgenic mice an increase in the surface expression of CD44, a marker for activated or memory T cells, was observed both in the CD4 and the CD8 SP population (figure 6). This phenomenon was also seen in the mature CD4$^+$ and CD8$^+$ T cells in the spleen (see Chapter 4). Finally, the expression of L-selectin, CD62-L, a marker which is expressed at high levels on naïve T cells and which is essential for homing to peripheral lymphoid organs (48), was comparable in transgenic and non-transgenic animals. Therefore, the decrease in peripheral CD8$^+$ T cell numbers in *CD2-GATA-3* transgenic mice cannot be explained by a reduced capacity of these cells to leave the thymus.

The increase of annexinV positive cells and the decrease of mature CD69^{lo}HSA^{lo} cells in the CD8 SP subpopulation, together with the low numbers of peripheral CD8$^+$ T cells, indicated that enforced GATA-3 expression resulted in a partial differentiation arrest of CD8$^+$ cells, associated with cell death in the thymus.

DISCUSSION

In this report we have used two different mouse models to study the role of GATA-3 in thymic T cell differentiation *in vivo*. We evaluated *GATA-3* directed *lacZ* expression in *GATA-3*$^{+/hlslacZ}$ mice, and examined the effects of enforced *GATA-3* expression throughout T cell differentiation in *CD2-GATA-3* transgenic mice, in which *GATA-3* transcription is driven by the *CD2* LCR.

Our findings implicate GATA-3 as a participant in the commitment process to the CD4 versus the CD8 lineage. First, we found that commitment to the CD8 T cell lineage coincided with downregulation of *GATA-3* expression. The most mature subpopulation of uncommitted thymocytes, the CD4$^+$CD8^{lo} subset, contained high numbers of *GATA-3* expressing cells. During the maturation of CD8$^+$ cells in the thymus, *GATA-3* expression was gradually lost. By contrast, *GATA-3* expression remained high during maturation of CD4 SP cells in the thymus. Second, enforced *GATA-3* expression inhibited the differentiation of CD8 SP cells. The CD8 SP fraction in the thymus contained increased numbers of apoptotic cells and exhibited a selective deficiency of mature CD69^{lo}HSA^{lo} cells. In the spleen and lymph nodes the numbers of CD8$^+$ T cells were significantly reduced. And third, a major fraction of the *CD2-GATA-3* transgenic mice developed thymic lymphomas at the DP CD69$^+$CD3$^+$ stage, which appeared to differentiate along the CD4 lineage.

The enforced expression of *GATA-3* did not directly influence the CD4 versus CD8 lineage cell fate decision, as in the *CD2-GATA-3* mice the percentages of CD4 and CD8 SP cells in the thymus were in the normal ranges. Although the molecular mechanisms underlying the developmental choice between CD4 and CD8 T cell fates are not known, they are thought to depend on differences in signal strengths of the MHC class I-CD8 and MHC class II-CD4 interactions. The influence of signaling molecules on lineage commitment is supported by the finding of differentiation towards the CD4 lineage in a gain-of-function extracellular signal-related kinase (Erk)-2 mutant, and in *Csk* or *c-Cbl* deficient mice (49-51). Activated Notch transmembrane receptor or *Bcl-2* overexpression were shown to promote differentiation to the CD8 lineage, probably by rescue from apoptosis and development along the CD8 lineage of cells that have a very low affinity MHC-interaction, which would normally die by neglect (52-54).

Our data point at a role for GATA-3 in the maturation of the cells once commitment has occurred. There is a progressive decline of *GATA-3* expression during CD8 lineage maturation, and the enforced *GATA-3* expression impaired cell survival in the most mature CD8 lineage cells. In this context, there is a striking parallel with Th1/Th2 differentiation, where *GATA-3* is expressed in naïve peripheral T cells, followed by a substantial increase during Th2 development and a gradual downregulation during Th1 development (22, 23). The Th2 phenotype is initiated by IL-4 signaling, and by the action of GATA-3 becomes stable over time and independent of extrinsic factors, such as IL-4 (24, 26, 55). It was recently shown that GATA-3 generates stability of Th2 commitment by chromatin remodeling of Th2-specific cytokine loci, associated with a positive autoactivation pathway, which is a recognized mechanism contributing to cell fate determination (26). Concomitantly, GATA-3 inhibits Th1

development by repressing IL-12Rβ expression and, as a result, IL-12 induced IFNγ production (56). Assuming a parallel role for GATA-3 in CD4/CD8 and Th1/Th2 development, we propose that GATA-3 is involved in the stabilization of the distinct gene expression profiles in committed CD4 cells, while for the full maturation of CD8 T cells, *GATA-3* expression needs to be downregulated.

The finding by Ouyang et al. (26) that GATA-3 may, either directly or indirectly, activate its own expression, could explain the modulated pattern of *GATA-3* expression levels during T cell development in *CD2-GATA-3* mice. Possibly, the elevated levels that we observed in these mice, may partly result from upregulation of endogenous *GATA-3*. This positive feedback mechanism could also account for the high GATA-3 protein levels present in the thymic lymphomas (figure 4). In addition, GATA-3 levels may be subject to posttranslational regulation, as indicated by the presence of caspase-mediated degradation of the closely related transcription factor GATA-1 in immature erythroid cells (46). A detailed analysis of GATA-3 protein and RNA levels at the various stages of T cell development in *CD2-GATA-3* mice is currently in progress.

It is clear that further experiments will be needed to identify the critical target genes for GATA-3 in early T cell differentiation. Intriguingly, GATA recognition sequences are present in the Notch4 promoter region (57). If Notch genes would be regulated by GATA-3, this could explain the parallels that exist between the *in vivo* function of GATA-3 and Notch. Both genes are essential for the development of the first stage of T cell differentiation, and not for any other haematopoietic lineage (21, 28, 58). Apart from the accelerated TCRαβ upregulation in developing CD69$^+$ thymocytes that progress from the DP to the CD4$^+$CD8^{lo} stage, we did not see any dramatic effects on the surface expression of presumed GATA-3 target loci, such as *TCRα*, *TCRβ*, and *TCRδ* or *CD8α*. In this context, the variable levels of surface CD8 on the thymic lymphoma cells with very high *GATA-3* expression levels would argue against a major function of GATA-3 in *CD8α* expression regulation.

Our previous finding of low *GATA-3* expression during the two waves of *TCR* gene rearrangement, separated by a stage of high *GATA-3* expression, suggested a role of GATA-3 in the regulation of proliferation events associated with the essential coupling of V(D)J recombination activity to cell cycle (21). However, the absence of any detectable effects of the *CD2-GATA-3* transgene on the cell cycle would argue against such an essential role for GATA-3. Nevertheless, all thymic lymphomas in the *CD2-GATA-3* mice characterized so far appeared to have originated at the DP stage, in which all *TCRα* gene rearrangements occur. Therefore, it remains possible that - in the presence of high levels of GATA-3 - oncogenic events, such as translocations, are mediated by aberrant use of the V(D)J recombination machinery, as has been found in V(D)J-recombination driven thymic lymphoma in mice deficient for the *ataxia telangiectasia* gene (59).

Alternatively, the oncogenic potential of GATA-3 could be related to the ability of GATA-3 to form a complex with the TAL-1 and LMO transcription factors, which are implicated in a large fraction of human T-cell acute lymphoblastic leukemias (60). Normally TAL-1 and LMO are not expressed in the T cell lineage, but expression is induced by translo-

cation events. It was recently shown that forced expression of *GATA-3 in vitro* potentiated the induction by the TAL-1 and LMO transcription factors of retinaldehyde dehydrogenase 2, which inhibits apoptosis of T cells by generating retinoic acid (60).

Finally, enforced *GATA-3* expression probably leads to increased basal transcription of the *RAD50* gene, which is involved in chromosomal double-stranded break repair. Because of the localization of the *RAD50* gene within the *IL-4/IL-5/IL-13* Th2 cytokine gene cluster, an increase of basal *RAD50* transcription is observed in Th2 cells (24). It is possible that in the *CD2-GATA-3* transgenic T cells the increase might be more extreme, thereby resulting in destabilization of the MRE11-RAD50-NBS1 protein complex, which is essential for chromosome stability (61). Further characterization of the tumor cells, including identification of chromosomal translocations, expression analysis of TAL-1, LMO, RAD50, NBS1 and the RAG proteins should identify the possible involvement of any of these oncogenic pathways in the origin of the thymic lymphomas in the *CD2-GATA-3* mice.

In conclusion, this study adds to our knowledge of the function of GATA-3 in early T cell development, as we have established a correlation between *GATA-3* expression and maturation towards the CD4 versus the CD8 lineage. We propose that in early T cell development, expression of *GATA-3* is essential for the maintenance of CD4 cell lineage fate commitment, but inhibits CD8 differentiation. Inferred from the recent findings that GATA-3 acts a key regulator of Th2 development by stabilizing patterns of gene expression, GATA-3 would then in early T cell development stabilize the unique gene expression profiles that are characteristic for the CD4 or the CD8 lineage by chromatin remodeling.

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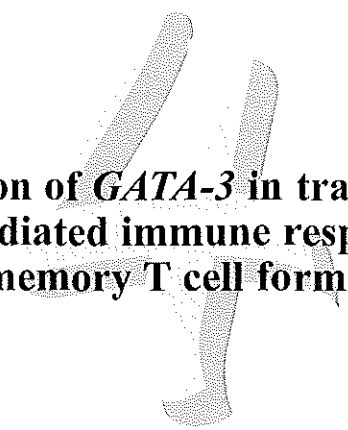
Chapter 4



Enforced expression of *GATA-3* in transgenic mice inhibits Th1-mediated immune responses and increases memory T cell formation

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ABSTRACT

The transcription factor GATA-3 is essential for development of the T cell lineage and is a key regulator of differentiation of naïve CD4+ T cells into Th2 effector cells. To study the functional role of GATA-3 during T cell mediated immune responses *in vivo*, we analyzed *CD2-GATA-3* transgenic mice, in which *GATA-3* is ectopically expressed in T cells under the control of the *CD2* locus control region. Analysis of these mice revealed increased numbers of T cells exhibiting a $CD44^{hi}CD45RB^{lo}$ memory phenotype in spleen and lymph nodes. Peripheral CD4+ T cells expressed elevated levels of the IL-1 receptor family member T1/ST2, indicating enhanced differentiation along the Th2 lineage. Purified CD4+ T cells from *CD2-GATA-3* transgenic animals could be induced to acquire the Th2 effector phenotype, but *in vitro* proliferation of Th1 cells was hampered and IFNγ production was reduced, when compared to wild-type CD4+ T cells. After immunization with TNP-KLH, *CD2-GATA-3* transgenic mice showed reduced serum levels of antigen-specific IgG2a, while IgG1 was within the normal range. T cells from *CD2-GATA-3* mice exhibited an increased recall response to TNP-KLH antigen *in vitro*. Enforced *GATA-3* expression severely reduced the Th1-mediated delayed type hypersensitivity response to KLH. Collectively, these observations indicate that enforced *GATA-3* expression is sufficient to selectively inhibit Th1 responses and to induce memory T cell formation *in vivo*.

INTRODUCTION

CD4+ T helper (Th) lymphocytes develop into two functionally distinct subsets that can be distinguished on the basis of their cytokine production profile (1, 2). Th1 cells are characterized by the production of interferon-γ (IFNγ) and tumor necrosis factor β (TNFβ), whereas Th2 cells typically produce interleukin-4 (IL-4), IL-5, IL-10 and IL-13. Each subset mediates distinct effector functions *in vivo*. Th1 cells are predominantly involved in immune responses against intracellular pathogens, and are associated with autoimmune disease. Th2 cells are of importance in the defense against extracellular pathogens, and are implicated in atopy and allergic diseases (3-5).

Both Th1 and Th2 cells are derived from a common naïve precursor (4-6). Signaling pathways initiated by cytokines play a dominant role in driving the differentiation of activated naïve CD4+ T cells into either effector phenotype (2, 7). For instance, IL-12 induces the differentiation of naïve Th cells to the Th1 effector phenotype (8-11), which requires induction of the IL-12 responsive transcription factor Stat4 (12-14). On the other hand, IL-4 directs the differentiation towards the Th2 effector phenotype (15-18). Th2 differentiation is mediated by Stat6 activation through IL-4 receptor engagement (19-21). In response to chronic antigenic stimulation *in vivo*, progressive polarization of the cytokine responses ultimately leads to the commitment of T helper cells to mutually exclusive Th phenotypes, which are thought to be maintained independently of extrinsic factors (22, 23).

Retroviral transfection of purified naïve CD4+ T cells *in vitro* with a constitutively activated form of Stat6 was shown to suppress IFNγ production and IL-12Rβ2 chain expression, and to induce the expression of Th2-specific cytokines (24). Stat6 also induces the expression of the transcription factors GATA-3 and c-Maf (24), which have been shown to be selectively expressed in a Th2-specific fashion (25-27). Recent experiments using *Stat6*-deficient cells have shown that, although IL-4 and Stat6 signaling may initially direct Th2 development, GATA-3 and c-Maf are capable of inducing the development of stable Th2 commitment, independent of Stat6 (28). The essential role of Stat6 in Th2 differentiation can be replaced by chromatin remodeling using pharmacological histone deacetylase- and cytosine methylation-inhibitors (29). It has been demonstrated that CD4+ T cell commitment to a particular Th phenotype is associated with the induction of DNAse I hypersensitive sites in the loci of effector cytokine genes. *In vitro* differentiation into Th1 cells induces chromatin remodeling of the *IFNγ* locus and, conversely, the differentiation into Th2 cells induces remodeling of the *IL-4/IL-5/IL-13* locus (30). The introduction of *GATA-3* into *in vitro*-cultured T cells was shown to generate Th2-specific DNAse I hypersensitive sites independently of Stat6, implicating GATA-3 in the process of chromatin remodeling (28).

The GATA-3 transcription factor is of critical importance for development of the T cell lineage (31). During T cell deifferentiation, *GATA-3* gene expression is required for the development of the earliest T cell progenitors (32, 33). GATA-3 levels are low during the two phases of *TCR* gene rearrangement, but are high in the fraction of rapidly proliferating cells that demarcates these two periods of *TCR* rearrangement (32). *GATA-3* expression remains high in CD4+ thymocytes, but progressively declines in CD8+ thymocytes. GATA-3 is detected in naïve CD4+ T cells and expression levels increase substantially during Th2 differentiation (26, 27). *GATA-3* expression has been shown to be indispensable for Th2 development, and is downregulated in response to IL-12-mediated Stat4 activation (27, 34). GATA-3 strongly transactivates the *IL-5* promoter, but appears to have only limited effects on *IL-4* gene transcription (27, 35, 36). Retroviral introduction of *GATA-3* during *in vitro* Th1 differentiation of naïve CD4+ T cells, resulted in an inhibition of IFNγ production, independently of IL-4 (34, 37), and a downregulation of IL-12Rβ2 (34), which normally accompanies Th2 differentiation (38).

The manipulation of Stat6 and *GATA-3* expression in Th1 and Th2 polarization cultures of wild-type or specific cytokine-deficient cells *in vitro* have added significantly to our understanding of the molecular basis of Th1/Th2 differentiation. However, limited data are available on the role of GATA-3 during immune responses in animal models, partly because the embryonic lethality of *GATA-3* deficiency in mice precluded *in vivo* studies (39). Analysis of transgenic mice with T cell-specific expression of a dominant-negative mutant of GATA-3 indicated that inhibition of GATA-3 activity reduced the key features of asthma, including Th2 cytokine levels, eosinophilia and IgE production (40).

In order to study the function of GATA-3 during T cell differentiation, we generated transgenic mice in which the expression of *GATA-3* is under the control of the human *CD2* locus control region (see Chapter 3). In these mice, the enforced *GATA-3* expression inhibit-

ed the maturation of CD8 single positive cells in the thymus and induced the development of thymic lymphomas of double positive cells, which appeared to differentiate along the CD4 lineage. These observations supported a role for GATA-3 in the regulation of CD4/CD8 lineage commitment.

To investigate how the enforced expression of *GATA-3* affected T helper cell differentiation, we analyzed T cell-mediated immune responses *in vivo* in *CD2-GATA-3* transgenic mice. The observations of a selective deficiency of antigen-specific IgG2a production and severely reduced delayed-type hypersensitivity responses in these mice showed that enforced *GATA-3* expression inhibited the differentiation of Th1 cells *in vivo*.

MATERIAL AND METHODS

MICE

The *CD2-GATA-3* mice are described in Chapter 3 and were crossed on an FVB background. To determine the genotype of the mice, tail DNA was analyzed by Southern blotting, as described in Chapter 3.

FLOW CYTOMETRIC ANALYSES

The preparation of single-cell suspensions, mAb incubations and three- or four-color cytometry have been described previously (41). The following mAb were purchased from Pharmingen (San Diego, CA): FITC-conjugated anti-CD3ε, PE-conjugated anti-CD4 (L3T4), anti-CD24/heat stable antigen (HSA), anti-CD25 (clone 3C7), anti-CD62L and anti-CD69, CyChrome-conjugated anti-CD4 (L3T4), anti-CD8 and anti-CD44, biotinylated anti-CD4 (L3T4), and anti-CD8, APC-labeled anti-CD3ε and anti-CD4. Anti-CD45RB (MB23G2) was a purified mAb conjugated to biotin, according to standard procedures. Secondary antibodies used were PE-, TriColor-, or APC-conjugated streptavidin (Caltag Laboratories, Burlingame, CA). The Th2-selective surface marker T1/ST2 (3E10, rat IgG1, kindly provided by A.J. Coyle, Cambridge, MA) was detected by secondary goat-anti-rat IgG-PE (Jackson Immunoresearch) (42).

For intracellular detection of GATA-3 protein, cells were fixed and permeabilized using paraformaldehyde and saponin, as described (43) and subsequently incubated with the Hg-3-31 anti-GATA-3 MoAb (Santa Cruz, Dan Diego, CA) and FITC-labeled anti-mouse IgG1 (Pharmingen) as a second step. For three-color analysis, 0.5-1 x 10^5 events were scored using a FACScan analyzer (Becton Dickinson, Sunnyvale, CA). For four-color analysis, 10^5 - $2x10^6$ events were scored using a FACS Calibur dual laser instrument (Becton Dickinson).

SERUM Ig DETECTION AND *IN VIVO* IMMUNIZATIONS

Total serum Ig levels were determined by subclass-specific sandwich ELISA, as described previously (44). Immunizations were done i.p. with 100 μg TNP-KLH precipitated on alum. Serum levels of TNP-specific Ig subclasses were determined by ELISA, using TNP-specific standards (IgG1, IgG2a and IgG2b) or TNP-specific reference serum samples

(IgM and IgG3), as described (45).

PURIFICATION OF CD4+ T CELLS AND *IN VITRO* CULTURES

Single cell suspensions from spleen were incubated with biotinylated mAb to CD8 (YTS-169), CD11b/Mac-1 (M1/70), CD40 (FGK-45.5), B220 (RA3-6B2) and IgM (M41), followed by streptavidin-conjugated microbeads (Miltenyi). Using a Vario-MACS magnetic-activated cell sorter, CD4+ T cells were purified according to the manufacturer's instruction to purity >95%. The CD4+ T cells were cultured for up to 5 days in the presence of IL-2 (50 U/ml) on 96 well plates pre-coated with 10 μg/ml anti-CD3 (145 2C11) mAb. Culture supernatants were harvested and cells were assayed for proliferation using standard tritiated thymidine incorporation methods.

Purified CD4+ T cells were polarized into Th1 and Th2 effector cells in a total volume of 200 μl for 4 days in the presence of 5 μg/ml anti-CD28 (37.51) and 50 IU/ml IL-2 on 96-well plates, which were pre-coated with 10 μg/ml anti-CD3ε (145 2C11) (32). Th1-polarizing cultures included 5 ng/ml rIL-12 (R&D Systems, Minneapolis, MN) and 10 mg/ml neutralizing mAbs to IL-4 (11B11). Th2 polarized cells were cultured in the presence of 10-50 ng/ml rIL-4 and 10 μg/ml neutralizing mAbs to IFNγ (XMG1.2). After 4 days of polarization cultures, the cells were thoroughly washed and transferred to new anti-CD3 coated 96-well plates, and cultured in the presence of IL-2, without addition of further cytokines or neutralizing antibodies. Default or non-polarizing cultures were performed as described above for the Th1 and Th2 polarization cultures, but in the absence of exogenously added cytokines or anti-cytokine antibodies. For the intracellular GATA-3 staining experiments (figure 3A), CD4+ cells were cultured in plates that were coated with 100 ng/ml anti-CD3ε, but otherwise the Th1 and Th2 culture conditions were identical to those described above.

To measure DNA synthesis, after 2-3 days of culture, cells were pulsed with [^{3}H]thymidine for ~16 hours, harvested and counted using standard methods. After three days of restimulation, culture supernatants were harvested to determine cytokine production, using IFNγ-, IL-4- and IL-5- specific ELISA systems, as previously described (46).

DTH RESPONSES

DTH responses were performed essentially as described by Cua et al. (47). In short, mice were immunized i.p. with 100 μg KLH in 250 μl PBS and on day 6 they were challenged with 150 μg of KLH in 25 μl PBS in the left hind footpad. The right hind footpad was in injected with a vehicle control (25 μl PBS). Responses were quantified 24 and 48 hours after the challenge, by measuring the difference in footpad thickness between the KLH- and the PBS-injected footpads.

RESULTS

INCREASED EXPRESSION OF MEMORY T CELL SURFACE MARKERS IN *CD2-GATA-3* TRANSGENIC MICE

To analyze the effect of enforced *GATA-3* expression on Th1 and Th2 differentiation, we investigated peripheral T cell differentiation and function in *CD2-GATA-3* mice, using non-transgenic littermates as controls. In these experiments, no essential differences were observed between two independent *CD2-GATA-3* transgenic lines investigated.

We characterized the T cell populations in spleen and mesenteric lymph nodes in 2-3 months old *CD2-GATA-3* mice by three or four color flow cytometry. The sizes of the $CD4^+$ T populations in *CD2-GATA-3* mice were within normal ranges, but $CD8^+$ T cells were reduced in number, to ~50% of normal. This decrease in size of the peripheral $CD8^+$ T cell population was associated with increased cell death and impaired maturation of CD8 single positive T cells in the thymus (see Chapter 3).

As GATA-3 is involved in the stabilization of the Th2 phenotype and the maintenance of Th2 cytokine expression, which are important features of T helper memory, we wanted to assess whether the enforced expression of *GATA-3* had an effect on the development of memory T cells. Antigen activation induces the expression of CD44 and decreases CD45RB expression on the cell surface of T cells. As this profile of cell surface marker expression is maintained, even after cells have reverted to a quiescent state, it can be used to define anti-

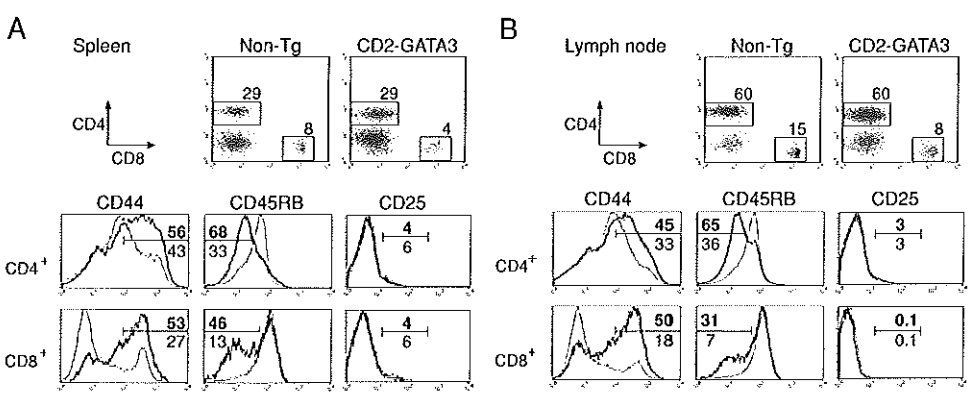

FIGURE 1

Enforced *GATA-3* expression is associated with increased numbers of T cells with a memory surface phenotype.

Single-cell suspensions from spleen (A) and mesenteric lymph nodes (B) from 3 month-old *CD2-GATA-3* transgenic mice and nontransgenic littermates were stained for CD4 and CD8, together with CD44, CD45RB or CD25. Surface CD4/CD8 profiles are shown as dot plots, in which the percentages of total lymphocytes within the indicated $CD4^+$ and $CD8^+$ gates are given. Total $CD4^+$ and $CD8^+$ T cell populations were analyzed for the indicated markers. Results are displayed as histograms of *CD2-GATA-3* transgenic mice (bold lines) and those of non-transgenic littermates (thin lines). For CD45RB and CD25, the percentages of positive cells are indicated above the marker line in bold (*CD2-GATA-3* transgenic mice) and below the marker line (non-transgenic littermates). Data shown are representative of at least 6 mice examined in each group.

gen experienced T cells (48). As shown in figure 1AB, in *CD2-GATA-3* transgenic mice both the $CD4^+$ and the $CD8^+$ T cell populations contained elevated proportions of $CD44^{hi}$ and $CD45RB^{lo}$ cells. This was found in the spleen and in the lymph nodes, and pointed to the presence of either recently activated or memory T cells (48). The expression levels of CD69 and IL-2Rα (CD25), which are markers of recently activated T cells (49), were low in *CD2-GATA-3* mice and non-transgenic littermates (Shown for CD25 in figure 1AB), arguing against the presence of major fractions of recently activated T cells in *CD2-GATA-3* mice. It has been shown that the proportions of T cells with a memory phenotype increases with age, probably as a result of progressive antigen experience (50, 51). When we compared CD44 expression at two different ages, 2 months and 6 months, we indeed found that the conversion to the $CD44^{hi}$ memory T cell phenotype increased with age, both in *CD2-GATA-3* transgenic mice and in control mice (data not shown).

Taken together, these results indicated that in the *CD2-GATA-3* mice both the $CD4^+$ and the $CD8^+$ T cell population contained an increased compartment of cells with a memory phenotype.

ENFORCED EXPRESSION OF *GATA-3* RESULTS IN INCREASED NUMBERS OF T1/ST2 POSITIVE $CD4^+$ T CELLS IN THE PERIPHERY

To investigate whether $CD4^+$ T cells in *CD2-GATA-3* mice exhibited preferential polarization towards Th2 development *in vivo* we evaluated surface expression of T1/ST2 (figure 2). This marker, which is an orphan receptor that belongs to the IL-1 receptor family, is specifically expressed on the surface of Th2 cells (42, 52, 53). In non-transgenic controls we found T1/ST2 expression on 5.1% ± 1.3 and 1.1% ± 0.1 (n=5) of $CD4^+$ T cells in spleen and mesenteric lymph nodes, respectively. In four-color labelings with CD4, CD8, CD44 and T1/ST2, it was shown that in non-transgenic mice T1/ST2 expression was largely confined to the $CD44^{high}$ fraction of activated/memory $CD4^+$ T cells (figure 2).

The *CD2-GATA-3* transgenic animals showed a significant increase in the proportions of $T1/ST2^+$ cells: 31% ± 1 and 23% ± 2 (n=3) in spleen and mesenteric lymph nodes, respectively. This increase could not be attributed solely to the increased proportion of $CD4^+$ T cells with a $CD44^{high}$ activated/memory phenotype, as T1/ST2 was also found to be expressed on naïve $CD4^+$ T cells with a $CD44^{low}$ surface profile (figure 2).

When we analyzed T1/ST2 expression in the thymic subpopulations, we found induction of T1/ST2 on a small fraction of the CD4 single positive cells in *CD2-GATA-3* transgenic mice: 6.0% ± 1.0 (n=3), compared with 0.4% ± 0.05 in non-transgenic mice (n=5). In contrast, T1/ST2 expression, which is reported to be absent on the surface of $CD8^+$ cells (42, 52), was not significantly induced on these cells in these SP thymocytes (<0.5%). Low expression of T1/ST2 was observed on $CD8^+$ T cells in spleen (3.0% ± 0.8) and lymph node (1.7% ± 0.6) from *CD2-GATA-3* transgenic mice (Shown in figure 2 for spleen and lymph node).

In summary, these data indicate that enforced expression of *GATA-3* resulted in significantly increased numbers of peripheral $CD4^+$ T cells with a Th2 polarized phenotype, not only in the $CD44^{hi}$ activated/memory T cell compartment, but also in $CD44^{lo}$ naïve T cells.

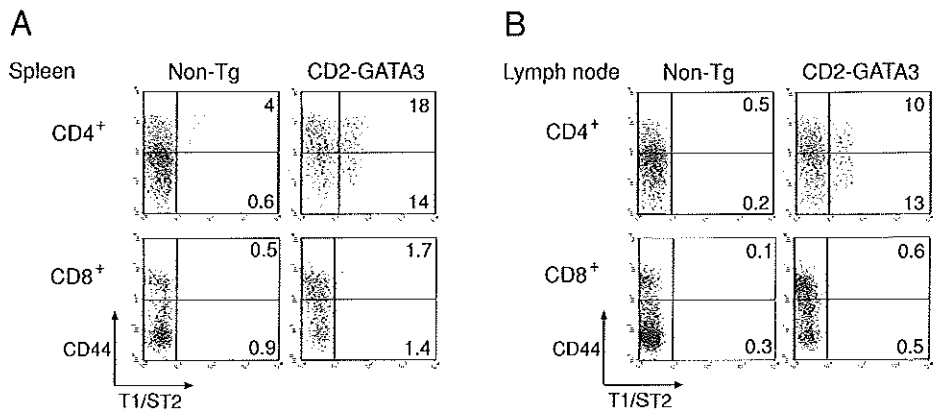

FIGURE 2

Aberrant T1/ST2 surface expression in *CD2-GATA-3* transgenic mice.

Single-cell suspensions from spleen (A) and mesenteric lymph nodes (B) from 2-3 month-old CD2-GATA-3 transgenic mice and nontransgenic littermates were stained for CD4, CD8, CD44 and T1/ST2. $CD4^+$ and $CD8^+$ T cells were gated and analyzed for CD44 and T1/ST2 expression. Data are displayed as dot plots, and the percentages of gated cells within the indicated $T1/ST2^+$ quadrants are shown.

INHIBITION OF Th1 DEVELOPMENT *IN VITRO* IN *CD2-GATA-3* MICE

The T1/ST2 positive $CD4^+$ T cells did not reflect *in vivo* activated cells with high-level Th2 cytokine production, as we could not detect the presence of IL-4, IL-5 or IL-10 by flow cytometric intracellular cytokine stainings. To investigate whether the differentiation potential of $CD4^+$ T cells into Th1 and Th2 effector phenotypes was altered in the *CD2-GATA-3* mice, we performed *in vitro* cell culture experiments on purified $CD4^+$ T cells from spleen and lymph nodes. These cells were stimulated with anti-CD3ε mAbs, either under Th1 polarizing conditions (in the presence of IL-12 and anti-IL-4 mAbs) or under Th2 polarizing conditions (in the presence of IL-4 and anti-IFNγ mAbs) for 4 days. Subsequently, the cells were washed and restimulated with anti-CD3ε mAbs for 3 days.

We evaluated the GATA-3 expression by intracellular flow cytometry, using a mouse monoclonal antisera specific for GATA-3 (figure 3A). The mean fluorescence intensities of GATA-3 expression were higher in the cultures of *CD2-GATA-3* transgenic mice, when compared to those of the nontransgenic littermates, both in the Th1 and in the Th2 cultures. Therefore, we conclude that the transgene is expressed both in Th1 and in Th2 cells.

In default (non-polarized) cultures, *CD2-GATA-3* transgenic $CD4^+$ T cells produced significantly lower levels of IFNγ and highly increased levels of IL-5 and IL-10 as compared to wild-type $CD4^+$ T cells, whereas IL-4 production was similar (figure 3B and data not shown). In Th1-polarized cultures, the production of IFNγ was significantly reduced and the production of IL-5 was strongly enhanced in the *CD2-GATA-3* transgenic $CD4^+$ T cells, when compared to those from non-transgenic littermates (figure 3B and data not shown). In the Th2-polarized cultures, the *CD2-GATA-3* transgenic $CD4^+$ T cells produced normal amounts of

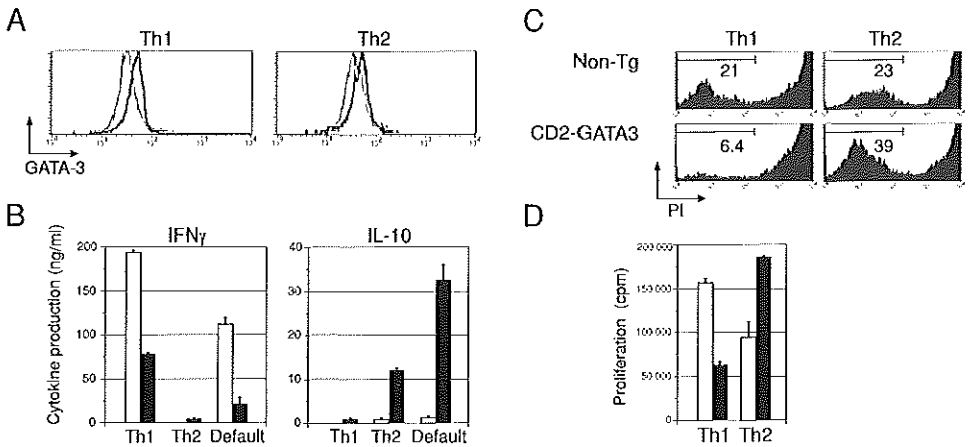

FIGURE 3

The effect of enforced *GATA-3* expression in CD4$^+$ T cell cultures under Th1 and Th2 conditions.

Expression of GATA-3 in *CD2-GATA-3* transgenic (bold lines) and non-transgenic (thin lines) purified CD4$^+$ T cells that were activated under Th1 or Th2 conditions as indicated. Cell suspensions were stained for surface CD4 and CD8 and subsequently for intracellular GATA-3 protein. Flow cytometry results are displayed as histograms of CD4$^+$CD8$^-$ cells.

Cytokine production in supernatants of 7-day cultures of purified CD4$^+$ T cells under default, Th1 and Th2 conditions, as indicated (Open bars: wild-type mice; closed bars: *CD2-GATA-3* transgenic mice).

Cell viability after 7 days of culture under Th1-priming or Th2-priming conditions of purified CD4$^+$ T cells of the indicated mice. Cell suspensions were stained for surface CD4 and CD8 and propidium iodide (PI). Results are displayed as histograms of CD4$^+$CD8$^-$ cells. The percentages of viable (PI$^-$) cells are indicated

Proliferation, determined by [^{3}H]thymidine incorporation, in response to stimulation with anti-CD3ε and IL-2 of purified CD4$^+$ cells of the indicated mice, under Th1 and Th2 conditions. Data are given as mean values ± SD (Open bars: wild-type mice; closed bars: *CD2-GATA-3* transgenic mice).

Data are representative of three experiments.

IL-4 (not shown), but higher levels of IL-10 (figure 3B).

The reduced IFNγ production by *CD2-GATA3* transgenic CD4$^+$ T cells cultured under Th1-polarizing conditions could either result from an inhibitory effect of GATA-3 on the differentiation of naïve cells into Th1 cells, or by an inhibition of the amount of IFNγ produced by differentiated Th1 effector cells. To distinguish between these possibilities, we assessed cell viability and proliferation in the T cell cultures. When analyzed by flow cytometry using propidium iodide, CD4$^+$ T cells from *CD2-GATA-3* transgenic mice showed increased cell death under Th1 culture conditions at day 7, as compared with non-transgenic littermates (figure 3C). When [^{3}H]thymidine incorporation was assessed at day 7, we observed a specific inhibitory effect of enforced GATA-3 expression on cell proliferation in Th1 cultures (figure 3D). By contrast, the presence of the *CD2-GATA-3* transgene enhanced viability and proliferation of CD4$^+$ T cells in the Th2 cultures (figure 3CD).

Collectively, these observations demonstrate that the presence of the *CD2-GATA-3* transgene inhibited the proliferation of Th cells under Th1 polarizing conditions. Furthermore, the

enforced *GATA-3* expression did not support significant proliferation and differentation of Th2 effector cells in the Th1 environment, as in these cultures IL-4 and IL-10 production was low.

ENFORCED *GATA-3* EXPRESSION INHIBITS SWITCHING TO IgG2A IN AN ANTIGEN-SPECIFIC IMMUNE RESPONSE

Serum levels of individual Ig isotypes are generally dependent on the Th1/Th2 balance. IL-4 primes mouse B lymphocytes for switching to IgG1 and IgE, while switching to these subclasses is inhibited by IFNγ (54). Conversely, IgG2a responses are induced by IFNγ and suppressed by IL-4. We analyzed total Ig serum levels, as well as the production of antigen-specific Ig isotypes during a humoral immune response, as these reflected the *in vivo* T helper cell polarization.

Total serum Ig levels were determined in 2-3 month-old *CD2-GATA-3* transgenic mice and non-transgenic littermates by ELISA. Serum levels of IgG1 were significantly higher in the *CD2-GATA3* transgenic animals, while the levels of all other isotypes were similar in the two groups of mice (figure 4A). Notably, no effect of the presence of the *CD2-GATA-3* transgene on Th2 driven total serum IgE levels was observed.

In order to analyze Ig class switching in a T cell-dependent response *in vivo*, mice were immunized i.p. with TNP-KLH, which was precipitated on alum. *CD2-GATA-3* transgenic mice expression showed a significantly decreased TNP-response for the INFγ-dependent IgG2a isotype on day 7 (figure 4B). The levels of all other Ig isotypes elicited in this response were comparable between transgenic animals and wildtype littermates. The decrease of TNP-specific IgG2a was not due to delayed kinetics of the response. On day 14, the TNP-specific IgG2a levels were still low, while TNP-specific IgG1 levels were somewhat elevated in the *CD2-GATA-3* transgenic mice. The day 14 levels for TNP-specific IgG1, IgG2a and IgG2b were 2550 ± 270, 76 ± 15 and 403 ± 51 μg/ml (mean values ± SEM) for the *CD2-GATA-3* transgenic mice and 1940 ± 530, 360 ± 120 and 550 ± 80 μg/ml for non-transgenic littermates, respectively.

Concomitantly, the levels of TNP-specific IgM were elevated in the *CD2-GATA-3* transgenic mice, both on day 7 (139 ± 14 U/ml in the *CD2-GATA-3* mice and 68 ± 10 U/ml in non-transgenic mice) and on day 14 (74 ± 9 U/ml and 49 ± 3 U/ml, respectively).

In conclusion, the observed increase in TNP-specific IgM production and the suppression of TNP-specific IgG2a production indicated that *GATA-3* expression is sufficient to inhibit IFNγ-mediated antigen-specific class switching. In the *CD2-GATA-3* transgenic mice an increased class switching to IgG1 was observed in the total serum levels, but not in the antigen-specific response.

ENFORCED *GATA-3* EXPRESSION SUPPORTS MEMORY T CELL FORMATION

Our *in vitro* experiments showed that *CD2-GATA-3* transgenic Th2 cells had an enhanced capacity to proliferate and survive *in vitro* (figure 3). We next tested the antigenic recall response *in vitro* after previous immunization with a TNP-KLH. $CD4^+$ T cells were

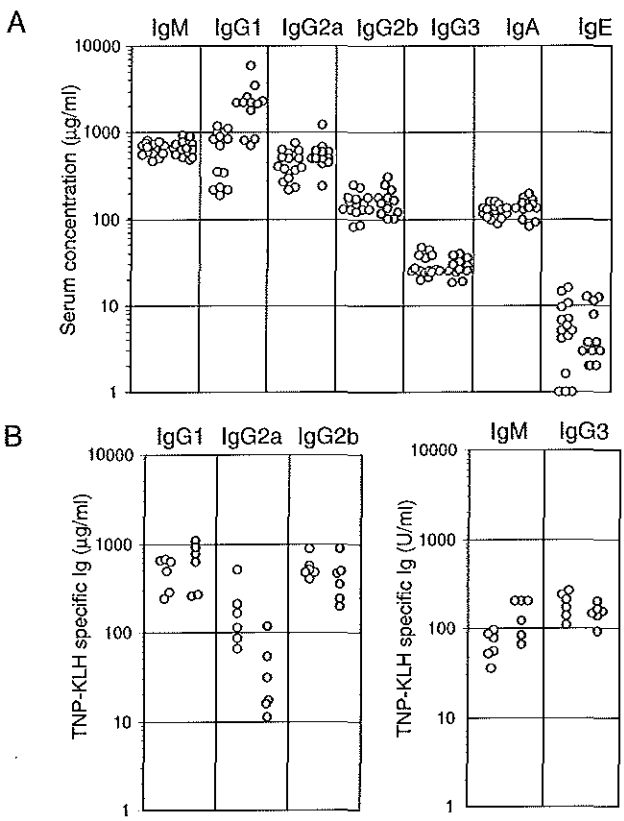

FIGURE 4

Expression of the *CD2-GATA-3* transgene affects Ig class switching *in vivo*.

Serum concentrations of Ig isotypes in unimmunized 2-3-month-old mice (open symbols: non-transgenic mice, n=15, closed symbols: *CD2-GATA-3* transgenic mice, n=12).

For Th cell dependent responses, serum concentrations of TNP-specific Ig were determined 7 days after i.p. injection of TNP-KLH (open symbols: non-transgenic mice, n=6, closed symbols: *CD2-GATA-3* transgenic mice, n=6). Pre-immune levels were <10 U/ml for IgM, <2 U/ml for IgG3, <10 mg/ml for IgG1, IgG2a and IgG2b. Levels were determined by ELISA.

In vitro recall response of splenic Th cells, 4 weeks after TNP-KLH immunization. Data shown are mean values for 4 wild-type mice (open squares), and 2 mice of each independent transgenic *CD2-GATA-3* line (closed symbols).

purified from spleens of immunized *CD2-GATA-3* transgenic mice and control littermates 4 weeks after i.p. injection with TNP-KLH precipitated on alum and stimulated *in vitro* using wild-type irradiated APCs, which had been pre-incubated with KLH for 4 hours. Proliferative responses were determined by [^{3}H]thymidine incorporation and showed that *CD2-GATA-3* transgenic mice had enhanced *in vitro* recall responses (figure 4C). However, CD4$^+$ T cells from *CD2-GATA-3* transgenic mice did not show increased proliferation when polyclonally stimulated with anti-CD3ε or ConA (data not shown).

ENFORCED *GATA-3* EXPRESSION DIMINISHES DELAYED TYPE HYPERSENSITIVITY RESPONSE TO KLH

To directly test whether enforced *GATA-3* expression suppresses Th1-dependent immune responses *in vivo*, we assayed delayed type hypersensitivity (DTH) responses to the protein antigen KLH. Two-month-old mice were primed by i.p. injection of 100 µg KLH, and challenged on day 6 by injection of 25 µl PBS alone and 25 µl PBS containing 150 µg KLH in the left and right hind footpad, respectively. Twenty-four hours after the injections, footpad

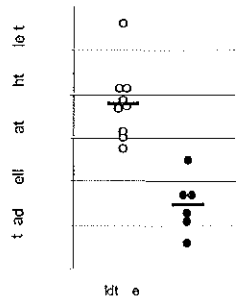

FIGURE 5
Diminished DTH responses in *CD2-GATA-3* transgenic mice.
Ratios of footpad swelling of KLH-injected over PBS-injected footpads, in non-transgenic mice (open symbols, n=9) and *CD2-GATA-3* transgenic mice (closed symbols, n=6) 24 hours after the injections.

thickness was measured and the difference between the two footpads was calculated (figure 5). The KLH-induced footpad swelling was significantly reduced in *CD2-GATA-3* transgenic animals, as compared to the wild type littermates. This reduction of footpad swelling did not reflect delayed kinetics of the DTH response, as also at 48 hours after the injections footpad swellings were still essentially absent in the *CD2-GATA-3* transgenic mice.

These observations demonstrate that GATA-3 expression has a severe inhibitory effect on the Th1-mediated DTH response *in vivo*.

DISCUSSION

Prior studies using Th1 and Th2 polarization cultures of wild-type and specific cytokine-deficient cells *in vitro* have identified GATA-3 as a master switch in Th2 development (26-28, 34, 37). GATA-3 does not only induce the expression of Th2-specific cytokines, but also acts as a repressor of Th1 differentiation. Introduction of GATA-3 by retroviral infection into naïve T cells strongly inhibited IFNγ production, independently of IL-4 expression (34, 37).

Our analysis of the *CD2-GATA-3* transgenic mice support the findings that *GATA-3* expression inhibits Th1 development. The enforced *GATA-3* expression inhibited Th1-mediated responses *in vivo*, including antigen-specific IgG2a production and DTH responses to protein antigen. In our Th1/Th2 polarization cultures, enforced *GATA-3* expression under Th1-inducing culture conditions resulted in a reduction in cell survival, proliferation and IFNγ production and an enhanced IL-5 production. The additional findings of increased T1/ST2-expression in $CD4^+$ T cells and elevated total IgG1 serum levels suggest that the presence of the *CD2-GATA-3* gene drives T cells preferentially towards differentiation along the Th2 pathway. Therefore, we conclude that GATA-3 plays a dual role *in vivo* in the differentiation of naïve T helper cells into Th2 cells, as it both represses Th1 differentiation and induced Th2 differentiation.

Our results also indicate that enforced expression of *GATA-3* enhanced memory cell formation. In *CD2-GATA-3* transgenic mice, the peripheral T cell compartment contained a high

proportion of cells with a memory cell surface profile, defined as $CD44^{hi}CD45RB^{lo}$ and negative for CD25 and CD69. The ratio of naïve versus memory-phenotype cells decreased with age, as normally seen in wild-type mice. Furthermore, T cells from *CD2-GATA-3* mice exhibited an increased recall response to TNP-KLH antigen *in vitro*. Finally, the selective increase of the total levels of the IL-4 dependent isotype IgG1 in the serum would also be consistent with increased Th2 memory formation.

In *CD2-GATA-3* transgenic mice the expression of the Th2-specific T1/ST2 marker within the $CD44^{hi}$ memory Th cell population in spleen and lymph nodes was increased by a factor ~6 and ~20, respectively. Therefore, it is conceivable that in these mice the $CD4^+$ memory T cell compartment mainly consists of committed Th2 cells. The increased proliferation and cell survival of *CD2-GATA-3* transgenic $CD4^+$ T cells in the *in vitro* Th2 polarization cultures could either reflect the presence of high proportions of memory Th2 cells, or be the result of efficient proliferation and further differentiation of naïve GATA-3 expressing $CD4^+$ T cells. Conversely, the increased cell death and limited proliferation of *CD2-GATA-3* $CD4^+$ T cells in the Th1 polarization cultures could be a result of reduced survival of the memory Th2 cells in a Th1 environment, or reduced proliferation of naïve *GATA-3* expressing $CD4^+$ T cells. These two possibilities are not mutually exclusive, but they both imply that the Th1 environment, i.e. the presence of IL-12 and anti-IL-4 antibodies, inhibits the survival of *GATA-3* expressing $CD4^+$ T cells. Therefore, we conclude that IL-12 or IL-4 signaling not only regulate differentiation of naïve cells into Th1 and Th2 effector cells (26-28, 32, 34), but also affect proliferation and survival of *GATA-3* expressing $CD4^+$ T cells. This would also be supported by the finding that *CD2-GATA-3* transgenic $CD4^+$ T cell populations, despite their large fractions of $CD44^{hi}$ $T1/ST2^+$ cells, did not produce significant amounts of IL-10 in our Th1 polarization cultures (in contrast with the high levels of IL-10 in default and Th2 polarization cultures).

GATA-3 therefore appears to facilitate the differentiation process of dividing effector cells to memory cells. We propose that GATA-3 regulates the fate decision of activated $CD4^+$ T cells, by reducing activation-induced cell death, in favor of Th2 memory cell formation. In this context, GATA-3 does not simply act as a survival factor supporting cell proliferation, because survival alone does not appear to be sufficient for memory cell formation, as was shown by the absence of increased memory formation in *Bcl-2* transgenic mice (55). Further experiments are required to define GATA-3 targets that are involved in Th2 memory cell formation.

One of the molecules involved in Th2 memory might be T1/ST2, as it is normally specifically expressed in the Th2 lineage within the compartment of $CD44^{hi}$ activated/memory T cells (figure 2). In *CD2-GATA-3* transgenic mice T1/ST2 was not only expressed in the compartment of $CD44^{hi}$ activator/memory T cells, but also in $CD44^{lo}$ naïve T cells. Enforced expression of *GATA-3* may induce T1/ST2 cell surface expression in $CD4^+$ T cells even before they had encountered antigen. This would argue for a direct regulation of *T1/ST2* transcription in T cells by GATA-3, independent of the process of stable polarization to the Th2 lineage after TCR stimulation. The recent identification of three GATA elements in the min-

imal GATA-responsive *T1/ST2* promoter in mast cells (56) would support this hypothesis of a direct regulation of *T1/ST2* expression by GATA-3, independent of Th2-specific cytokines.

Collectively, the observations reported to date provide evidence for a key role of *GATA-3* in nearly all major fate decisions during T cell development. The lack of even the earliest CD44$^+$CD25$^-$ DN T cell precursor subpopulation in the absence of GATA-3 shows that this factor is required for commitment to the T cell lineage (32,33). Our findings in the transgenic mice with enforced expression of *GATA-3* under the control of the *CD2* promoter implicated GATA-3 in CD4 versus CD8 lineage development (see Chapter 3), in Th2 differentiation and in memory T cell formation. In case of the Th1/Th2 fate decision, it has been shown that GATA-3 can support the differentiation of one cell lineage while inhibiting the development of the other (34). In this context, GATA-3 parallels the transcription factor pax-5, which was recently shown to play an essential role in B-lineage commitment by suppressing alternative lineage choices (57).

In conclusion, this study shows that enforced expression of *GATA-3* inhibits Th1 function *in vivo*. At the same time, the effects on allergy associated Th2 differentiation were limited, as we did not observe elevated total serum IgE levels (figure 4) or increased eosinophilic bronchoalveolar inflammation in an ovalbumin-induced model for allergic asthma (B.L., unpublished results). Therefore, our findings could possibly be useful when considering enhancement of GATA-3 activity as a potential therapy for diseases that reflect an unbalanced activity of Th1 cells (58). This would require the development of strategies to manipulate the activity of GATA-3 in patients with specific autoimmune diseases.

ACKNOWLEDGEMENTS

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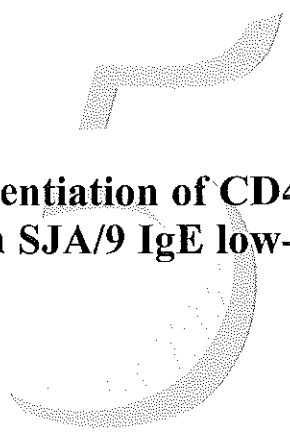
Chapter 5



Dysregulated differentiation of CD4$^+$ T cells into effector phenotypes in SJA/9 IgE low-responder mice

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This paper is dedicated to the memory of Tom Lorrijnen

ABSTRACT

SJA/9 mice mount humoral immune responses to protein antigen and helminth parasites that are characterized by low IgE levels. IgE levels of SJA/9 mice can be restored either by injection of thymocytes obtained from congenic SJL mice or by infusion of recombinant IL-4 prior to or during the immune response. Therefore, we examined the capacity of SJA/9 $CD4^+$ T cells to differentiate into IL-4-producing effector cells. SJA/9 $CD4^+$ T cells can be induced efficiently to differentiate *in vitro* into IL-4-producing cells, yet they fail to do so *in vivo* during the response to either polyclonal goat anti-IgD or TNP-KLH. Instead, $CD4^+$ T cells purified from SJA/9 mice after these responses, produce large amounts of IL-10. This altered $CD4^+$ T cell differentiation is not due to a defect in IL-4R-mediated signals, as both IL-4 receptor-mediated activation of STAT-6 and IL-4-induced proliferation are normal in SJA/9 $CD4^+$ T cells. In addition, we show that neutralization of IL-10 levels during the *in vivo* response to TNP-KLH results in abrogation of the differentiation of SJA/9 $CD4^+$ T cells into an IL-10-producing phenotype. However, the *in vivo* neutralization of IL-10 could not rescue the IgE response of SJA/9 mice, indicating that the production of IL-10, in itself, was not responsible for the low IgE responses in SJA/9 mice. We conclude that the differentiation of $CD4^+$ T cells into mature effector phenotypes is dysregulated in SJA/9 mice, resulting in both an attenuated differentiation into IL-4-producing cells and an enhanced differentiation into IL-10-producing cells.

INTRODUCTION

SJA/9 mice were derived from breeding the BALB/c immunoglobulin (Ig) H chain allotype (Ig^a) into the SJL background (1). SJA/9 mice are characterized by the absence of a significant IgE response to helminth parasites, such as *Nippostrongylus brasiliensis*, or to protein antigens which induce strong IgE responses in both BALB/c and SJL mice (2, 3). This defective response is Ig isotype-specific, as IgG1 responses to *Nippostrongylus brasiliensis* and tri-nitro-phenol conjugated to Keyhole Limpet hematocyanin (TNP-KLH) are normal (4). When stimulated *in vitro* with LPS and IL-4, SJA/9 B cells produce levels of IgE comparable to those produced by SJL and BALB/c B cells, indicating that there is no intrinsic defect in SJA/9-derived B cells to class-switch to IgE in response to IL-4 (5-7). The antigen-specific IgE response of SJA/9 mice could be reconstituted by adoptive transfer of unprimed SJL-derived T cells, suggesting that a T cell-intrinsic defect might be responsible for the absence of IgE responses in SJA/9 mice (2, 4, 8). Moreover, the infusion of recombinant IL-4 during the first week of the immune response to *Nippostrongylus brasiliensis* or TNP-KLH completely restored the IgE response in SJA/9 mice (4). These data indicate that a deficient IL-4 production by Th2 cells might be responsible for the low IgE levels observed during humoral immune responses in SJA/9 mice. However, the presence of a functional, IL-5-producing Th2 population in these mice was suggested by the observation of a normal of

blood eosinophilia during the response to *Nippostrongylus brasiliensis* in SJA/9 mice, which could be blocked by the infusion of IL-5-neutralizing antibodies (4).

CD4+ T helper (Th) lymphocytes can differentiate into two functionally distinct subsets which can be distinguished based on their cytokine production profile (9, 10). Th1 cells are characterized by the production of IFNγ and TNFβ, whereas Th2 cells typically produce IL-4, IL-5, IL-9, IL-10 and IL-13. Each of these helper T cell subset mediates distinct effector functions *in vivo*. For instance, Th1 cells are involved in immune responses against intracellular pathogens, and are associated with autoimmune disease. Th2 cells on the other hand are important in the defense against extracellular pathogens, and are implicated in atopy and allergic diseases (11-13). The ability of CD4+ T cells to differentiate into Th2 or Th1 cells is critical in conferring a susceptible or resistant phenotype to *Leishmania major* infection in mice, reflecting the *in vivo* relevance of polarized Th cell responses (14-16).

Both Th1 and Th2 cells are derived from a common naïve precursor (12, 13, 17). Cytokine-induced signaling pathways are essential in driving the differentiation of activated naïve CD4+ T cells into either mature effector phenotype (10, 18). IL-12 induces the differentiation of naïve Th cells to the Th1 effector phenotype (19-22), by activating the transcription factor Stat4 (23-25). IL-4 directs CD4+ T cell differentiation towards the Th2 effector phenotype (26-29), which is mediated by Stat6 activation (30-32). Differentiated effector Th cells negatively regulate the activity of the other effector subset. For instance, the production of IFNγ by Th1 effector cells inhibits Th2 cell differentiation and proliferation (33, 34), whereas the Th1 cells themselves have lost responsiveness to IFNγ at the receptor level (35, 36). The production of cytokines by Th1 cells is indirectly inhibited by the production of IL-10 by Th2 effector cells (37).

As the natural occurring defect in IgE responses of SJA/9 mice was shown to be T-cell dependent and could be reconstituted by infusion of rIL-4, we analyzed the differentiation of SJA/9 CD4+ T cells into effector phenotype. In this study, we show that while SJA/9 CD4+ T cells have no intrinsic defect in Th2 polarization, they are not able to fully differentiate into IL-4-producing effector cells *in vivo* following immunization protocols that normally elicit Th2-mediated responses. This low IL-4 production is accompanied by an enhanced IL-10 production by SJA/9 Th cells, indicating that the differentiation of CD4+ T cells in SJA/9 into effector cells is dysregulated.

MATERIALS AND METHODS

MICE

SJA/9 and SJL mice were bred and maintained under specific pathogen free (SPF) conditions at the Erasmus Center of Animal Research, Rotterdam. All experiments with live animals were performed according to the regulations of the institutional animal care and use committee.

T CELL PURIFICATION

CD4+ T cells were purified by negative selection from spleen and lymph nodes as described (38). In brief, single cell suspensions were prepared from spleens and mesenteric and lingual lymph nodes. Erythrocytes were depleted by standard ammonium-chloride lysis. Cell suspensions were labeled with biotinylated monoclonal antibodies (mAb) to CD40 (FGK45.5), CD11b/Mac-1 (M1/70), B220 (RA3 6B2), IgM (M41) and CD8 (YTS169). After thorough washing, cells were incubated with streptavidin-coated microbeads (Miltenyi Biotec GMBH, Bergisch Gladbach, Germany). Cells were then separated using a vario-MACS (Miltenyi) cell purification protocol according to the manufacturer's instructions. The negative cell fraction was collected and purity was confirmed by flow cytometry. Purity of CD4+ T cells was routinely ~95 % CD4+.

CD4+ T CELL CULTURES AND POLARIZATION

Purified CD4+ T cells were cultured at 10^5 cells/well for up to 4 days in flat bottom 96-wells culture plates (NUNC, Nalge-Nunc Int., Denmark), which had been pre-coated with 5 µg/ml anti-CD3ε mAb (145 2c11), in the presence of 10 U/ml rIL-2 (a kind gift of Dr. Besemer). Culture medium was RPMI 1640 (BioWhittaker, Verviers, Belgium), containing 5% heat-inactivated FCS (BioWhittaker), 25 mM HEPES buffer (GibcoBRL, Life Tech LTD, Paisley, Scotland), 100 IU/ml penicillin (GibcoBRL), 100 mg/ml streptomycin (GibcoBRL), 1mM pyruvate (Sigma, Zwijndrecht, The Netherlands) and 50 µM 2-ME (Sigma). Proliferation of CD4+ T cells was measured by adding 0.5 µCi [^{3}H]-thymidine per well and incubating the cultures for another 16 hours. Th1 and Th2 polarization cultures were performed by culturing the cells as described above in the additional presence of 5 µg/ml anti-CD28 mAb (37.51) and exogenously added cytokines and anti-cytokine mAbs. Th1 polarization was induced by adding in 5 ng/ml recombinant IL-12 (R&D Systems, Minneapolis, MN) and 40 µg/ml anti-IL-4 mAb (11B11). Th2 polarization was induced by the addition of 10 ng/ml recombinant IL-4 (affinity-purified from culture supernatants of plasmacytoma transformant of the X63-Ag8-653 cell line carrying the IL-4 cDNA (39)) and 40 µg/ml anti-IFNγ mAb (XMG1.2). After 4 days of culture, cells were washed and restimulated on anti-CD3ε (145 2c11; 5 µg/ml) pre-coated 96 well plates in the absence of any exogenously added cytokines. Culture supernatants were collected at day 2 of the restimulated cell culture. Cytokine (IL-4, IFNγ) concentrations in supernatants of replicate cultures were determined using a sandwich ELISA system as descibed before (40). Differences are considered and referred to as significant if $p \leq 0.05$ according to Student's t-test.

SEQUENCE ANALYSIS

IL-4 cDNA was amplified from mRNA extracted from purified, polyclonally activated CD4+ T cells by RT-PCR, using the IL-4- specific primer pair: 5' GGG ATT TGT TAG CAT CTC TTG AT 3' and 5' ATA AGT TAA AGC ATG GTG GCT CA 3'. Subsequent *IL-4* cDNA sequence analysis was performed with Dye terminator reagents (PE biosystems) using the ABI377 fluorescent cycle sequencer (PE biosystems) according to the manufacturer's

instructions.

CT.4S IL-4-SPECIFIC BIOASSAY

CT.4S cells (a kind gift of Dr. D. Gray) were maintained in the presence of rIL-4 (41). SJL and SJA/9 T cell culture supernatants were titrated in two series of triplicate serial dilutions using 96 well round-bottom plates (1/1 though 1/128). The samples were pre-incubated 1 hour at 37°C with the following monoclonal antibodies: S4B6 (IL-2 specific; 40 μg/ml), 2A5.1 (IL-10 specific; 40 μg/ml), and either the IL-4-specific mAb 11B11 or isotype control mAb (β-Gal specific) GL113 (both at 40 μg/ml). CT.4S cells were added at 10^5 cells/well. The plates are incubated 24 hours at 37°C, at which time [^{3}H]-thymidine was added at 0.5 μCi/well and cells were incubated for another 18 hours at 37°C. [^{3}H]-thymidine incorporation was measured by scintillation counter (β-plate counter, Beckman, Mijdrecht, The Netherlands). IL-4-specific CT.4S proliferation was calculated by subtracting the 11B11-values from the GL113-values.

FLOW CYTOMETRIC ANALYSES

Single-cell suspensions were harvested from tissue culture plates and stained for analysis by flow cytometry as described previously (42). The following mAb were purified and conjugated to FITC or biotin according to standard procedures: anti-CD4 (GK1.5), anti-B220 (RA3 6B2), anti-CD25 (PC61), anti-CD44 (PGP-1), anti-CD45RB (MB23G2) and anti-CD62L (Mel-14). As secondary antibodies were used: PE- or Tricolor-conjugated streptavidin (Caltag Laboratories, Burlingame, CA). Stained cells were analyzed using a FACScan analyzer (Becton Dickinson, Sunnyvale, CA).

ANTI-IGD IMMUNIZATION

Polyclonal goat IgG raised against mouse IgD (Nordic, Tilburg, The Netherlands) was reconstituted at 4 mg/ml in PBS. Groups of 5 mice were injected intravenously (i.v.) in the lateral tail vain with 200 μl (800 μg) goat IgG reconstituted in PBS or with saline control. Serum was collected at days 0 (prior to injection), days 4, 7 and 11. Total serum IgE levels were determined using a sandwich ELISA system as previously described (43).

TNP-KLH IMMUNIZATION

Immunizations were performed by intra-peritoneal (i.p.) injection of with 10 μg TNP-KLH precipitated on alum. Sera were collected on days 0 (prior to immunization), 14 and 28. Serum levels of TNP-specific immunoglobulin (Ig) subclasses were determined by ELISA, using TNP-specific standards, as previously described (40, 43).

ALGINATE-ENCAPSULATION AND INJECTION OF HYBRIDOMA CELLS.

GL113 and 2A5.1 hybridoma cells were harvested from culture during logarithmic growth phase. Cells were counted, and $2x10^6$ hybridoma cells were resuspended in 500 μl of PBS. Cells were then encapsulated in alginate as described (44). Alginate-encapsulated cells were injected i.p. using a 21G needle ($2x10^6$/mouse).

DTH RESPONSES TO KLH

Delayed-type hypersensitivity (DTH) responses were performed essentially as described (45). Groups of 6 mice were immunized i.p. with 100 μg KLH. On day 6, the mice were challenged by subcutaneous injection of 150 μg KLH in 30 μl PBS (5 μg/μl) in the left hind footpad and 30 μl of PBS in the right hind footpad. Footpad thickness was measured at days 0 (prior to injection), 1 and 2 using an engineer's micrometer (Mitutoyo Digimatic, Veenendaal, The Netherlands). Day 1 values were used to calculate specific swelling by calculating the ratio of left versus right hind footpad thickness.

ELECTRIC MOLBILITY SHIFT ASSAYS (EMSA)

$CD4^+$ T cells purified from spleen and lymph nodes were incubated with a dilution series of rIL-4 for 1 hour at 37°C. Cells were washed and cell extracts were prepared by resuspending the pellet in a lysis buffer containing NP-40 (0.5%; Boehringer, Petersburg, VA), NaCl (150 μM; Fischer Scientific, Chicago, IL), PMSF (0.4 mM), Leupeptin (1 μg/ml), Pepstatin (1 μg/ml), Apoprotinin (3 μg/ml), DTT (Di-thio-treitol, 1mM) (all Boehringer). Protein content of the cell lysate was measured using standard Bradford techniques. DNA binding of activated Stat factors present in the lysate was analyzed by incubating cell lysate containing 5 μg of total protein for 30 minutes with a 0.5 ng of a [^{32}P]-labeled dsDNA probe containing a single GAS element (interferon-gamma activated site) as previously described (46). Reaction mixtures were separated on a 0.11x TBE 5% poly-acrylamid (PAA) gel. PAA gels were dried and exposed to X-ray film.

RESULTS

SJA/9 $CD4^+$ T CELLS CAN BE INDUCED TO PRODUCE IL-4 *IN VITRO*

SJA/9 mice have an T cell-intrinsic defect resulting in low IgE responses which can be reversed by infusion of rIL-4 early during the immune response (4). These data raise the possibility that SJA/9 $CD4^+$ T cells have a reduced potential to differentiate into IL-4-producing effector cells. In order to investigate the potential of SJA/9 $CD4^+$ T cells to differentiate into IL-4-producing cells, we purified $CD4^+$ T cells from SJA/9 and SJL spleens and lymph nodes and cultured them under Th1- and Th2-inducing conditions for 4 days, and restimulated the polarized Th cells. Cytokine levels were determined on T cell culture supernatants harvested at day 2 after restimulation of the polarized Th cells. As shown in figure 1, Th2-polarized SJA/9 $CD4^+$ T cells produced high levels of IL-4 upon restimulation, comparable to the levels produced by SJL $CD4^+$ T cells. In addition, IFNγ production by Th1-polarized $CD4^+$ T cells was comparable between the two strains of mice (figure 1). Therefore, it appears that SJA/9 $CD4^+$ T cells have a normal potential to differentiate into IL-4- and IFNγ-producing cells *in vitro*.

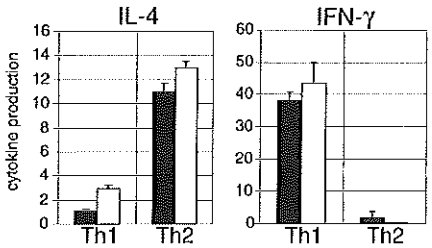

FIGURE 1
Cytokine production by *in vitro* polarized CD4$^+$ T cells.
Cytokine production as detected by sandwich ELISA in culture supernatants from purified splenic SJA/9 (black bars) and SJL (white bars) CD4$^+$ T cells cultured in the presence of recombinant IL-12 and IL-4-neutralizing monoclonal antibodies ('Th1') or in the presence of recombinant IL-4 and IFNγ-neutralizing monoclonal antibodies ('Th2'). Values (ng/ml) represent mean ± 1 SD of 4 replicate cultures. Differences are considered and referred to as significant if p≤0.05 according to Student's t-test.

IL-4 PRODUCED BY SJA/9 T CELLS IS BIOACTIVE

We show that *in vitro* Th2-polarized SJA/9 CD4$^+$ T cells produce similar levels of IL-4 as compared to SJL CD4$^+$ T cells. Also, infusion of rIL-4 during *Nippostrongylus brasiliensis* infection restores the IgE response in SJA/9 mice (4). We therefore tested the possibility that the SJA/9 IL-4 protein is not functional. To this end, we first derived the cDNA sequence from the SJA/9 IL-4 mRNA. The nucleotide sequence for the SJA/9 IL-4 cDNA was identical to the published murine IL-4 cDNA sequence (not shown) (47). In addition to the cDNA sequence, the specific bioactivity of the SJA/9 IL-4 protein was determined. IL-4-containing supernatants from SJA/9 and SJL CD4$^+$ T cell cultures were tested for induction of IL-4-specific proliferation of CT.4S cells. Dilution series of these CD4$^+$ T cell culture supernatants were added to cultures of CT.4S cells, both in the presence and in the absence of neutralizing antibodies to IL-4. Units of IL-4-activity (1 unit equals half-maximal IL-4-induced CT.4S proliferation) in the Th cell culture supernatants were compared to ELISA values for total IL-4 protein content of these supernatants. Thus, the specific IL-4 bioactivity was determined to be 20.7 units/ng for SJA/9 IL-4 and 19.2 units/ng for SJL IL-4. Taken together, these results show that SJA/9 IL-4 is encoded for by a non-mutated mRNA and that the SJA/9 IL-4 protein has full bioactivity, as compared to SJL IL-4.

SJA/9 CD4$^+$ T CELLS PRODUCE LOW LEVELS OF IL-4 WHEN RE-STIMULATED AFTER THE *IN VIVO* RESPONSE TO ANTI-IgD

SJA/9 T cells are capable of producing IL-4 *in vitro* and the SJA/9 IL-4 protein has full bioactivity. Still, IgE levels during humoral immune responses can be restored by infusion of rIL-4, indicating a possible defect in IL-4 production by SJA/9 CD4$^+$ T cells *in vivo*. Therefore, we wanted to analyze IL-4 production by purified CD4$^+$ T cells during the immune response to polyclonal Goat anti Mouse IgD (GAM-IgD), a response which is characterized by high IL-4 production *in vivo* (48). CD4$^+$ T cells, purified at day 7 of the anti-IgD response, were polyclonally restimulated *in vitro*, and cytokine production was measured by ELISA. In this *ex vivo* stimulation, SJA/9 CD4$^+$ T cells produced significantly lower levels of IL-4 and significantly higher levels of IFNγ as compared to SJL CD4$^+$ T cells (figure 2A). These low levels of *ex vivo* IL-4 production by SJA/9 CD4$^+$ T cells might reflect a decreased Th2 polarization *in vivo* or a decreased induction of *IL-4* gene expression *ex vivo*.

Interestingly, SJA/9 CD4$^+$ T cells purified from GAM-IgD immunized animals dis-

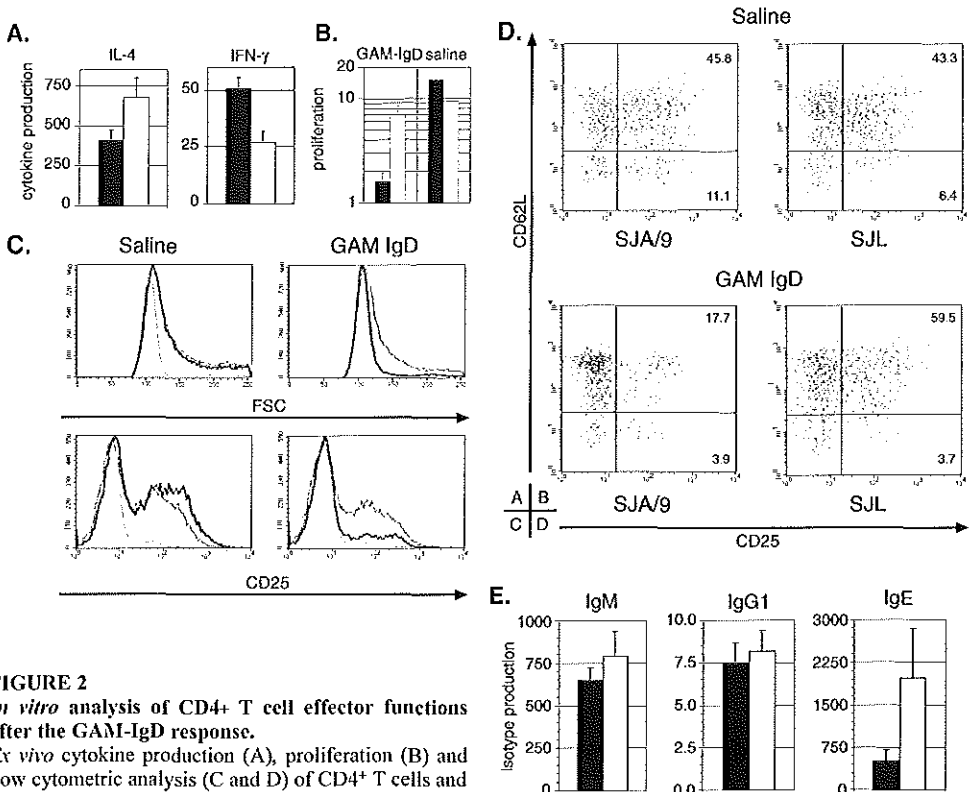

FIGURE 2
***In vitro* analysis of CD4+ T cell effector functions after the GAM-IgD response.**
Ex vivo cytokine production (A), proliferation (B) and flow cytometric analysis (C and D) of CD4+ T cells and immunoglobulin isotype production (E) during the goat-anti-mouse IgD immunization.
CD4+ T cells, purified from spleen at day 7 of the GAM-IgD response were restimulated *in vitro* on immobilized CD3ε-specific mAb. (A) Levels of IL-4 (pg/ml) and IFNγ (ng/ml) in SJA/9 (black bars) and SJL (white bars) Th cell culture supernatants harvested at day 4 of *ex vivo* restimulation. Values are mean cytokine concentration ± 1 SD of four replicate cultures. (B) Proliferation (in $1x10^4$ cpm) of SJA/9 (black bars) and SJL (white bars) CD4+ T cells at day 3 of the *in vitro* restimulation. Values ($1x10^3$ cpm) are mean cpm values ± 1 SD of three replicate cultures. (C) Flow cytometric analysis of cell size (forward scatter) and CD25 expression in SJA/9 (fat curve) and SJL (thin curve) CD4+ T cells at day 2 of the *in vitro* restimulation. Control plots derived from unstimulated SJA/9 cells are represented by a dotted curve. (D) Flow cytometric analysis of CD25 expression on $CD62L^{hi}$ and $CD62L^{lo}$ CD4+ T cell subsets of SJA/9 (A and C) and SJL (B and D) mice, at day 2 of the *in vitro* restimulation. (E) Serum values of total IgM (μg/ml), IgG1 (μg/ml) and IgE (ng/ml) in SJA/9 (black bars) and SJL (white bars), determined by isotype-specific sandwich ELISA at day 7 of the GAM IgD response. Values are mean Ig-isotype serum ± 1 SD of groups of five mice each.

played significantly lower proliferation during this *ex vivo* culture as compared to SJL CD4+ T cells. No differences were observed between in two strains in the *in vitro* proliferation of CD4+ T cells purified from saline injected control animals (figure 2B). To confirm these results, the cells from the *ex vivo* cultures were analyzed on expression of activation markers using flow cytometry. Analysis of CD4+ T cells stimulated *in vitro*, revealed a significantly decreased number of CD25-expressing CD4+ T cells from the GAM-IgD-immunized SJA/9 mice (22.3%) as compared to GAM-IgD-immunized SJL mice (44.2%) or saline injected SJA/9 (58.0%) or SJL (50.5%) mice (figure 2C). In addition, cell size analysis indicated an

absence of blast-like cells in *ex vivo* polyclonally stimulated SJA/9 $CD4^+$ T cells, only when they were purified from GAM-IgD treated animals (Figure 2C). Taken together, these data suggest that the activation of SJA/9 $CD4^+$ T cells by polyclonal stimulation *in vitro* is less pronounced shortly after the *in vivo* response to GAM-IgD.

To distinguish between activated ($CD25^+$) and resting ($CD25^-$) naïve ($CD45RB^{hi}$, $CD44^{lo}$ and $CD62L^{hi}$) and antigen-experienced Th cells ($CD45RB^{lo}$, $CD44^{hi}$ and $CD62L^{lo}$), $CD4^+$ T cells were stained for expression of the cell surface markers CD45RB, CD44, CD62L and CD25 (shown for CD62L vs. CD25 in figure 2D). Levels of CD25 expression on antigen-experienced $CD4^+$ T cells ($CD62L^{lo}$) were comparable between GAM IgD treated SJL and SJA/9 mice (3.7% and 3.9%, respectively). Conversely, expression of CD25 on naïve ($CD62L^{hi}$) $CD4^+$ T cells was reduced on $CD4^+$ T cells purified from GAM-IgD immunized SJA/9 mice (17.7%; dot-plot C) as compared to GAM-IgD immunized SJL (59.5%; dot-plot D) or saline-injected SJA/9 (45.8%; dot-plot A) and SJL (43.3%; dot-plot B) mice (figure 2D). Therefore, it seems likely that the low proliferative response of SJA/9 $CD4^+$ T cells to polyclonal activation *ex vivo* could mainly be attributed to the naïve subset of Th cells.

We next analyzed immunoglobulin (Ig) serum levels to determine the magnitude and the nature of the humoral immune response to the anti-IgD immunization. Isotype-specific sandwich ELISA revealed that SJA/9 mice produced significantly lower levels of IgE in response to GAM-IgD immunization as compared to SJL mice, whereas serum levels of IgM and IgG1 were comparable between the two strains (figure 2E).

In summary, we show that SJA/9 $CD4^+$ T cells have a potential to differentiate into IL-4-producing cells *in vitro* comparable to that of SJL $CD4^+$ T cells. However, the *in vivo* differentiation of SJA/9 $CD4^+$ T cells into IL-4-producing cells is impaired during the GAM-IgD-induced response. In addition, SJA/9 $CD4^+$ T cells purified from GAM-IgD immunized animals were only partially activated in response to polyclonal stimulation *in vitro*. This impaired activation seemed to be mainly restricted to $CD4^+$ T cells with a naïve phenotype.

SJA/9 $CD4^+$ T CELLS PRODUCE LOW IL-4 AND HIGH IL-10 AFTER TNP-KLH IMMUNIZATION

It is possible that the low *ex vivo* IL-4 production and impaired proliferation to polyclonal activation of SJA/9 $CD4^+$ T cells purified during the GAM-IgD response is unique to this type of stimulation. Alternatively, it could be reflective of a more general characteristic of SJA/9 Th cell-mediated immune responses. To distinguish between these possibilities, we analyzed SJA/9 and SJL responses to immunization with the protein antigen TNP-KLH. $CD4^+$ T cells were purified at day 28 following immunization with TNP-KLH, and restimulated *in vitro* on anti-CD3 coated plates. Under these conditions, SJA/9 $CD4^+$ T cells produced significantly smaller amounts of IL-4 as compared to SJL $CD4^+$ T cells, similar to those produced by Th cells purified from untreated SJA/9 or SJL mice (figure 3A). In addition, SJA/9 $CD4^+$ T cells produced significantly higher levels of IL-10 as compared to SJL $CD4^+$ T cells. $CD4^+$ T cells purified from both strains produced equal levels of IFNγ.

In the GAM-IgD responses, SJA/9 $CD4^+$ T cells were only partially activated by poly-

clonal *in vitro* restimulation. This phenomenon seemed to be restricted to the naïve subset of CD4+ T cells. Therefore, we analyzed both polyclonal and KLH-specific activation of CD4+ T cells purified from TNP-KLH immunized mice. The proliferation of polyclonally restimulated SJA/9 CD4+ T cells was strongly decreased as compared to their SJL counterparts (figure 3B). In contrast, no differences were seen between the proliferation of SJA/9 and SJL CD4+ T cells when they were cultured with KLH-pulsed spleen cells derived from either SJA/9 or SJL mice (figure 3B). This result indicates that the KLH-specific SJA/9 CD4+ T cells proliferate normally in response to antigen.

To check for potential altered kinetics of the IgE response in SJA/9 mice, total IgE serum levels were determined at various days during the TNP-KLH response (figure 3C). The IgE levels of SJA/9 mice were significantly lower than those of SJL mice at days 11 and 14 after initial immunization. The kinetics of the response, however, were similar between the two strains of mice.

Taken together, these data indicate that the low proliferation of *in vitro* polyclonally stimulated Th cells seen in SJA/9 mice is not reflective of an intrinsic proliferative defect of antigen-experienced CD4+ T cells purified from the SJA/9 mice. Rather, the naïve (non-antigen-experienced) fraction of the CD4+ T cells seems to have a low proliferation in response to *ex vivo* polyclonal activation, but only after a recent immune response. The high production of IL-10 *in vitro* by polyclonally stimulated SJA/9 CD4+ T cells purified from immu-

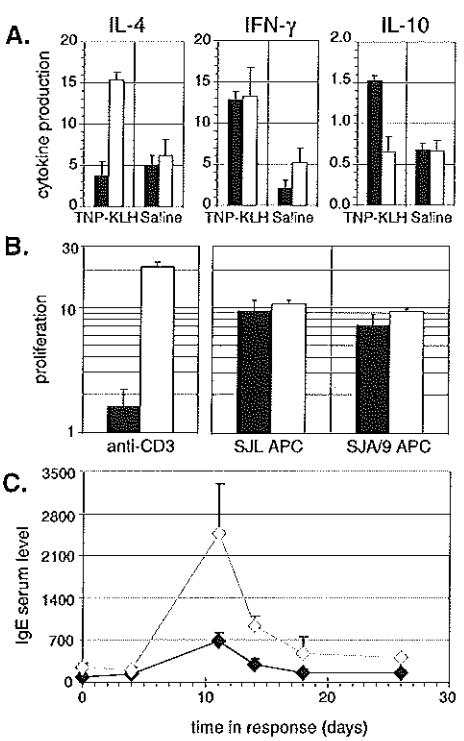

FIGURE 3

***In vitro* analysis of the CD4+ T cell effector functions after the TNP-KLH response.**

(A) *Ex vivo* cytokine production and (B) proliferation of CD4+ T cells and (C) total IgE serum values during the response to TNP-KLH.

CD4+ T cells were purified at day 28 of the primary immune response from TNP-KLH and saline immunized animals and re-stimulated on immobilized CD3ε-specific mAb *in vitro*. (A) Culture supernatants from both groups (as indicated) were harvested at day 4, and IL-4 (ng/ml), IFNγ (ng/ml) and IL-10 (OD) production by SJA/9 (black bars) and SJL (white bars) CD4+ T cells was measured by sandwich ELISA. Values are mean ± 1 SD of four replicate cultures. (B) Polyclonal (immobilized CD3ε-specific mAb) and antigen-specific (KLH-pulsed spleen cells from SJA/9 and SJL mice) proliferation of SJA/9 (black bars) and SJL (white bars) CD4+ T cells on at day 3 of the *in vitro* restimulation. Values ($1x10^3$) are mean cpm values ± 1 SD of three replicate cultures (C) Total SJA/9 (solid diamonds) and SJL (open diamonds) IgE serum values were measured by isotype-specific sandwich ELISA on days 0, 4, 11, 14, 18 and 26 of the TNP-KLH response. Values are mean IgE serum levels ± 1 SD of groups of 5 mice each.

nized mice might indicate an altered differentiation of SJA/9 CD4$^+$ T cells during the immune response.

SJA/9 CD4$^+$ T CELLS HAVE FUNCTIONAL IL-4 RECEPTOR SIGNAL TRANSDUCTION

During the generation of immune responses in SJA/9 mice, CD4$^+$ T cells tend to differentiate into a population of helper T cells characterized by high IL-10 and low IL-4 production. *In vitro*, however, SJA/9 CD4$^+$ T cells can be polarized efficiently into IL-4 producing cells. These observations could be explained by a diminished activation of Stat6 in response to IL-4 (which is a limiting factor *in vivo* but not *in vitro*) in SJA/9 CD4$^+$ T cells. Therefore, we wanted to analyze IL-4 receptor (IL-4R)-mediated signal transduction in SJA/9 CD4$^+$ T cells. To this end, CD4$^+$ T cells were purified from SJA/9 and SJL spleens, and incubated with a dilution series of recombinant IL-4. STAT-6 activation in whole cell extracts was analyzed by electrophoretic mobility shift assays (EMSA) (46). In addition, IL-4-induced proliferation of CD4$^+$ T cells was measured using standard [^{3}H]-thymidine incorporation methods. No differences were observed in either recombinant IL-4-dependent STAT-6 activation (figure 4A) between SJL and SJA/9 CD4$^+$ T cells, or in IL-4-induced proliferation between SJL and SJA/9 CD4$^+$ T cells (figure 4B). These results indicate that there are no differences in IL-4-induced signal transduction leading to STAT-6 activation and cellular proliferation between SJA/9 and SJL CD4$^+$ T cells.

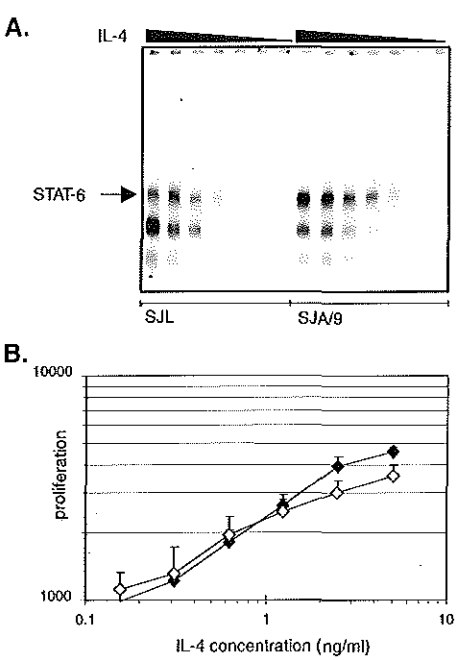

FIGURE 4
IL-4 responsiveness of CD4$^+$ T cells.
Stat-6 activation (A) and proliferation of CD4$^+$ T cells (B) in response to an rIL-4 dilution series. (A) Stat-6 activation in response to a dilution series of rIL-4 was analyzed in SJA/9 and SJL whole cell lysates by EMSA using a [^{32}P]-labeled dsDNA probe containing a single GAS element. (B) Proliferation of SJA/9 (solid diamonds) and SJL (open diamonds) CD4$^+$ T cells in the presence of a dilution series of rIL-4. Values are mean values of three replicate cultures ± 1 SD.

IN VIVO NEUTRALIZATION OF IL-10 DOES NOT RESCUE THE IgE RESPONSE TO TNP-KLH IN SJA/9 MICE

CD4+ T cells which are purified from recently immunized SJA/9 mice, produce high amounts of IL-10 when restimulated *ex vivo*. Therefore, we wanted to examine the role of this cytokine in both CD4+ T cell differentiation and in the IgE response of SJA/9 mice *in vivo*. To this end, SJA/9 and SJL mice were transplanted with alginate-encapsulated hybridoma cells. These cells produce monoclonal antibodies (mAb) specific for IL-10 (2A5.1) or, as an isotype-matched control, for β-Gal (GL-113). This method was shown to be effective in neutralizing serum levels of IL-4 and of IL-6 when mice were transplanted with alginate-encapsulated hybridoma cells producing the IL-4-specific mAb 11B11 and the IL-6-specific mAb 20F3, respectively (49). Alginate-encapsulated hybridoma cells ($2x10^6$) were injected i.p. 3 days prior to the TNP-KLH immunization. At the time of TNP-KLH immunization, serum levels of rat IgG1 were between 1 and 5 μg/ml, and remained constant for approximately three weeks (data not shown). Serum levels of total and TNP-specific immunoglobulin isotypes were determined prior to immunization and at days 14 and 28 of the response.

Total IgE levels from both strains of mice were relatively low during the TNP-KLH response. SJA/9 mice treated with the control antibody GL113 had significantly lower IgE responses as compared to SJL mice treated with GL113 (figure 5A). In SJA/9 mice, treatment with IL-10-neutralizing mAb (2A5.1) had little effect on total IgE responses as compared to GL113-treated SJA/9 mice. Total IgE levels in SJL mice treated with 2A5.1 mAb were non-significantly enhanced as compared to GL113-treated SJL mice. In addition, 2A5.1-treated SJA/9 mice had similar levels of TNP-specific IgE as compared to those treated with GL113

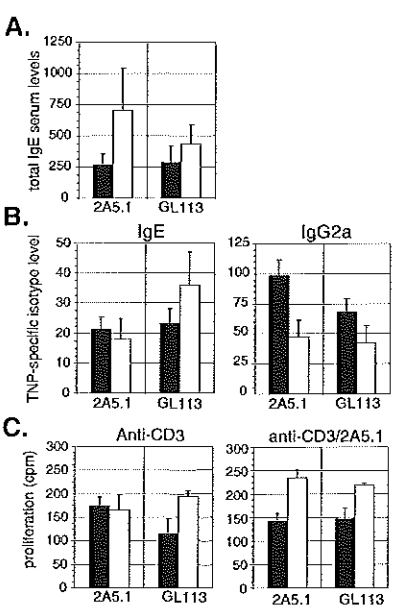

FIGURE 5

Neutralization of IL-10 during the TNP-KLH response *in vivo*.

Total IgE (A) and TNP-specific IgE and IgG2a (B) levels and *ex vitro* proliferation of CD4+ T cells (C) during the immune response to TNP-KLH under neutralization of IL-10.

Mice (5 per group) were transplanted i.p. with $2x10^6$ alginate encapsulated hybridoma cells, producing mAb specific for IL-10 (2A5.1) or β-Gal (GL113), and a TNP-KLH immunization was performed. (A) SJA/9 (black bars) and SJL (white bars) total IgE serum levels (depicted as mean ± 1 SD) were determined at day 14 of the TNP-KLH response by sandwich ELISA. (B) SJA/9 (black bars) and SJL (white bars) TNP-specific IgE and IgG2a serum values (depicted as mean ± 1 SD) were measured on day 14 of the response by TNP-specific sandwich ELISA. (C) CD4+ T cells were purified and restimulated *in vitro* on immobilized CD3ε-specific mAb in the absence (marked as anti-CD3) or presence of IL-10 neutralizing mAb (marked as anti-CD3/2A5.1). At day 3 of the culture, proliferation was measured by [^{3}H]-thymidine incorporation. Values ($1x10^3$) are mean cpm values ± 1 SD of three replicate cultures.

(figure 5B). TNP-specific IgE levels were non-significantly reduced in SJL mice treated with 2A5.1 mAb as compared to GL113-treated SJL mice. No significant differences in TNP-specific IgE responses were observed between SJL and SJA/9 mice.

TNP-specific IgG2a serum levels were elevated in 2A5.1-treated SJA/9 mice as compared to GL113-treated animals, whereas levels were comparable between 2A5.1 and GL113-treated SJL mice (figure 5B). TNP-specific IgG2a levels were significantly enhanced in SJA/9 mice as compared SJL mice only within the 2A5.1-treated groups. TNP-specific IgG1 levels could not be measured accurately, due to the interference of the rat IgG1 mAb (GL113 or 2A5.1) with the ELISA assay (data not shown).

$CD4^+$ T cells were purified on day 28 of the response from all groups of animals, and their ability to proliferate in response to polyclonal stimulation *in vitro* was determined. $CD4^+$ T cells purified from SJA/9 mice treated with GL113 show a significantly decreased proliferation to polyclonal restimulation *in vitro* as compared to GL113-treated SJL mice (figure 4C). The *in vitro* proliferation of SJA/9 $CD4^+$ T cells was restored by neutralizing IL-10 in the cultures (figure 4C). The *in vitro* proliferation of $CD4^+$ T cells purified from SJA/9 mice which had been treated with 2A5.1 *in vivo*, was only slightly reduced as compared to cultures of $CD4^+$ T cells purified from *in vivo* 2A5.1-treated SJL mice. Interestingly, *in vitro* neutralization of IL-10 in the cultures of 2A5.1-treated SJA/9 $CD4^+$ cells did not enhance the proliferation, suggesting that these cultures did not contain IL-10-producing T cells.

Taken together, these data indicate that the *in vivo* treatment on SJA/9 mice with IL-10-neutralizing antibodies could prevent the differentiation of $CD4^+$ T cells into IL-10-producing cells, but could not restore the IgE response of the SJA/9 mice to levels comparable to that of the SJL strain of mice.

SJA/9 MICE ELABORATE NORMAL DTH RESPONSES TO PROTEIN ANTIGEN

SJA/9 mice elaborate altered Th cell-dependent immune responses *in vivo*. Specifically, differentiation of $CD4^+$ T cells into IL-4-producing cells is affected. To determine whether the Th1 compartment in SJA/9 mice is functional, we induced a typical Th1-mediated immune response in SJA/9 and SJL mice, the delayed-type hypersensitivity (DTH) response to the protein antigen KLH. Mice were injected i.p. with 100 µg KLH. At day 6, mice were rechallenged by injection of 150 µg KLH in 30 mg PBS in the left-hind footpad and 30 µg PBS in the right-hind footpad. Footpad thickness was measured at 24 hours after the challenge, and KLH-specific swelling was calculated by determining the ratio of left versus right

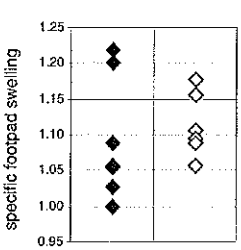

FIGURE 6
DTH responses.
SJA/9 and SJL mice were challenged subcutaneous in the left-hind footpad with 150 µg KLH at day six after initial KLH immunization (i.p. 100 µg). Right hind footpads were injected with equal volumes (30 µl) of PBS. Footpad thickness of SJA/9 (solid diamonds) and SJL (open diamonds) right and left hind footpads were determined at 24 hours after challenge and left versus right hind footpad ratios were calculated.

hind footpad thickness. No differences were observed between SJA/9 and SJL mice in specific footpad swelling in response local challenge with KLH (figure 6). These results indicate that the Th1 compartment in SJA/9 mice is fully functional as compared to SJL animals.

DISCUSSION

SJA/9 mice display defective IgE but normal IgG1 responses to helminth parasites and protein antigens (2-4). IgH class switching of activated B cells to the IgE isotype is largely dependent on IL-4 (6, 50, 51). The isotype-specific defect of SJA/9 mice could, therefore, be reflective of a specific defect in IL-4-responsiveness. It has been shown, however, that SJA/9 B cells are capable of IgE production when stimulated *in vitro* with rIL-4 and LPS (5-7). Also, congenic SJL thymocytes have been shown to revert the IgE response to *Nippostrongylus brasiliensis* and TNP-KLH (2, 4, 8), indicating that the SJA/9 defect in IgE responses resides within the T cell compartment. In addition, infusion of rIL-4 early during the immune response also restored the IgE response of SJA/9 mice (4), indicating the possibility of a defective IL-4 production by SJA/9 $CD4^+$ T cells to be the responsible factor.

Here we show that SJA/9 $CD4^+$ T cells can be induced to produce IL-4 *in vitro* efficiently (figure 1), but produce only modest amounts of IL-4 when restimulated *ex vivo* following the immune response to polyclonal anti-IgD goat serum (figure 2), or to TNP-KLH (figure 3). These results indicate that the T cell defect responsible for the defective IgE responses of SJA/9 mice does not result from their capacity to produce IL-4, but from the *in vivo* induction of SJA/9 $CD4^+$ T cells to become IL-4-producing effector cells. $CD4^+$ T cells purified from SJA/9 mice after an immune response produce high levels of IL-10 and low levels of IL-4 as compared to congenic SJL $CD4^+$ T cells (figures 2, 3), which could explain the inhibition of *in vitro* proliferation in response to polyclonal activation.

It seems likely that the antigen-experienced subset of SJA/9 $CD4^+$ T cells is responsible for the high IL-10 production. SJA/9 $CD4^+$ T cells purified from non-immunized animals proliferate normally and do not produce IL-10 in response to polyclonal activation *in vitro*. Also, polyclonal, but not antigen-specific proliferation is strongly decreased in SJA/9 $CD4^+$ T cell cultures from previously immunized animals (figure 3B). Finally, the defective induction of IL-2Rα upon polyclonal *ex vivo* stimulation is largely confined to the naïve ($CD62L^{hi}$) $CD4^+$ T cells (figure 2C) (52). Taken together, these findings indicate that during the response to GAM-IgD or TNP-KLH, antigen-experienced SJA/9 $CD4^+$ T cells differentiate into a population of $CD4^+$ T cells producing low amounts of IL-4 and high levels of IL-10, suggesting a dysregulation of $CD4^+$ T cell differentiation in SJA/9 animals.

Th-derived production of IL-10 may be accounted for by IL-10-producing Th2 cells, or by regulatory T cells 1 (Tr-1), a regulatory subset of Th effector cells which produce IL-10 but no IL-4 (53). The *in vivo* counterpart of these *in vitro* generated Tr-1 cells, are contained within the $CD45RB^{low}$ subset of $CD4^+$ T cells (54). It has been reported that the differentiation of these IL-10-producing $CD4^+$ T cells is dependent on the presence of IL-10 (53, 55).

We show that during the immune response to GAM-IgD or TNP-KLH, SJA/9 $CD4^+$ T cells differentiate into IL-10-producing $CD4^+$ T cells to a higher extent than do SJL $CD4^+$ T cells. The IL-10 production after *ex vivo* re-stimulation of SJA/9 $CD4^+$ T cells could be derived from both Th2 and Tr-1 cells. Neutralizing IL-10 during the *in vivo* immune response inhibited the suppression of the proliferation in response to polyclonal activation *in vitro* (figure 3). This indicates the importance of IL-10 for the differentiation of SJA/9 $CD4^+$ T cells into the IL-10-producing phenotype. These data suggests that at least part of the IL-10-producing cells in SJA/9 $CD4^+$ T cells might be Tr-1 cells. Importantly, however, neutralization of IL-10 during the immune response did not result in increased levels of TNP-specific IgE. Therefore, the SJA/9 $CD4^+$ T cells were are incapable of producing sufficient amounts of IL-4 to induce isotype-switching to IgE, even when the differentiation into an IL-10-producing phenotype was inhibited. So, IL-10-producing SJA/9 $CD4^+$ T cells do not actively inhibit the differentiation of SJA/9 $CD4^+$ T cells into IL-4-producing cells. More likely, some regulatory mechanism acting on SJA/9 $CD4^+$ T cell differentiation is responsible for both their propensity to differentiate into an IL-10-producing phenotype and their lack of differentiation into an IL-4-producing phenotype.

There are a number of possible explanations for the dysregulated SJA/9 $CD4^+$ T cell differentiation. IL-4-induced STAT-6 activation in SJA/9 $CD4^+$ T cells might be diminished, leading to a deficiency in inducing Th2 differentiation in response to IL-4. However, we show that IL-4-dependent activation of STAT-6 and IL-4-induced proliferation is similar between $CD4^+$ T cells derived from SJA/9 and SJL mice. Moreover, SJA/9 $CD4^+$ T cells can be induced to differentiate into IL-5 producing Th2 effector cells *in vivo* (4). Also, we show that SJA/9 $CD4^+$ T cells can readily be differentiated *in vitro* into IL-4-producing Th2 cells.

It remains possible that *IL-4* gene expression is specifically affected in SJA/9 $CD4^+$ T cells, independent of the expression of other Th2 cytokine genes, such as *IL-5* and *IL-13*. We show that Th2 polarized SJA/9 $CD4^+$ T cells are capable of producing IL-4 to similar levels as SJL $CD4^+$ T cells. However, a putative defect in IL-4 production by SJA/9 $CD4^+$ T cells might be too subtle to detect under these optimized conditions *in vitro*. Under physiological conditions, not all the factors essential for the optimal induction *IL-4* gene transcription might be available to SJA/9 $CD4^+$ T cells, resulting in an IL-4-specific defect in cytokine production by these cells. For instance, an absence of factors essential for the induction of c-Maf, a transcription factor acting on the *IL-4* promotor specifically (56), could account for such a defect in induction of *IL-4* gene transcription. These issues remain to be addressed in future studies.

The SJA/9 strain was originally derived from breeding the BALB/c immunoglobulin H chain allotype (Ig^a) into the SJL background (1). It can be anticipated, however, that SJA/9 and SJL mice differ on additional chromosomal loci, which could contain genes important for the regulation of Th-mediated immune responses. Such differences might then account for the observed differences in $CD4^+$ T cell differentiation and the subsequent regulation of the IgE response. A more detailed genetical analysis of the SJA/9 and SJL strains could therefore be an interesting approach to determine these genetic loci that putatively affect Th cell differen-

tiation.

In conclusion, we show that CD4+ T cells derived from SJA/9 mice -which are characterized by a specific defect in the induction of IgE responses- fail to differentiate into IL-4-producing Th2 effector cells *in vivo*. This defective differentiation into an IL-4-producing phenotype is associated with a preferential *in vivo* differentiation into an IL-10-producing phenotype. The acquisition of an IL-10-producing phenotype in itself is not responsible for the impaired induction of an IgE response. The molecular mechanisms responsible for the dysregulated differentiation of SJA/9 CD4+ T cells remain to be elucidated and could be very informative on the regulation of Th cell differentiation into IL-10-producing phenotypes.

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Chapter 6

Suppressor of cytokine signaling (SOCS)-3 inhibits activation of nuclear factor of activated T cells (NFAT)p

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ABSTRACT

Recent studies have suggested that signaling initiated by the activation of antigen receptors and signaling activated through cytokine receptors may be regulated by a common set of inhibitory proteins. SOCS-3, which has previously been demonstrated to inhibit cytokine signaling, is induced upon T cell receptor (TCR) ligation. Overexpression of *SOCS-3* can inhibit transcription driven by the IL-2 promoter in response to T cell activation. This inhibitory activity correlates with the suppression of calcineurin dependent dephosphorylation and activation of the transcription factor NFATp. Infection of primary murine T cells with a retrovirus encoding *SOCS-3* blocks their IL-2 production in response to activation. Interestingly, SOCS-3 was found to co-immunoprecipitate with the catalytic subunit of calcineurin. These studies suggest that SOCS-3 may regulate T cell function as part of a negative feedback loop.

INTRODUCTION

Regulation of T cell activation is critical for the maintenance of immune homeostasis and the prevention of autoimmune disease (1). This homeostatic regulation can be implemented at several levels. Regulation of T cell activation at the level of cell-cell interaction involving negative regulatory receptors such as CTLA-4 and Fas are well characterized (1). Much less is known about the negative regulation of T cell activation through TCR mediated signaling pathways. Mechanisms involved in this regulation include recruitment of the inhibitory protein tyrosine kinase, Csk (2), and protein tyrosine phosphatases such as SHP-1 and SHP-2 (3, 4). Interestingly, the SHP-1 and SHP-2 phosphatases also regulate cytokine receptor signaling (5). There are many similarities between TCR mediated signaling and cytokine receptor mediated signaling. Early events in both cases involve clustering of receptor subunits and the activation of tyrosine kinases. The most well studied tyrosine kinases activated by cytokine receptors are the janus kinase (JAK) family (6), whereas TCR ligation leads to activation of the Src family kinases Lck and Fyn, followed by activation of ZAP70 (7). Activated tyrosine kinases proceed to phosphorylate receptor chains and associated scaffolding proteins, which then bind further downstream effectors. One of the most well-characterized downstream effectors of TCR mediated signal transduction is calcineurin, a calcium dependent serine/threonine phosphatase that dephosphorylates, and activates the transcription factor NFAT upon TCR ligation (8). Important downstream effectors of cytokine receptor activation include the signal transducer and activator of transcription (STAT) family of transcription factors (6).

In addition to protein tyrosine phosphatase recruitment, another mechanism in the regulation of cytokine signaling involves members of the SOCS gene family (9-11). CIS, SOCS-1 and SOCS-3 have all been shown to inhibit cytokine signaling by interfering with the JAK-STAT pathway (12). The existence of parallels between cytokine receptor mediated sig-

naling and signaling through the TCR has led to interest in the potential involvement of SOCS family members in TCR mediated signaling. CIS has been shown to be induced by TCR stimulation and to enhance TCR mediated MAP kinase activation, possibly through its interaction with protein kinase C (PKC)-Θ (13). While the effects of SOCS-1 on TCR signaling remain unclear, SOCS-1 has recently been shown to inhibit the activation of NFAT in 293T cells expressing CD8 and Syk (14). Whether SOCS-3 plays a role in the regulation of TCR mediated signaling has not previously been reported. In this report, we show that SOCS-3 is induced in T cells upon TCR ligation and can inhibit TCR mediated activation of the *IL-2* promoter when overexpressed. Furthermore, SOCS-3 is shown to interact with calcineurin and inhibit the activation of NFATp in response to calcium signaling. Our results suggest a role for SOCS-3 in the regulation of NFATp activity in T cells.

MATERIALS AND METHODS

NORTHERN BLOT ANALYSIS

RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). A total of 5 μg of total RNA was denatured for 5 min at 68YC, separated by electrophoresis in 1% agarose/formaldehyde gel and transferred to nylon membrane. *SOCS-3* cDNA fragment was labeled using Rediprime (Amersham Pharmacia, Piscataway, NJ).

CELL CULTURE AND TRANSFECTION

Jurkat cells were grown in RPMI 1640 supplemented with 10% heat inactivated FCS. Jurkat cells were transfected by elecroporating $2x10^7$ cells in 400 μl media at 250 V and 960 μF. Stably transfected cells were selected by resistance to 1 mg/ml G418 (Gibco BRL, Gaithersburg, MD). 293T cells were grown in DMEM supplemented with 10% heat inactivated FCS. 293T cells were transfected by the calcium phosphate method. $CD4^+$ T cells were isolated from spleens and/or lymph nodes of C57BL/6 mice by negative selection. Briefly, single cell suspensions containing no red blood cells were incubated with rat anti-mouse mAbs (Pharmingen, San Diego, CA) against B220, MAC-1, and CD8 at 20 μg/ml each. After washing, suspensions were incubated with anti-rat IgG Dynabeads (Dynal, Lake Success, NY). Antibody coated cells were removed using a magnetic concentrator (Dynal). Remaining cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FCS, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 0.3 μg/ml glutamine, 0.1 mM non-essential amino acids and 20 U/ml human IL-2.

INTRACELLULAR CYTOPLASMIC STAINING

Cells were fixed in 4% paraformaldehyde in PBS, permeabilized with staining buffer (PBS with 1% BSA and 0.2% NaN_3) containing 0.1% saponin (Sigma, St. Louis, MO), blocked with normal rat IgG, and stained with PE conjugated rat anti-IL-2 (Pharmingen, San Diego, CA). Cells were analyzed on a FACScan.

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Probe used was derived from the distal NFAT binding site from the human *IL-2* promoter, and its sequence is 5'-GGA GGA AAA ACT GTT TCA TAC AGA AGG CGT-3' Double stranded oligonucleotides were prepared and labeled by filling in recessed ends with Klenow enzyme (New England Biolabs, Beverly, MA). Nuclear extracts were prepared as previously described (15). Binding reactions were carried out in a 20 ml volume containing 10 mM Tris pH 7.5, 50 mM NaCl, 1mM EDTA, 1μM β-mercaptoethanol, 300 ng of poly-dIdC, and 1% Ficoll. Probe was incubated with 5 μg nuclear extract for 20 minutes at room temperature, after which reaction mixture was run on a prerun 5% polyacrylamide gel for 2 hours at 200 V. Gel was dried onto Whatman paper and autoradiographed.

IMMUNOPRECIPITATION AND WESTERN BLOTTING

Cell extracts were made using lysis buffer consisting of 0.5% Nonidet P-40, 50 mM Tris (pH 8.0), 200 mM NaCl, 50 mM NaF, 0.5 mM $NaVO_4$, 20mM $Na_4P_2O_7$, 10% glycerol, 0.1 mM EDTA, 0.4 mM PMSF, and 1mM DTT. Extracts were precleared with normal mouse IgG or normal rabbit IgG before incubation with NFATp antisera (Santa Cruz Biotechnology, Santa Cruz, CA) or HA antisera (Santa Cruz Biotechnology). Immune complexes were captured with Protein A sepharose, washed with lysis buffer, washed with PBS, and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membrane. Antisera used in western blotting included NFATp (Santa Cruz Biotechnology), HA (Santa Cruz), and Xpress (Invitrogen, San Diego, CA).

GST PRECIPITIONS

Plasmid encoding GST-SOCS3 fusion protein was constructed by cloning SOCS-3 into the BamHI site of pGEX-3X (Amersham Pharmacia, Piscataway, NJ). GST and GST-SOCS-3 proteins were harvested from bacterial cultures after 3 hours of induction with 0.2 mM IPTG at 30°C. Bacteria were lysed using lysozyme, followed by addition of sarcosyl and sonication. Extracts were incubated with glutathione-agarose beads. Beads were washed and stored at 4°C. Precipitation experiments were performed by incubating cell extracts with beads coupled to 15 μg GST or GST-SOCS-3 and washing beads with lysis buffer described above, washing beads with PBS, and resolving associated proteins by SDS-PAGE.

RETROVIRAL INFECTIONS

Infections were performed as previously described (16). Briefly, retroviruses were generated by transient transfection of 293T cells with pCL-Eco, and either pMIG or pMIG-SOCS-3. 48 and 72 hours after transfection, retrovirus containing medium was harvested from plates of transfected cells and used for infection. Infections were performed by incubating cells in retrovirus containing media in 24 well plates. Plates were spun at 2500 rpm at 32YC for 90 minutes, after which retrovirus containing media was removed and fresh T cell media was added.

RESULTS

TCR-MEDIATED T CELL ACTIVATION REGULATES *SOCS-3* EXPRESSION

Several cytokines, including IL-2, IL-3, IL-6, leukemia inhibitory factor, growth hormone, and leptin can induce *SOCS-3* mRNA (9, 17). To examine the effects of TCR stimulation on *SOCS-3* expression, RNA from purified murine T cells stimulated with CD3-specific antibodies (αCD3) was analyzed by Northern blotting. *SOCS-3* mRNA levels were increased by stimulation with αCD3, reaching peak levels after 8 hours of stimulation and remaining elevated for at least 24 hours (figure 1). SOCS-3 induction by TCR ligation is less rapid and of much greater duration than induction by the other stimuli listed above. SOCS-3 can also be induced with αCD3 in polarized Th1 and Th2 cell populations (data not shown).

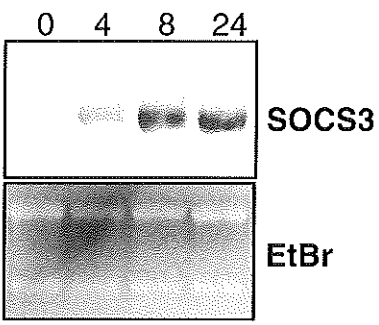

FIGURE 1
Changes in *SOCS-3* mRNA levels on TCR ligation.
RNA was isolated from splenic T cells of C57BL/6 mice stimulated with plate bound αCD3 for the amount of time indicated. The RNA was then subjected to Northern blot analysis with probe specific for *SOCS-3* (top panel). Ethidium bromide staining of RNA gel is shown to demonstrate equal loading of lanes (bottom panel).

INHIBITION OF *IL-2* PROMOTER ACTIVITY BY SOCS-3

To investigate the effects of *SOCS-3* expression on signaling downstream of the TCR, Jurkat cells were cotransfected with a luciferase reporter driven by the proximal *IL-2* promoter and either *SOCS-3* or a vector control. SOCS-3 inhibited the activation of the *IL-2* promoter by PMA and ionomycin (figure 2A). Further cotransfection experiments utilizing a luciferase reporter driven by 3 copies of the distal NFAT/AP-1 site from the *IL-2* promoter revealed the ability of SOCS-3 to inhibit transcriptional activation driven by this element (figure 2B). In contrast, SOCS-3 had no effect on the PMA/ionomycin induced activation of a third luciferase reporter driven by multimerized AP-1 binding sites (figure 2C). The results of these transient transfection experiments suggest that SOCS-3 suppresses NFAT-dependent transcriptional activation. Furthermore, as PMA and ionomycin bypass the initial tyrosine phosphorylation events involved in T cell activation, the mechanism of SOCS-3 in blocking gene induction by NFAT lies downstream of the tyrosine kinases lck and ZAP70.

SOCS-3 INHIBITS NFATp DEPHOSPHORYLATION

To further study the effects of SOCS-3 on NFAT function, Jurkat cells constitutively expressing *SOCS-3* were generated. Jurkat cells expressing *SOCS-3* were deficient in their ability to produce IL-2 as compared to Jurkat cells transfected with vector alone when stimulated with PMA and ionomycin (figure 3A). When Jurkat cells expressing *SOCS-3* were

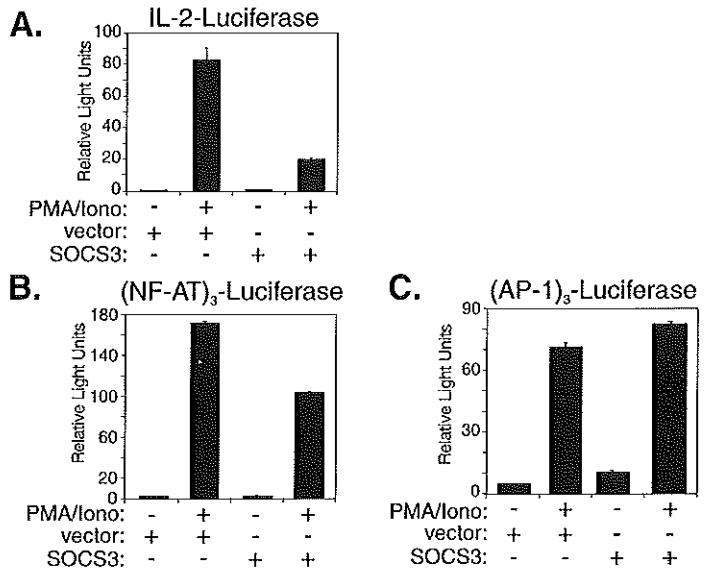

FIGURE 2
SOCS-3 suppresses NFAT mediated transcriptional activation.
(A) Jurkat cells were transfected with an *IL-2-Luc* reporter (20 μg) and either pcDNA3 or pcDNA3-*SOCS-3* (10 μg). 12 hours after transfection, cells were cultured with or without PMA (100nM) and ionomycin (2μM) for 8 hours, after which cells were harvested and analyzed for luciferase activity. Transfections were repeated as above using a 3XNFAT-Luc reporter (B) and a 3XAP-1-Luc reporter (C).

stimulated with αCD3, the pattern of tyrosine phosphorylation seen in whole cell extracts was similar to that observed in cells transfected with empty vector (data not shown), again suggesting that SOCS-3 does not interfere with the activation of tyrosine kinases through the TCR. To determine if the overexpression of SOCS-3 altered NFAT activation in these cells, NFAT activity was investigated directly by electrophoretic mobility shift assay (EMSA).

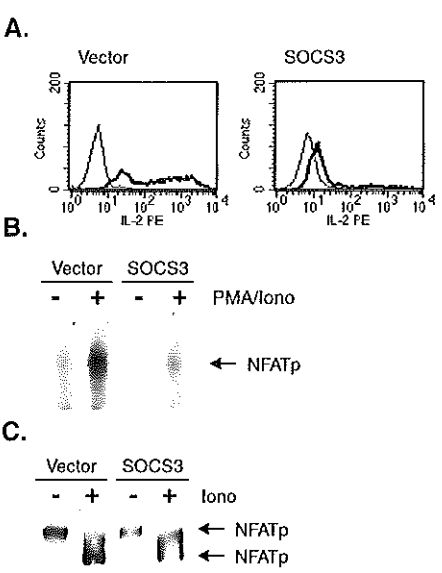

FIGURE 3
SOCS-3 inhibits NFATp activation.
(A) Intracellular cytoplasmic staining for IL-2. Jurkat cells stably transfected with pcDNA3 or pcDNA-*SOCS3*, as indicated, were left unstimulated (thin line) or stimulated for 20 hours with PMA and ionomycin (thick line). After stimulation samples were fixed, permeabilized, and stained with PE conjugated rat anti-murine IL-2 (Pharmingen) for analysis by flow cytometry. (B) Examination of NFAT activation by EMSA. Nuclear extracts were prepared from stably transfected Jurkat cells unstimulated or stimulated for 4 hours with PMA (100 nM) and ionomycin (2 mM). Extracts were assayed for binding to the distal NFAT binding site of the human *IL-2* promoter. (C) Analysis of NFATp dephosphorylation. Stably transfected Jurkat cells were unstimulated or stimulated with ionomycin (2 mM) for 2 minutes. Whole cell extracts were analyzed by immunoblot for NFATp.

NFAT binding to its distal binding site from the *IL-2* promoter in response to four hour culture in the presence of PMA and ionomycin was reduced in nuclear extracts from Jurkat cells expressing SOCS-3 (figure 3B). These data demonstrate that overexpression of SOCS-3 can inhibit NFAT activation and IL-2 production in Jurkat cells.

Possible explanations for the decrease in NFAT activation observed by EMSA include lower total levels of NFAT, reduced nuclear entry of NFAT, or inhibition of NFAT DNA binding activity. Total levels of NFATp in the cells were analyzed by immunoblotting. No significant differences in levels of NFATp were seen between Jurkat cells transfected with *SOCS-3* and those transfected with empty vector (data not shown). Because the nuclear entry of NFAT depends on dephosphorylation of multiple serine/threonine residues in response to sustained increases in intracellular calcium, the dephosphorylation of NFATp induced by a 2 minute stimulation with ionomycin was examined. Dephosphorylation of NFATp leads to a 10-20 kD decrease in its apparent molecular weight by immunoblot (18, 19). NFATp was immunoprecipitated from stimulated or unstimulated stably transfected Jurkat cells and analyzed by immunoblotting for a decrease in apparent molecular weight. Less efficient dephosphorylation of NFATp was observed in Jurkat cells expressing *SOCS-3* when compared with Jurkat cells transfected with empty vector (figure 3C). This result suggests that SOCS-3 interferes with the activation of NFATp by inhibiting its dephosphorylation.

INTERACTION OF SOCS-3 WITH CALCINEURIN

NFAT dephosphorylation by the cellular phosphatase calcineurin is activated by increases in intracellular calcium concentration (20). Stimulation of the TCR leads to tyrosine phosphorylation of receptor components, the recruitment of molecular scaffolds, and, among other events, the recruitment and activation of phospholipase C (PLC)γ, which in turn leads to increased intracellular calcium levels. Ionomycin bypasses PLCγ, directly increasing the intracellular calcium concentration. The observation that SOCS-3 inhibited NFATp dephosphorylation induced by ionomycin raised the possibility that SOCS-3 could interact with NFATp or calcineurin.

To determine whether SOCS-3 interacts with these molecules, whole cell extracts from 293T cells transfected with calcineurin, NFATp, or empty vector were incubated with agarose beads coupled to glutathione S-transferase (GST) or a GST-SOCS-3 fusion protein. Although there is a slight interaction between calcineurin and beads coupled to GST, a much stronger interaction was observed between calcineurin and beads coupled to GST-SOCS-3 (figure 4A). The association of calcineurin with beads coupled to GST-SOCS-3 was also much stronger than that seen between NFATp and beads coupled to GST-SOCS-3 (figure 4A). The reverse interaction of SOCS-3 with calcineurin was tested by transfecting 293T cells with combinations of SOCS-3, calcineurin, and empty vector. SOCS-3 co-immunoprecipitated with calcineurin only in cells which had been transfected with both (figure 4B).

The ability of SOCS-3 to associate with calcineurin led to the question whether SOCS-3 affects the phosphatase activity of calcineurin. Calcineurin phosphatase activity

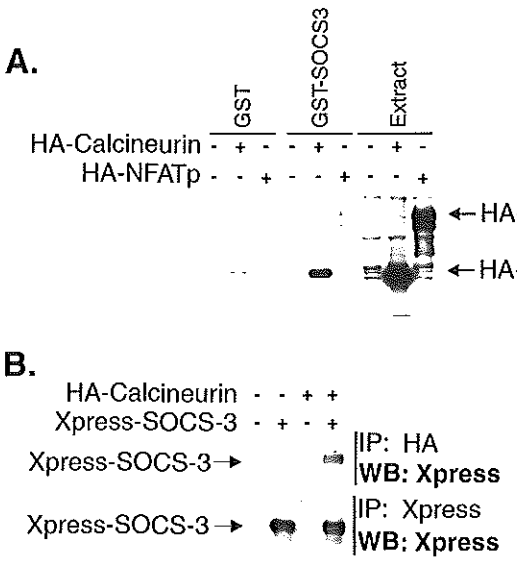

FIGURE 4
Interaction of SOCS-3 and Calcineurin.
(A) Agarose beads coupled to GST or GST-SOCS-3 were incubated with extracts from 293T cells untransfected or transfected with HA tagged Calcineurin or HA tagged NFATp. Proteins associated with the beads were analyzed by immunoblot for HA. Extract lanes represent 10% of total input. (B) 293T cells were untransfected or transfected with HA tagged calcineurin, Xpress tagged *SOCS-3*, or both, as indicated. Whole cell extracts were immunoprecipitated with antisera to HA (top panel) or Xpress (bottom panel), and immunoblotted for Xpress.

towards an *in vitro* labelled RIIK peptide was similar in extracts from Jurkat cells stably transfected with SOCS-3 and extracts from Jurkat cells transfected with empty vector (data not shown). Further phosphatase assays utilizing 293T cell extracts demonstrated that recombinant GST-SOCS-3 does not inhibit the dephosphorylation of the RIIK peptide by calcineurin (data not shown). These experiments demonstrate that SOCS-3 does not affect the phosphatase activity of calcineurin towards all substrates.

SOCS-3 INHIBITS IL-2 PRODUCTION IN PRIMARY CD4$^+$ T CELLS

As the initial observation of *SOCS-3* mRNA modulation by αCD3 was made in primary murine T cells, the ability of SOCS-3 to alter NFAT dependent responses in these cells was examined. CD4$^+$ T cells isolated from lymph nodes of C57BL/6 mice were infected with retroviruses encoding either *SOCS-3* and green fluorescent protein (*GFP*) or *GFP* alone. Three days after infection, the cells were stimulated for 4 hours with PMA and ionomycin, and analyzed for IL-2 production by intracellular cytoplasmic staining. While there was a

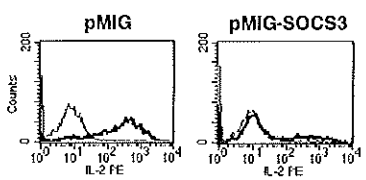

FIGURE 5
Intracellular Cytoplasmic Staining for IL-2 in primary T cells.
CD4$^+$ T cells were isolated from lymph nodes of C57BL/6 mice and stimulated with plate bound αCD3 and αCD28. 24 and 48 hours after stimulation, cells were transduced with either pMIG or pMIG-SOCS-3 retroviruses. Five days after stimulation, cells were stimulated with PMA (100 nM) and ionomycin (2 μM) for 4 hours. Subsequently, stimulated (*thick lines*) and unstimulated (*thin lines*) cells were stained with CyChrome conjugated αCD4, fixed and stained for intracellular IL-2 as in figure 2A.
Cells expressing both CD4 and GFP by flow cytometry were analyzed for intracellular IL-2 content.

marked activation of IL-2 synthesis in cells expressing *GFP* alone, this activation was greatly reduced in cells also expressing *SOCS-3* (figure 5). The failure of T cells expressing *SOCS-3* to synthesize IL-2 resembles that seen in T cells from transgenic mice expressing a dominant negative NFAT (21).

DISCUSSION

The data presented demonstrate a role for SOCS-3 in the regulation of TCR mediated signal transduction. Expression of *SOCS-3* suppresses the activation of the *IL-2* promoter in activated T cells. Further analysis revealed the ability of SOCS-3 to suppress transcription driven by the NFAT/AP-1 element of the *IL-2* promoter with no effect on transcription driven by AP-1 alone. SOCS-3 is shown to interact with calcineurin and to inhibit activation of NFATp by preventing its calcium dependent dephosphorylation.

The preponderance of studies on the *SOCS* gene family have addressed their role in cytokine signal transduction, although other functions are beginning to emerge. CIS and SOCS-3 have so far been implicated in TCR mediated signaling (13). In the case of cytokine signaling, both CIS and SOCS-3, in addition to SOCS-1, are negative regulators of JAK-STAT signaling (12). In contrast, CIS and SOCS-3 have opposing effects on TCR mediated signals, with CIS being a positive regulator associated with PKCΘ and SOCS-3 being a negative regulator associated with calcineurin. As the SH-2 domain and SOCS box motif are well conserved among the SOCS family (22), differences in function between family members, such as that seen between CIS and SOCS-3 in T cells, may help to elucidate the role of other regions of the proteins which are not conserved within the family.

The inability of SOCS-3 to block calcineurin phosphatase activity towards an RIIK peptide substrate leaves open the question of the mechanism of SOCS-3 in the inhibition of NFATp activation. One possibility is that SOCS-3 alters the interaction between NFATp and calcineurin. It is also possible that SOCS-3 simultaneously associates with calcineurin and a serine/threonine kinase, bringing a kinase capable of phosphorylating NFATp to the NFATp/calcineurin complex. In support of this possibility, SOCS-3 has been shown to interact with the Pim family of serine/threonine kinases (B. Vuong and P. Rothman, unpublished data), and Pim family kinases can phosphorylate NFAT *in vitro* (23).

Clinically, the activation of NFAT can be blocked by the drugs Cyclosporin A or FK506, which act in conjunction with immunophilin receptors to bind to and inhibit the phosphatase activity of calcineurin (24). Side effects of these drugs have generated interest in obtaining more specific inhibitors of NFAT activation. Synthetic peptides based on the calcineurin binding site of NFAT are interesting candidates and have already been shown to block NFAT activation in cell lines (25, 26). The experiments above demonstrate that SOCS-3 inhibits

the activation of NFATp without grossly effecting calcineurin phosphatase activity, suggesting that synthetic peptides based on sequences within SOCS-3 may also be of interest in this regard.

Increases in *SOCS-3* mRNA levels in T cells activated through the TCR were also observed, suggesting the involvement of SOCS-3 in a classical feedback loop of TCR mediated signal transduction. A recent report showing the ability of SOCS-3 to inhibit IL-2 signaling in lymphocytes adds an interesting dimension to the loop, as IL-2 cooperates with TCR mediated signals to stimulate T cell proliferation (27). SOCS-3 may have a dual role in regulating T cell responses, inhibiting the activation of both NFAT by calcineurin and JAK1 by the IL-2 receptor.

ACKNOWLEDGEMENTS

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Chapter 7

General discussion

THE ROLE OF GATA-3 IN CELL-FATE DECISIONS DURING THYMIC T CELL DIFFERENTIATION

7.1. INTRODUCTION

The transcription factor GATA-3 plays a key regulatory role at two points in T cell differentiation. GATA-3 has been shown to be indispensable for development of the T cell lineage, and plays an essential role in the differentiation into Th2 cells. Targeted deletion of the *GATA-3* gene results in early embryonic lethality and is associated with neural abnormalities as well as anemia and abdominal hemorrhages (1). *Rag2*$^{-/-}$ complementation analysis revealed contribution of *GATA-3*$^{-/-}$ embryonic stem (ES) cells to all hematopoietic lineages except the T lymphocyte lineage (2). The thymocytes present in the *GATA-3*$^{-/-}$/*Rag2*$^{-/-}$ chimeras had the *Rag2*$^{-/-}$ genotype, as determined by Southern blotting analysis, and were as a consequence blocked at the CD25^{+}CD44^{-} double negative (DN) stage of T cell differentiation. Therefore, it was concluded that development of *GATA-3*$^{-/-}$ T lymphocytes was blocked at or before the DN progenitor stage (2). In addition, targeting *GATA-3* mRNA by an antisense approach in fetal thymic organ cultures (FTOC) inhibited generation of thymocytes from fetal liver progenitors but not from fetal thymic cells (3). Taken together, these data indicate a crucial role for GATA-3 in early T cell development, but it is unclear in which developmental stage(s) GATA-3 is essential. Furthermore, the level of *GATA-3* gene expression in the subsequent stages of T cell development is unknown. The only data available are from a Northern blot analysis of various T cell lines, which revealed expression of *GATA-3* in cell lines derived from fetal prothymocytes (CD25^{-}CD44^{+}) and in thymic T cell lines but not in cell lines derived from fetal prethymocytes (CD25^{+}CD44$^{+/-}$) (4).

In order to identify the stages of T cell development that are dependent on *GATA-3* expression *in vivo*, we generated ES cells in which the *GATA-3* locus was targeted by an in-frame insertion of a *lacZ* reporter gene, containing a nuclear localization signal (nls). Using these ES cells, we generated chimeric mice and subsequently obtained germline transmission of the *lacZ* reporter. In heterozygous *GATA-3*$^{+/nlslacZ}$ animals we analyzed the proportion of *lacZ*-expressing cells at the various stages of T cell development and differentiation. Flowcytometric measurement of lacZ activity in developing *GATA-3*$^{+/nlslacZ}$ T cells provided a powerful means of determining the levels of *GATA-3* genetrabscription. In fact, it has been shown that observed patterns of *lacZ* expression in neural tissues correlated to a high degree with previously characterized patterns of *GATA-3* gene transcription (5). In addition, *GATA-3*-deficient ES cells (*GATA-3*$^{neo/nlslacZ}$) were generated next to the *GATA-3*$^{+/nlslacZ}$ cells. The presence of the *lacZ* reporter gene in these two types of ES cells allowed us to specifically trace the fate of individual *GATA-3*$^{neo/nlslacZ}$ and *GATA-3*$^{+/nlslacZ}$ ES cell-derived T cell progenitors in the thymus of ES cell chimeric mice.

7.2. GATA-3 IN EARLY THYMIC T CELL DIFFERENTIATION

To investigate the contribution of *GATA-3*$^{+/nlslacZ}$ and *GATA-3*$^{neo/nlslacZ}$ ES cells to early T cell progenitors, we generated chimeric mice by injection of ES cells of both genotypes into wild-type blastocysts. In this *in vivo* competition analysis we confirmed the contribution of *GATA-3*$^{+/nlslacZ}$ ES cells to the T cell lineage from the earliest T cell progenitors onwards. In contrast, no contribution to the T cell lineage was observed when *GATA-3*$^{neo/nlslacZ}$ ES cells were injected. Therefore, we show that in the absence of *GATA-3*, T cell development is blocked from the earliest thymic (CD25$^-$CD44$^+$ DN) stage onwards. Using *GATA-3*$^{-/-}$ ES cells in *Rag-2*$^{-/-}$ complementation studies, we observed a contribution of *GATA-3*$^{-/-}$ ES cells to the B cell lineage but not to the T cell lineage (A.K. and R.W.H, unpublished observations) confirming previously published results (2). The finding, that *GATA-3*$^{-/-}$ ES cells do not contribute to even the earliest detectable thymic progenitors implies that *GATA-3*$^{-/-}$ progenitors either do not reach the thymus or die upon arrival within the CD25$^-$CD44$^+$ stage. As we could trace our ES cell-derived progenitors by the expression of *lacZ* activity, we could define the developmental block of *GATA-3*$^{-/-}$ T cell precursors more accurately than in the *Rag*-complementation studies (2). From these latter experiments, it was only clear that the developmental block of *GATA-3*$^{-/-}$ cells was at or before the DN stage, as chimerism was not determined within the 4 subpopulations of the DN fraction (2).

Detailed analysis of *lacZ* expression during T cell differentiation in the thymus revealed a complex pattern of *GATA-3* gene transcription (figure 1). *GATA-3* gene expression is low during phases of *TCR* gene rearrangement (CD25$^+$CD44$^-$ DN and DP cells). Expression of *GATA-3* was strongly induced after β-selection (transition from CD25$^+$CD44$^-$ to CD25$^-$CD44$^-$ DN stage). In addition, upon successful completion of *TCRα* gene rearrangement at the DP stage of T cell development and the initiation of positive selection, *GATA-3* was strongly induced to reach a maximal expression level at the CD4$^+$CD8^{lo} CD69$^+$ stage, at which the T cells make the CD4/CD8 lineage commitment decision.

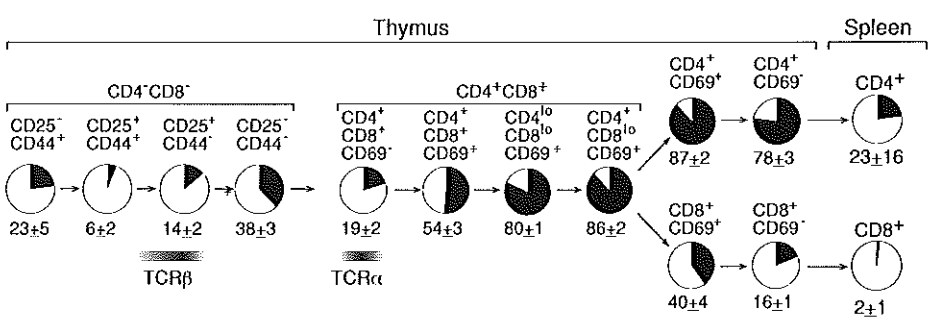

FIGURE 1

Levels of *GATA-3* transcription during T cell differentiation.

GATA-3 gene expression levels at various stages during T cell differentiation, represented as the fraction of *lacZ*$^+$ cells in the *GATA-3*$^{nsllacZ/+}$ thymus. Phases during which *TCR* gene rearrangements occur are indicated.

At the $CD25^{+}CD44^{-}$ DN and the $CD69^{-}$ DP stages T lymphocytes are small, resting cells rearranging their *TCRβ* and *TCRα* gene loci, respectively. The low levels of *GATA-3* expression detected at these stages of T cell differentiation correlated to cell size, indicating that the residual *GATA-3*-expressing cells at these maturational stages might already have completed *TCR* gene rearrangement and have initiated cellular proliferation. We therefore conclude that *GATA-3* transcription seems to be inhibited during phases of *TCR* gene rearrangement. The observed pattern of *GATA-3* transcription during early T cell development might be compatible with two alternative, but not necessarily mutually exclusive, potential roles for GATA-3 in T cell differentiation. First of all, GATA-3 might be involved in *TCR* enhancer-dependent gene transcription of *TCRβ* and *TCRα* genes. An incompatibility of the processes of *TCR* gene rearrangement and enhancer-dependent *TCR* gene transcription could then explain the observed regulation of *GATA-3* gene transcription. Alternatively, GATA-3 might be involved in the induction of cellular proliferation immediately after the successful rearrangement of *TCR* genes. The incompatibility of *TCR* gene rearrangement with the process of cellular proliferation could then explain the strict regulation of *GATA-3* gene transcription (6).

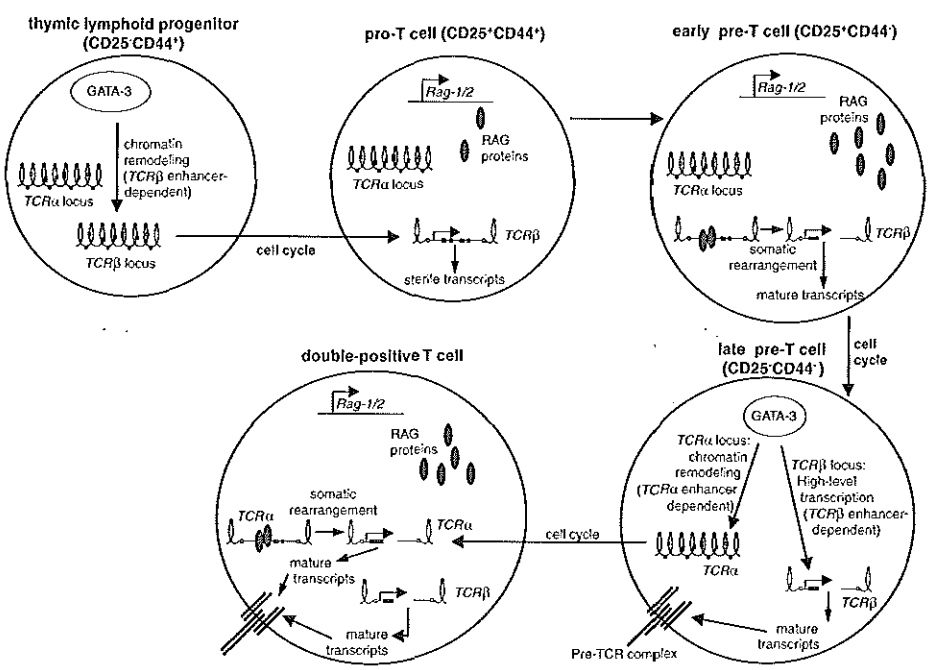

FIGURE 2
Model for GATA-3 in early T cell differentiation.
We hypothesize a role for GATA-3 both in enhancer-dependent chromatin remodeling at the *TCRβ* and *TCRα* loci prior to the induction of gene rearrangement, and in enhancer-dependent high-level transcription after gene rearrangement of the *TCR* loci. See text for details.

The presence of GATA-3 binding sites has been described in the *TCRα*, *TCRβ* and *TCRδ* enhancers. Binding of GATA-3 to the *TCRα* and *TCRβ* enhancer sites induces *TCRα* and *TCRβ* enhancer activity (7-11). Targeted deletion of the *TCRβ* enhancer results in a partial block of T cell development at the pre-T cell stage (12). The regulation of chromatin remodeling at the *TCRβ* locus by the *TCRβ* enhancer was shown to be critical for T cell differentiation (13). T cell development in mice homozygous for a targeted deletion of the *TCRα* enhancer (*Eα*) region is arrested at the DP stage, associated with a lack of *TCRα* gene rearrangement. Expression of germline or mature (rearranged) *TCRα* transcripts is dependent on *Eα* (14). Therefore, we assume a dual function for the *TCR* gene enhancers during T cell development. Firstly, the *TCR* enhancers regulate chromatin accessibility at the *TCR* loci prior to the induction of *TCR* gene rearrangement, thereby facilitating the interaction of RAG proteins with the chromatin. Secondly, the *TCR* gene enhancers regulate high-level transcription from the *TCR* loci after successful completion of the rearrangement process.

It has been shown, that the chromatin structure at the *TCRβ* locus at the $CD25^+CD44^+$ pro-T cell stage is accessible for transcription factors, as reflected by the production of germline transcripts (15, 16). As expression of the *Rag* genes and *TCRγ* gene rearrangements can already be detected at this stage, it can be assumed that Rag proteins are present and functional (17-19). The low level of Rag proteins at this stage and a putative difference in affinity for Rag proteins between the *TCRβ* and *TCRγ* recombination signal sequences could then explain why *TCRβ* gene rearrangement only occurs at the $CD25^+CD44^-$ early pre-T cell stage.

Upon successful rearrangement of the *TCR* genes, high-level transcription of the newly rearranged loci will be induced. This is also regulated by the *TCR* enhancers. The differential regulation of chromatin structure at the *TCR* loci during phases of V(D)J recombination versus phases of high-level transcription might reflect a different context of transcription factors at the *TCR* enhancer. Binding of GATA-3 could play a role in either function of the *TCR* enhancer (figure 2). The observed enhanced levels of TCRαβ expressed at the cell surface of *CD2-GATA-3* DP T cell immediately after the successful rearrangement of the *TCRα* gene (DP $CD69^+$) would indicate a role for GATA-3 in *TCR* enhancer-dependent high-level transcription of the *TCR* genes. Based on our observations, we cannot draw conclusions about a role for GATA-3 in *TCR* enhancer-dependent chromatin remodeling at the *TCR* loci prior to phases of V(D)J recombination. In this context, the function of GATA-3 in early T cell development may well parallel its function in the regulation of the *IL-4/IL-5/IL-13* locus, where GATA-3 acts by inducing chromatin remodeling during cellular proliferation (20, 21). The observed levels of *GATA-3* transcription would indicate that *TCR* enhancer-dependent transcription of the *TCR* genes is absent during *TCR* gene rearrangement. Residual activity of the proximal promotor of the *TCR* genes might still result in low-level transcription of the locus during these stages, resulting in the production of germline transcripts.

The alternative explanation for the observed stringent regulation of *GATA-3* gene transcription during phases of *TCR* gene rearrangement hypothesizes a role for GATA-3 in the induction of cellular proliferation after the successful completion of *TCR* gene rearrange-

ment. This hypothesis seems reasonable, as other GATA-3 family members have been implicated in cell-cycle regulation as well. GATA-1 was shown to affect the regulation of cell cycle in a study, in which overexpression of GATA-1 dysregulated terminal erythroid differentiation associated with an enhanced cellular proliferation, implicating GATA-1 in the regulation of proliferation versus differentiation (22). Also, *GATA-1*$^{-/-}$ megakaryocytes are retarded in their differentiation and display enhanced proliferation (23). Using *GATA-2*$^{-/-}$ ES cells and *GATA-2*$^{-/-}$ yolk sack-derived hematopoietic progenitor cells, it was shown that GATA-2 is indispensible for the expansion of multipotent progenitors, but is not required for erythroid or myeloid terminal differentiation (24). In addition, activity of estrogen-inducible GATA-2 has been shown to promote proliferation and block terminal differentiation in chicken primary erythroblasts (25). Together, these data implicate a role for GATA-factors in cell fate decisions by the induction of proliferation at the expense of potential differentiative developmental steps.

CD2-GATA-3 transgenic mice developed T cell lymphomas from 3 months of age onwards. The T cell lymphomas consisted of lymphoblasts of a DP, CD4^{+}CD8^{lo} or CD4 SP phenotype. Regardless the coreceptor expression profile, the T cell lymphomas expressed high levels of CD3ε on the cell surface, as well as high levels of GATA-3. These T cell lymphomas metastasized to secondary lymphoid organs (spleen, lymph nodes) and non-lymphoid organs such as the liver and kidneys. The observed lymphoma induction at the DP CD69^{+} lymphoblastoid stage of T cell development also argues for a role for GATA-3 in the induction of cell proliferation upon the completion of *TCRα* gene rearrangement. The oncogenic event might be a result of aberrant use of the V(D)J recombination machinery, of complex formation of GATA-3 with the TAL-1 and LMO transcription factors, which are associated with human acute lymphoblastic leukemias (26), or of an increased basal transcription of the *RAD50* gene, resulting in destabilization of the MRE11-RAD50-NBS1 protein complex, which is essential for chromosome stability (27). Future cytogenetic studies of the lymphoma cells in the *CD2-GATA-3* transgenic mice should demonstrate the presence of translocations involving the *TCR* loci or the *TAL-1* gene.

7.3. GATA-3 IN CD4 VERSUS CD8 LINEAGE COMMITMENT

After the initiation of positive selection at the DP stage, T cells undergo lineage commitment and differentiate into CD4^{+} SP or CD8^{+} SP T cells. We show that CD4^{+} SP T cells remain a high expression level of *GATA-3*, whereas CD8^{+} SP T cells markedly downregulate *GATA-3* expression levels during SP maturation. A more detailed analysis revealed that transcription of *GATA-3* is strongly induced directly after the initiation of positive selection (CD69^{+} DP cells). The highest expression of *GATA-3* was observed at the CD4^{+}CD8^{lo}CD69^{+} stage of T cell development. This is the stage at which the CD4/CD8 lineage commitment decision is made by differentiating T cells, which will continue to downregulate CD8 expression and develop into CD4 SP cells, or which will undergo coreceptor reversal induced by IL-7R-mediated signals and develop into CD8 SP cells (28). As we show a progressive downregulation of GATA-3 transcription during CD8 SP maturation from this point onward, it can

be assumed that switching off of *GATA-3* transcription is part of the genetic program of CD8 differentiation.

It seems unlikely that GATA-3 directly affects the lineage commitment decision, as overall numbers of thymic CD4 and CD8 SP populations are comparable between *CD2-GATA-3* transgenic animals and littermate controls. Rather, the final maturation of CD8 SP T cells is disrupted in *CD2-GATA-3* transgenic animals, leading to low numbers of peripheral CD8$^+$ T cells. Using the *GATA-3$^{+/nlslacZ}$* mice, we show a progressive decline of *GATA-3* transcription during CD8 SP maturation. Therefore, it can be hypothesized that the downmodulation of *GATA-3* expression is essential for the acquisition or implementation of the genetic program associated with final CD8 maturation. The enforced expression of *GATA-3* during CD8$^+$ SP T cell maturation in the *CD2-GATA-3* transgenic animals might then interfere with this genetic program, ultimately resulting in the induction of programmed cell death due to a failure to downmodulate GATA-3 levels.

In this context, there is a striking parallel with Th1/Th2 differentiation, where *GATA-3* is expressed in naïve peripheral T cells, followed by a substantial increase during Th2 development and a gradual downregulation during Th1 development (29, 30). The acquisition of the Th2 phenotype is initiated by IL-4-dependent Stat6 activation, but becomes independent of extrinsic factors, such as IL-4 over time, as a result of GATA-3 autoregulation (21, 31-33). It was recently shown that GATA-3 generates stability of Th2 commitment by chromatin remodeling of Th2-specific cytokine loci, associated with a positive autoactivation pathway, which is a recognized mechanism contributing to cell fate determination (21). Concomitantly, GATA-3 inhibits Th1 development by repressing IL-12Rβ expression and, as a result, IL-12 induced IFNγ production (34). Assuming a parallel role for GATA-3 in CD4/CD8 and Th1/Th2 development, we propose that GATA-3 is involved in the stabilization of the distinct gene expression profiles in committed CD4 cells, while for the full maturation of CD8 T cells, *GATA-3* expression needs to be downregulated.

REGULATION OF HELPER T CELL DIFFERENTIATION INTO TH1 AND TH2 PHENOTYPES

7.4. INTRODUCTION

On the basis of a large number of studies using a variety of approaches, a generalized model for Th cell differentiation has emerged (figures 10, 11 of the general introduction; reviewed in 35-38). In order to enter the genetic program leading to Th cell differentiation, the resting naïve Th cell needs to receive concomitant activating and differentiation-inducing signals, derived from the TCR and from cytokine receptors, respectively (39). Upon activation, naïve Th cells will enter the cell cycle (39). Full differentiation into Th1 or Th2 effector cells requires several cell divisions (35). Cytokine-mediated signals resulting in the activation of Stat factors are essential for the induction of Th cell differentiation (40-45).

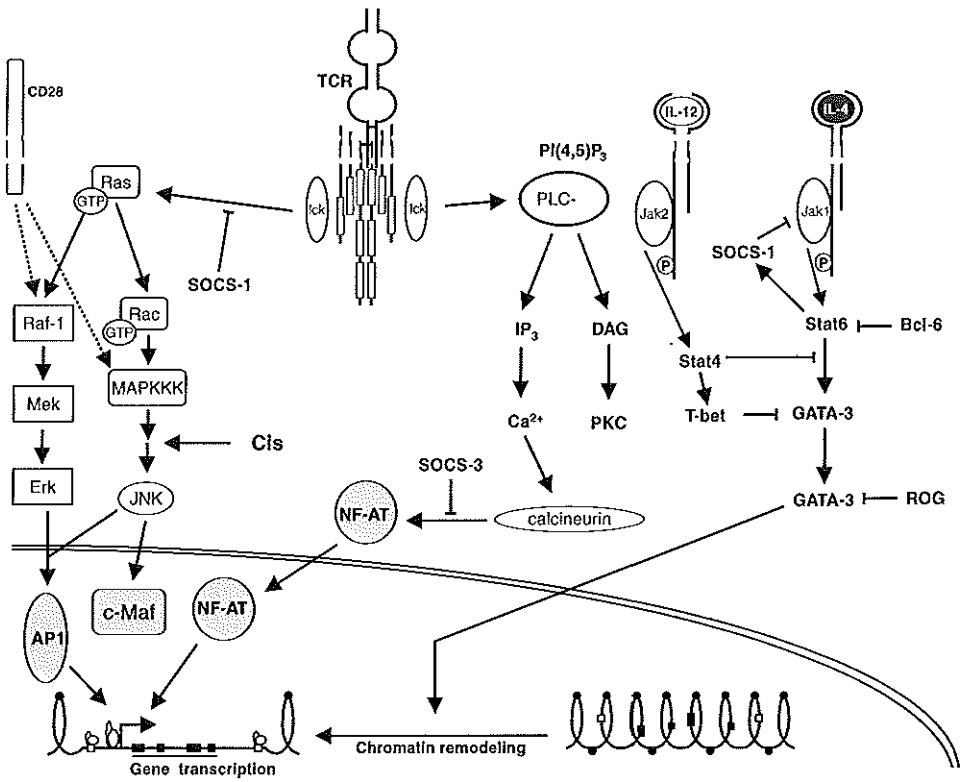

FIGURE 3
Regulation of Th2 differentiation.
Regulation of the induction of Th2 differentiation occurs at various levels of the cascade of events leading to the acquisition of an Th2 phenotype. Regulation of this process of cellular differentiation is implemented at multiple levels. See text for details.

IL-12-dependent Stat4 activation induces the differentiation into the Th1 phenotype (40-42, 46-49). IL-4-dependent Stat6 activation induces differentiation towards the Th2 phenotype (43-45, 50-53). Activated Stat factors differentially induce the Th1-specific transcription factor T-bet or the Th2-specific transcription factor GATA-3, which are thought be the key regulators of Th cell differentiation of several genes (35, 36, 38, 54, 55). These transcription factors induce chromatin remodeling, regulating the accessibility of the cytokine loci, which is essential for high-level expression of these genes. In addition, these transcription factors can act directly on the proximal promotor regions (20, 21, 33, 56). Eventually, expression of individual cytokine genes is dependent on the accessibility of the locus as well as the presence of both cell-type specific and activation-inducible transcription factors (35, 36, 38).

Regulation of Th1 versus Th2 differentiation is exerted at multiple levels. This is schematically depicted in figure 3 for Th2 differentiation. First of all, expression of cytokine receptors determines the responsiveness of the Th cell to differentiation-inducing signals in

the micro-environment. Secondly, regulation of cytokine-induced signal transduction regulates the sensitivity of the cell for these differentiation-inducing signals. Thirdly, the induction and cross-regulation of the critical transcription factors T-bet and GATA-3 determine the outcome of the differentiation process. Fourthly, regulation at the induction of the target genes of these transcription factors provides a final level of regulation.

7.5. CYTOKINE-DEPENDENT REGULATION OF Th SUBSET ACTIVITY IN SJA/9 MICE

We investigated cytokine-mediated regulation of T cell differentiation in great detail in the SJA/9 strain of mice, which is characterized by a specific Th2 defect. The Th cells from this mouse strain fails to produce sufficient IL-4 during the immune response, resulting in the observed IgE deficiency. The IL-5-dependent eosinophilia is normal in this strain, indicating a partially functional Th2 compartment. We show that the $CD4^+$ T cells of this strain acquire an alternative effector phenotype during the immune response. SJA/9 $CD4^+$ T cells acquire an IL-10-producing phenotype during *in vivo* responses to anti-IgD and TNP-KLH precipitated on alum, responses which are usually associated with strong Th2 differentiation. The observed IL-10 production, however, is not mechanistic in the deviated differentiation of the SJA/9 $CD4^+$ T cells, as neutralization of IL-10 did not restore the IgE response. Therefore, SJA/9 Th cells have a deficiency in their capacity to differentiate into IL-4-producing cells. The IgE responses of SJA/9 mice can be restored by adoptive transfer of (congenic) SJL thymocytes, indicating that the defective IL-4 production is caused by a T-cell intrinsic defect (57). The defect in SJA/9 Th cells does not reside in IL-4 receptor signaling, as IL-4-dependent induction of Stat6 activation and cellular proliferation were comparable between SJL and SJA/9 $CD4^+$ T cells. Also, the SJA/9 IL-4 protein is fully bioactive as compared to SJL IL-4 protein. We therefore hypothesize an altered transcriptional regulation of the *IL-4*-gene in SJA/9 $CD4^+$ T cells.

7.6. ROLE OF SOCS-3 IN T CELL ACTIVATION

Th2 development is also regulated at the level of IL-4R-mediated signal transduction, for instance by phosphotyrosine phosphatases, such as SHP-1,2 and SHIP, and the adapter molecule SOCS-1 (58, 59). This effect of SOCS-1 on Jak-1-mediated Stat6 activation could be due to shielding of the critical phosphotyrosine in Jak-1, or alternatively, to targeting Jak-1 for proteosomal breakdown. We went on to investigate the role of the SOCS family member SOCS-3 in T cell activation and differentiation. We show that *SOCS-3* mRNA levels in peripheral T cells are regulated by TCR-mediated T cell activation. Interestingly, when introduced into a Jurkatt T cell line, SOCS-3 inhibited TCR-dependent NF-ATp activation, by direct interaction with the serine-phosphatase calcineurin. In this manner, SOCS-3 prevented the calcium-dependent dephosphorylation, and subsequent nuclear translocation and induction of gene transcription, of NF-ATp. As SOCS-3 directly affects TCR-mediated signal transduction, it seems unlikely that SOCS-3 plays a role in the regulation of cytokine receptor-mediated signal transduction. Rather, as *SOCS-3* transcription is induced by TCR-mediated signal transduction, SOCS-3 seems to be implicated in a classical negative feed-

back loop of TCR-dependent T cell activation.

Interestingly, a positive regulatory role in T cell activation has been described for the family member CIS (60). *CIS* transcription was shown to be induced by TCR-mediated T cell activation. Enforced expression of *CIS* in $CD4^+$ T cells resulted in enhanced proliferative responses, increased cytokine production and prolonged survival of Th cells in response to CD3 cross-linking. CIS was shown to bind PKC, resulting in enhanced MAP kinase and JNK activity (60). In addition, SOCS-1 was shown to suppress CD3ζ and Syk-mediated NF-AT activation in the 293T kidney cell line (61).

Additional evidence for a role of SOCS-1 in TCR-mediated signal tranduction was derived from *SOCS-1*$^{-/-}$ mice. Targeted deletion of SOCS-1 was shown to induce the presence of a population of large blastoid DP thymocytes at day 4, whereas the DP population had disappeared by day 10. At both timepoints, relatively large SP populations were present, indicating a possible enhanced selection of DP T cells, which could be the result of deregulated TCR-dependent NF-AT activation (62).

Together, these data implicate SOCS family members in the regulation of TCR-mediated T cell activation, in addition to their initially characterized role in the regulation of cytokine receptor signal transduction. As both GATA-3 and T-bet are induced by both TCR-mediated and cytokine receptor-mediated signals, the SOCS family members might be involved in the regulation of Th cell differentiation (figure 3). Additional experiments would be needed to further analyze the functional role of SOCS family members in Th cell differentiation.

7.7. ROLE OF GATA-3 IN PERIPHERAL T CELL DIFFERENTIATION

Using a cDNA representational difference analysis (RDA) between Th1 and Th2 cells, *GATA-3* was identified as a gene preferentially expressed in Th2 cells (30). *GATA-3* expression was shown to be downregulated during Th1 differentiation, whereas it is induced during Th2 differentiation (29, 30). The role of GATA-3 in Th cell differentiation has been analyzed using a variety of *in vitro* assays. Forced expression of *GATA-3* under control of the *CD4* promotor induced Th2 cytokine gene expression in $CD4^+$ T cells after *in vitro* Th1 differentiation (30). In addition, retrovirally introduced *GATA-3* inhibited IFNγ production and induced IL-4 and IL-5 production during Th1 differentiation of naïve Th cells (63). Ectopic expression of *GATA-3* by retroviral transfection of Th1 cells after polarization and of Th1 clones resulted in Th2 gene expression and chromatin remodeling at Th2 gene loci (20). So, in these *in vitro* assays GATA-3 seems to induce Th2 differentiation and to repress Th1 differentiation.

Very few data are available on the role of GATA-3 in Th cell differentiation during *in vivo* responses. In one study, an attempt was made to analyze the effect of transgenic expression *in vivo* of the KRR dominant-negative form of GATA-3 on the Th2-mediated allergic response (64). The authors show a marked reduction of eosinophils in the broncho-alveolar lavage, an inhibition of OVA-specific IgE in serum and an inhibited mucus production in the lungs of KRR mice. However, analysis of cytokine production by isolated $CD4^+$ T cells

revealed an inhibition of the production of both Th1 cytokines (IFNγ) and Th2 cytokines (IL-4, IL-5, IL-13), indicating that the Th cells in the KRR mice might have a more generalized defect in proliferation, differentiation or cytokine production. Furthermore, the study lacks the control of the expression of wild-type *GATA-3* in a similar transgenic fashion (64). Therefore, this study only revealed limited information about the role of GATA-3 during *in vivo* immune responses.

In a more recent study, the KRR mutation of GATA-3 was more thoroughly analyzed (65). The authors show that the KRR mutation heavily disrupts the acetylation of GATA-3, resulting in a strongly decreased transcriptional activity of wild-type GATA-3 in the presence of the KRR-mutated GATA-3. Forced expression of *KRR-GATA-3* under transcriptional control of the *Lck* distal promotor did not appear to affect thymic T cell differentiation. Peripheral T cell populations displayed an altered distribution over the secondary lymphoid organs, resulting in enhanced numbers of $CD4^+$ T cells in the spleen and in the circulation, whereas $CD8^+$ T cell numbers were strongly reduced in the lymph nodes and in the circulation, indicating a complex effect of enforced expression of *KRR-GATA-3* on peripheral T cell homing. Finally, forced expression of *KRR-GATA-3* was shown to prolong T cell survival and IL-2Rα expression as well as IL-2-induced proliferation (65).

We investigated the role of GATA-3 during peripheral Th cell differentiation *in vivo*. Using the *GATA-3*$^{+/nlslacZ}$ chimeric mice, we characterized the regulation of *GATA-3* gene transcription. During Th1 differentiation, *GATA-3* transcription was downmodulated, whereas *GATA-3* transcription during Th2 differentiation was enhanced. We analyzed the functional role of GATA-3 during *in vivo* Th cell-mediated immune responses using the *CD2-GATA-3* transgenic animals. Our findings on the role of GATA-3 *in vivo* support the *in vitro* observations suggesting that *GATA-3* expression inhibits Th1 development (34, 63). First of all, the IFNγ-dependent KLH-specific IgG2a production was strongly decreased in *CD2-GATA-3* transgenic mice. In addition, the Th1-induced DTH response to KLH in *CD2-GATA-3* transgenic mice was significantly decreased. These results indicate a decreased induction of the Th1-dependent local inflammation. This could be the result of decreased Th1 cell extravasation at the site of KLH challenge and a decreased production of cytokines (IFNγ, TNFβ/LT, IL-3, GM-CSF) and chemokines (MIF, MCF) by the activated Th1 cells. In addition, enforced expression of *GATA-3* resulted in a reduced Th cell survival, proliferation and IFNγ production of cells cultured *in vitro* under Th1-inducing conditions. The observed increased number of T1/ST2-expressing $CD4^+$ T cells and elevated total IgG1 serum levels suggest that the presence of the *CD2-GATA-3* gene drives differentiating Th cells preferentially towards the Th2 pathway. However, the Th2-dependent total serum IgE levels or eosinophilic bronchoalveolar inflammation in the mouse model of allergic asthma were not significantly different between *CD2-GATA-3* transgenic mice and wild-type littermates. These data indicate that the putative increased differentiation of *CD2-GATA-3* $CD4^+$ T cells into Th2 cells did not result in enhanced Th2-dependent immune responses *in vivo*.

It is difficult to estimate the exact levels of GATA-3 protein present in the *CD2-GATA-3* Th cells. First of all, GATA-3 protein levels might be regulated by post-tran-

scriptional mechanisms. It has been shown that caspase-mediated cleavage of GATA-1 protein plays a functional role in the negative regulation of erythroid development (66). GATA-2, however, was insensitive to CD95L-induced caspase activity in erythroid precursors. Substitution of a critical aspartate residue at the major caspase cleavage site (EDLD at position 125) of GATA-1 rendered a caspase-insensitive form of the protein (66). It is unknown whether GATA-3 is also subject to caspase-mediated cleavage. Secondly, several post-translational modifications might affect the functional activity of GATA-3. GATA-1 and GATA-3 have been shown to be subject to acetylation by CBP/p300 at multiple well-conserved lysine residues positioned in between and C-terminally adjacent to the Zinc fingers (65, 67, 68). This post-translational modification of GATA-3 has been reported to increase its activity (65, 67, 68). Also, GATA-1, GATA-2 and GATA-4 have been shown to be subject to phosphorylation (69-71). Phosphorylation of GATA proteins has been shown to increase affinity for DNA and functional activity of the GATA proteins. Phosphorylation of GATA-3 has not been reported yet.

Finally, a transcriptional repressor, repressor of GATA (ROG), has been reported to negatively regulate transcriptional activity of GATA-3 by a direct, physical interaction. ROG is lymphoid-specific and is induced in T cells by TCR-mediated activation. A differential TCR-dependent induction of ROG between polarized Th1 and Th2 cells was shown (72). Therefore, it could be possible that in *CD2-GATA-3* $CD4^+$ T cells significantly higher levels of ROG are induced upon activation than in wild-type Th cells, thereby partially antagonizing the effect of the enforced expression of *GATA-3*. A more detailed analysis of GATA-3 protein levels and the degree of post-translational modification of the GATA-3 protein would allow for a more accurate interpretation of the phenotype of the *CD2-GATA-3* transgenic mouse model.

Two possible mechanisms by which the enforced expression of *GATA-3* affects Th cell differentiation need to be considered (figure 4). Firstly, GATA-3 might directly influence the cell-fate decision of Th cell differentiation, resulting in enhanced numbers of Th2-differentiating cells and reduced numbers of Th1-differentiating cells. Also, *in vitro* analysis of the effect of the introduction of GATA-3 into differentiating Th cells by several groups would argue for this role of GATA-3 (20, 21). Alternatively, the enforced expression of *GATA-3* might have no effect on the differentiation decision of Th cells, but might interfere with the execution of the genetic program associated with the Th1 phenotype. This might then result in Th1 cells which are compromised in their viability, in their proliferation capacity and/or in their effector functions. We observed increased cell death and decreased proliferation of *CD2-GATA-3* $CD4^+$ T cells cultured under Th1 conditions. Either way, the net result would be an increased number of Th2 cells in the periphery, consistent with the observed increase in T1/ST2-expressing cells in *CD2-GATA-3* transgenic mice and the increased total serum levels of the IL-4-dependent IgG1.

The increased T1/ST2 expression might also be caused by a direct effect of GATA-3 on T1/ST2 gene expression, resulting in a non-Th2-specific T1/ST2 expression. The identification of three GATA elements in the minimal T1/ST2 promoter in mast cells (73) would sup-

port this hypothesis of a direct regulation of T1/ST2 expression by GATA-3, independent of the acquisition of a Th2 phenotype.

The absence of an enhanced Th2-dependent response *in vivo* might also be attributed to the altered kinetics of the GATA-3 expression in *CD2-GATA-3* Th cells. As we did not detect enhanced production levels of IL-4 by *CD2-GATA-3* Th cells, whereas we did observe strongly enhanced IL-10 production, it might be hypothesized that the increased expression of GATA-3 early in the process of Th cell differentiation alters the cell-fate decision of these cells. In addition to the observed suppression of Th1 differentiation, the *CD2-GATA-3* Th cells might acquire an IL-10-producing phenotype, resulting in suppression of Th2 differentiation and activity.

7.8. GATA-3 IN MEMORY T CELL DEVELOPMENT

Our results indicate that enforced expression of *GATA-3* resulted in an enhanced memory Th cell formation. In *CD2-GATA-3* transgenic mice relatively high numbers of peripheral T cells displayed a memory cell surface profile ($CD44^{hi}CD45RB^{lo}$ and $CD25^-$, $CD69^-$). In addition to a phenotypical characterization, we also performed *in vitro* recall responses. T cells from *CD2-GATA-3* mice exhibited a significantly increased recall response to KLH. In *CD2-GATA-3* transgenic mice the expression of the Th2-specific T1/ST2 marker within the $CD44^{hi}$ Th cell population in spleen and lymph nodes was strongly increased. In addition, serum levels of total IgG1 were significantly increased. Therefore, we conclude that *CD2-GATA-3* mice have a $CD4^+$ memory T cell compartment with strong Th2 characteristics. Our *in vitro* data (increased proliferation in Th2 cultures and decreased proliferation in Th1 cultures) support of this notion.

Two alternative explanations must be considered (figure 4). Firstly, GATA-3 might facilitate the differentiation process of dividing effector cells to memory cells. GATA-3 would then regulate the cell-fate decision of activated $CD4^+$ T cells, possibly by reducing activation-induced cell death in favor of (Th2) memory cell formation. In this model, GATA-3 does not simply act as a survival factor supporting cell proliferation, because survival alone does not appear to be sufficient for memory cell formation, as was shown by the absence of increased

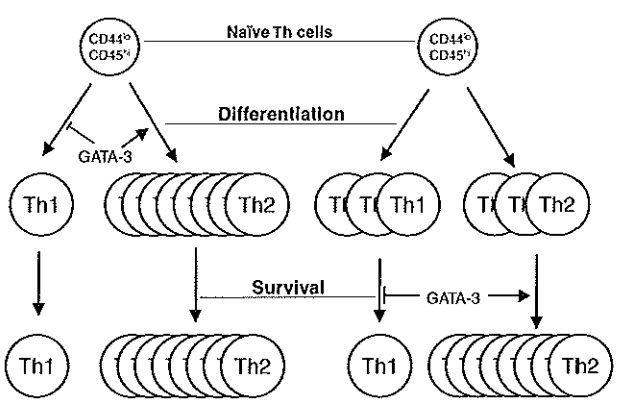

FIGURE 4
The role of GATA-3 on Th cell differentiation.
Enforced expression of *GATA-3* affects the differentiation of Th1 cells, either by influencing the developmental decision of the naive Th cell in favor of the Th2 pathway, or by affecting the survival of Th1-committed cells. See text for details.

memory formation in *Bcl-2* transgenic mice (74).

Alternatively, GATA-3 might be responsible for a higher level of cell division of activated T cells, leading to a higher number of Th2 effector cells. An increased survival and acquisition of a memory phenotype by these effector cells could then be caused by the cytokines present in the local microenvironment in the *CD2-GATA-3* transgenic mice. In the case of so-called homeostatic proliferation, sustained cell division of naïve T cells has been shown to be accompanied by a phenotypical and functional acquisition of a memory phenotype (75, 76). Other GATA family members have also been shown to induce proliferation at the expense of terminal differentiation, and we have hypothesized this role for GATA-3 in early T cell development and SP T cell maturation in the thymus as well.

7.9. CONCLUDING REMARKS AND FUTURE DIRECTIONS

In conclusion, our data implicate GATA-3 in nearly all major fate decisions during T cell development (figure 5). The lack of even the earliest $CD44^+CD25^-$ DN T cell precursor subpopulation in the absence of GATA-3 shows that this factor is essential for development of the T cell lineage. It remains to be elucidated whether GATA-3 is essential for commitment of pluripotent precursors to the T cell lineage. Our findings in transgenic mice with enforced expression of *GATA-3* under the control of the *CD2* locus control region implicate GATA-3 in CD4 versus CD8 lineage development, in Th2 versus Th1 differentiation and in memory T cell development. The mechanism by which GATA-3 regulates cell fate decisions during T cell differentiation may be different between subsequent developmental stages. At the com-

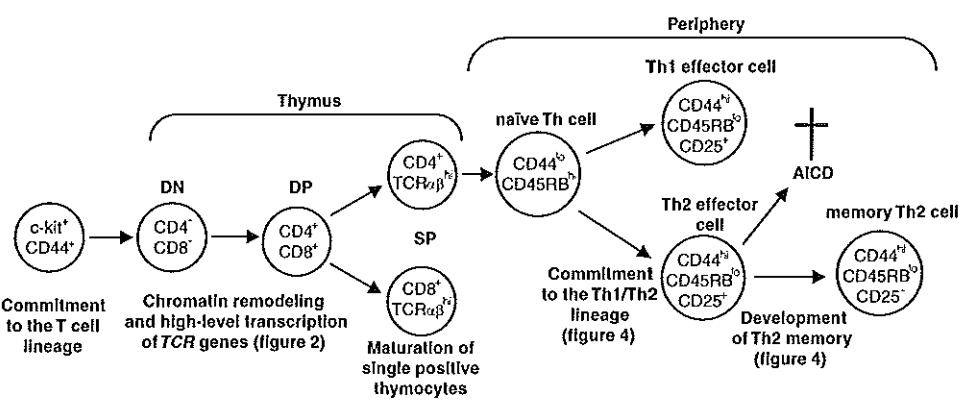

FIGURE 5.

Effects of GATA-3 on T cell development and differentiation.

The stages of T cell differentiation at which we show or hypothesize GATA-3 to regulate T cell differentiation are indicated. The hypothesized role of GATA-3 in early T cell differentiation in the thymus is discussed in detail in figure 2. After the initiation of positive selection at the $TCR\alpha\beta^{lo}$ DP stage of T cell differentiation, GATA-3 seems to be implicated in the high-level expression of $TCR\alpha\beta$. At the $CD4^+CD8^{+/lo}$ $TRC\alpha\beta^{hi}CD69^+$ stage of T cell differentiation, GATA-3 regulates the process of lineage commitment by affecting CD8 SP maturation. At the differentiation of naive Th cells into Th1 or Th2 cells, GATA-3 positively influences Th2 development (see also figure 4). Finally, differentiation of memory Th cells is enhanced by GATA3. AICD = activation-induced cell death. See text for details.

mitment to the T cell lineage and the induction of memory formation, GATA-3-mediated regulation of cell fate decisions may well involve the induction of proliferation. How induction of proliferation would guide the differential induction of one out of two potential cell fates remains to be elucidated. It is a possibility that the induction of proliferation is mechanistic in effecting a cell-fate decision. It has been observed, for instance, that the initiation of *IL-4* gene transcription in naïve Th cells requires at least three cell divisions, whereas *IFN*γ gene transcription can already be detected after one cell division (77). In this sense, the induction of proliferation could be favorable for the induction of Th2 differentiation. In addition, the induction of GATA-3 during Th2 differentiation has also been correlated to changes in chromatin structure at the Th2 cytokine locus (33), a process which is also strictly dependent on entry of cell cycle. The alternative explanation would be, that the induction of cellular proliferation by GATA-3 is a parallel effect of GATA-3 activity and independent of GATA-3-regulated cell fate decisions. The mechanism by which GATA-3 induces cell fate decisions might then involve the regulation of chromatin accessibility at relevant genetic loci. This has been shown to occur at the Th2 cytokine locus by the introduction of GATA-3 into naïve and Th1-polarized $CD4^+$ T cells. Also, in early T cell development, GATA-3 might act in this manner on *TCR* gene loci, regulating accessibility of these loci for RAG proteins.

In case of the Th1/Th2 fate decision, it has been shown that GATA-3 can support the differentiation of one cell lineage while inhibiting the formation of the other (34). In this context, GATA-3 parallels the transcription factor pax-5, which was recently shown to play an essential role in inducing B-lineage commitment by suppressing alternative lineage choices (78). Interestingly, pax-5 and GATA-3 have been implicated in respectively the B- and T-lymphocyte-specific transcription of *Rag2* (79, 80). *Rag* expression is essential for somatic rearrangement of the antigen receptor genes, and induction of *Rag* expression might be the hallmark of commitment to B- and T-lymphocyte lineages. In this sense, GATA-3 might be the equivalent factor in determining T lymphocyte lineage commitment as pax-5 has been shown to be for B lymphocyte development (81, 82). A more detailed analysis of GATA-3 target genes and an analysis of the effect of ectopic *GATA-3* expression on commitment to other hematopoietic lineages are needed to substantiate this potential function of GATA-3 in the T cell lineage. In addition, generation of knockout mice with conditional or inducible mutations in the *GATA-3* gene could be very informative to further study the role of GATA-3 in T cell differentiation.

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Summary

Samenvatting

SUMMARY

T cell development is a tightly regulated process of cellular differentiation. The unique thymic microenvironment facilitates efficient development of T cells in an ordered sequence of alternating differentiative and proliferative phases. Thymocytes which have successfully rearranged their *TCRα* and *TCRβ* genes commit to either the CD4 (helper T cell) or the CD8 (cytotoxic T cell) lineage. Mature T cells exit the thymus and circulate as naïve T cells in the periphery. Upon activation, naïve T cells will differentiate into one of the various effector phenotypes, such as the Th1, Th2 and Tr-1 phenotypes, which are characterized by production of IFNγ, IL-4 and IL-10, respectively. Eventually, activated T cells differentiate into memory cells or enter apoptosis.

This thesis is focussed around the question how cell fate decisions during differentiation of T lymphocytes are regulated. This issue is addressed using several approaches. Firstly, the presence and functional role of the transcription factor GATA-3 is analyzed throughout thymic and peripheral T cell differentiation. Secondly, the regulation of peripheral T cell differentiation by cytokines and adapter proteins which act on the cytokine signaling pathway is studied.

The transcription factor GATA-3 is essential for development of the T cell lineage and for T cell differentiation into the Th2 phenotype. We wanted to analyze the role of GATA-3 in T cell differentiation *in vivo*. Targeted insertion of a *lacZ* reporter gene into the *GATA-3* locus allowed us to analyze *GATA-3* gene transcription levels during T cell differentiation in the mouse. These analyses revealed a modulated pattern with strict down-regulation at phases of *TCR* gene rearrangement, insulated by proliferative phases in which *GATA-3* transcription was higher. In addition, *GATA-3* gene expression was progressively downregulated during CD8 single positive maturation, whereas most CD4 cells maintained *GATA-3* expression. In peripheral T cells, a significant proportion of $CD4^+$ T cells expressed *GATA-3*, whereas *GATA-3* expression was absent in $CD8^+$ T cells.

We have also generated chimeric mice, by injecting *lacZ*-expressing embryonic stem cells into wild-type blastocysts, and followed these cells during T cell development. In this *in vivo* competition assay, *GATA-3*-deficient embryonic stem cells did not contribute to even the earliest detectable progenitor stage of the T cell lineage, whereas *GATA-3*$^{+/-}$ ES cells did.

Subsequently, we modified *GATA-3* gene transcription levels troughout T cell differentiation using transgenic mice, in which the *GATA-3* gene was placed under transcriptional control of the CD2 locus control region. We observed no effect of the modified *GATA-3* gene transcription levels *in vivo* on *TCRβ* and *TCRα* gene rearrangement. Nevertheless, enhanced levels of TCRαβ membrane expression were observed after the initiation of positive selection. Maturation of CD8 single positive thymocytes was impaired and associated with a markedly increased induction of apoptosis. As a result, peripheral $CD8^+$ T cell numbers were strongly decreased in *CD2-GATA-3* transgenic mice.

Within 9 months, ~50% of the *CD2-GATA-3* animals developed thymic lymphomas, mainly of a $CD4^+CD8^+$ lymphoblastoid phenotype. In each case, the lymphoma cells dis-

played a single *TCRβ* gene rearrangement, indicating that the oncogenic event had taken place in one T cell precursor. Metastases of these monoclonal lymphomas were observed in the spleen, lymph nodes, kidney and liver.

In the *CD2-GATA-3* transgenic mice, the numbers of peripheral $CD4^+$ T cells were within normal ranges, but an increased proportion of these cells was positive for the Th2-specific marker T1/ST2. Th1-mediated responses *in vivo* were significantly reduced. Analysis of *in vitro* Th1-polarized transgenic $CD4^+$ T cells revealed a decreased IFNγ production, an increased IL-5 production and an increased induction of apoptosis. *In vitro* Th2-polarized transgenic $CD4^+$ T cells produced similar amounts of IL-4 and increased amounts of IL-5 and IL-10, when compared to wild-type $CD4^+$ T cells. In addition, peripheral $CD4^+$ T cells in *CD2-GATA-3* transgenic animals displayed an increased size and function of the memory subset.

Taken together, these findings show that GATA-3 regulates several important cell-fate decisions during T cell differentiation. First of all, GATA-3 is indispensable for development of the T cell lineage from the earliest thymic progenitors onwards. During T cell differentiation in the thymus, GATA-3 enhances the membrane expression of TCRαβ. In addition, enforced expression of *GATA-3* inhibits the maturation of CD8-committed T cells. In the periphery, GATA-3 plays an important role in the negative regulation of Th1 differentiation. The increase of the size and function of the memory compartment in *CD2-GATA-3* transgenic mice implicate GATA-3 in the induction of Th memory development. We propose a model in which GATA-3 regulates T cell fate decisions at the CD4/CD8 and the Th1/Th2 commitment, as well as in memory cell development versus the induction of activation-induced cell death (AICD). In this model, GATA-3 acts by positively regulating one differentiation pathway (CD4, Th2 and memory development) whilst negatively regulating its alternative choice (CD8, Th1 and AICD, respectively).

In addition, we analyzed the regulation of peripheral T cell differentiation by cytokine-mediated signals. First we studied the regulation of Th2 differentiation by cytokines in SJA/9 mice. The SJA/9 strain of mice has a T cell-intrinsic defect which results in low IgE responses. We show that SJA/9 $CD4^+$ T cells do not acquire an IL-4-producing phenotype during *in vivo* Th2 immune responses. Instead, SJA/9 Th cells produce large quantities of IL-10 when restimulated *in vitro*, indicating a possible deviation of the SJA/9 Th cell differentiation into a Tr-1 phenotype. *In vivo* neutralization of IL-10 did not rescue the IgE response. The defect in SJA/9 Th cells could not be attributed to a defect in IL-4-induced activation of Stat6. We therefore hypothesize that the Th2 defect in SJA/9 mice is due to an altered transcriptional regulation of the *IL-4* gene.

Secondly, we studied the role of the SOCS-3 adapter protein in the regulation of Th cell differentiation. Members of the SOCS family of adapter proteins have been implicated in the negative regulation of cytokine-mediated signal transduction, indicating a regulatory role for SOCS family members in Th1/Th2 differentiation. We analyzed the effect of SOCS-3 on T cell activation and differentiation in Jurkat T cells. We show, however, that SOCS-3 is involved in negative regulation of TCR-mediated activation of the transcription factor

NF-AT by a direct interaction with calcineurin. As SOCS-3 transcription is induced by TCR-mediated T cell activation, we conclude that SOCS-3 is involved in a classical negative feedback loop on T cell activation, with probably equal effects on Th1 and Th2 cells.

In summary, our findings implicate GATA-3 as a key regulator of cell fate decisions during T cell differentiation, by supporting commitment to the T cell lineage, differentiation into CD4 T cells, Th2 cells and memory T cells. We also show, that the cell-fate decision in Th/Th2 differentiation is not directly regulated by SOCS-3 or the genetic markers implicated in the Th cell intrinsic defect of SJA/9 mice.

SAMENVATTING VOOR LEKEN

INLEIDING

Het immuunsysteem is belangrijk voor de bescherming van ons lichaam tegen infecties door ziekteverwekkers zoals bacteriën, virussen, schimmels en parasieten. Delen van deze ziekteverwekkers, de zogenaamde antigenen, worden herkend door de cellen van het immuunsysteem. Het immuunsysteem wordt gevormd door vele verschillende soorten witte bloedcellen, die allemaal hun eigen functie hebben. T en B cellen hebben de unieke eigenschap dat ze op hun celoppervlak receptoren hebben, die antigenen specifiek kunnen herkennen. Deze antigeen-receptoren kunnen signalen doorgeven naar het inwendige van de cel, met als gevolg, dat de cellen, die de juiste antigenen herkennen, gaan delen en uitrijpen tot effectorcellen. Geactiveerde killer T cellen kunnen virus-geinfecteerde cellen doden. Geactiveerde helper T cellen kunnen signaalstoffen (cytokines) maken die andere witte bloedcellen activeren en die B cellen helpen uit te rijpen. B cellen kunnen uitrijpen tot plasmacellen, die antistoffen maken die in het bloed kunnen circuleren en het antigeen kunnen neutraliseren.

T cellen ontwikkelen in een speciaal nabij het hart gelegen orgaan, de thymus, of wel de zwezerik. Tijdens de uitgroei in de thymus maken de T cellen hun antigeenreceptor, de T celreceptor (TCR). Deze TCR mag geen antigenen herkennen die afkomstig zijn van de eigen lichaamscellen, want dan zouden deze T cellen een afweerreactie tegen het eigen lichaam kunnen aansturen. Daarom worden in de thymus die T cellen geselecteerd, die een TCR hebben die wel functioneel is, maar niet lichaamseigen antigenen herkent. Slechts een klein gedeelte van de T cellen die in de thymus uitgroeien worden uiteindelijk goed genoeg bevonden, en verlaten de thymus als rijpe T cellen.

Infecties door verschillende soorten ziektekiemen worden door het afweersysteem met een verschillende reactie beantwoord. Er bestaan dan ook verschillende typen helper T cellen, die zorg dragen voor het aansturen van deze verschillende soorten afweerreacties. Het meest bekend zijn de type-1 helper T cellen (Th1) en de type-2 helper T cellen (Th2). Th1 cellen zijn belangrijk voor de afweer tegen ziekteverwekkers die zich in cellen van het lichaam huisvesten. Th2 cellen zijn van belang voor de afweer tegen ziekteverwekkers die niet de lichaamscellen ingaan, maar zich tussen de cellen in de weefsels bevinden. Bij het eerste contact met een ziekteverwekker zijn de helper T cellen nog niet uitgerijpt tot één van de verschillende typen. Het soort ziekteverwekker bepaalt de uitrijping van de helper T cel tot het juiste type. Het is van groot belang dat het lichaam tegen iedere infectie de juiste soort afweerreactie heeft. Het proces van uitrijping van naive helper T cellen tot Th1 of Th2 cellen noemen we helper T cel differentiatie. Een proces, waarbij een niet-gespecialiseerde cel zich ontwikkelt tot één van meerdere mogelijke gespecialiseerde celtypen, noemen we in het algemeen differentiatie. Zo is de hele ontwikkeling van T cellen in de thymus en in het bloed een opeenvolging van fasen van cellulaire differentiatie en fasen van celgroei.

Cellen die differentiëren, verwerven hierbij nieuwe functies en nieuwe kenmerken, maar

ze verliezen ook bepaalde funkties en kenmerken. Een naive T cel is een rustende cel, die maar weinig cytokines aanmaakt. Na differentiatie tot Th2 cel gaat deze cel een bepaalde set van cytokines maken, die nodig zijn om zijn specialistische functie uit te voeren. Een Th1 cel maakt een andere set cytokines, die voor zijn specifieke functie belangrijk zijn.

De veranderingen, die gepaard gaan met differentiatie van cellen, zijn gebaseerd op het gebruik van een nieuwe set genen. Een gen is een stukje erfelijk materiaal (DNA) waarop de informatie voor de aanmaak van een eiwit is gecodeerd. Het DNA van het gen wordt vertaald in RNA (transcriptie). Dit RNA wordt weer vertaald in het eiwit (translatie). In iedere cel is het DNA aanwezig voor alle menselijke eiwitten. Toch heeft iedere cel slechts een gedeelte van al deze eiwitten nodig. Daarom is er in de cel een strikte regulatie, welke genen mogen worden afgelezen en welke niet. Deze regulatie van de transcriptie van genen wordt verzorgd door de transcriptiefactoren. Dit zijn eiwitten, die bepaalde stukjes DNA herkennen en ervoor zorgen dat een gen wel of juist niet wordt vertaald in RNA en eiwit. Op deze manier reguleren transcriptiefactoren de differentiatie van een cel: in aanwezigheid van bepaalde transcriptiefactoren ontwikkelt de naive helper T cel zich tot Th1 cel, terwijl in de aanwezigheid van andere transcriptiefactoren de naive T cel zich tot Th2 cel ontwikkelt.

Het onderzoek dat in dit proefschrift wordt beschreven geeft meer duidelijkheid over de manier waarop de T cel differentiatie wordt gereguleerd. Hiertoe is er onderzoek gedaan naar de rol van de transcriptiefactor GATA-3 in de T cel ontwikkeling. Bovendien is de rol van cytokines onderzocht in de regulatie van de differentiatie van naive helper T cellen. Tenslotte is er gekeken naar de rol van het eiwit SOCS-3 in helper T cel activatie en differentiatie. SOCS-eiwitten dempen de reactie van cellen op cytokines.

SAMENVATTING VAN HET ONDERZOEK

De transcriptiefactor GATA-3 is essentieel voor de ontwikkeling van T cellen in de thymus en voor de differentiatie van naive helper T (Th) cellen in Th2 cellen. T cel ontwikkeling is te onderscheiden in verschillende stadia op basis van de aanwezigheid van bepaalde eiwitten op het celoppervlak. Zo begint de ontwikkelende T cel als CD4 en CD8 dubbelnegatief, en wordt vervolgens dubbel-positief. Op dit stadium differentieert de T cel tot CD4 enkel positieve helper T cel, of tot CD8 enkel positive killer T cel. Rijpe Th cellen in het bloed differentiëren na activatie tot Th1 of Th2 effector cel. Tenslotte differentieert de geactiveerde T cel zich tot geheugen cel of sterft in een proces dat activatie-geïnduceerde celdood heet.

In de beschreven studie is onderzocht in welke fase van de T cel ontwikkeling de transcriptiefactor GATA-3 nu precies van belang is. Hiertoe is de transcripie van het *GATA-3* gen, dat codeert voor het GATA-3 eiwit, bestudeerd tijdens de T cel ontwikkeling. Hierbij bleek dat de transcriptie van het *GATA-3* gen sterk wisselde tussen de verschillende fases van de T cel ontwikkeling. In fasen waarin de cellen rustend waren, werd er weinig *GATA-3* gen-transcriptie gemeten, terwijl in fasen van celdeling er veel *GATA-3* gen-transcriptie plaatsvondt.

Om te bestuderen voor welke fase van de vroege T cel ontwikkeling GATA-3 essentieel

is, werden GATA-3-deficiënte embryonale stamcellen vergeleken met embryonlae stamcellen die wel GATA-3 hadden. Hierbij bleek, dat GATA-3-deficiënte embryonale stamcellencellen in het geheel niet bijdroegen tot de T cel ontwikkeling vanaf het vroegst detecteerbare ontwikkelingsstadium in de thymus.

Om het belang van de sterk wisselende niveau's van *GATA-3* gen-transcriptie tijdens de T cel ontwikkeling te bestuderen, werden trasngene muizen gemaakt. De cellen van deze transgene muizen bevatten een extra DNA-fragment met daarop het *GATA-3* gen, waarvan de transcriptie op een andere manier gereguleerd wordt dan die van het eigen *GATA-3* gen. Hierdoor was de *GATA-3* gentranscriptie gedurende de hele T celontwikkeling op een hoog niveau. Deze geforceerde *GATA-3* gen-expressie had tot gevolg, dat in dubbel-positieve T cellen meer TCR op de celmembraan werd gedetecteerd. Bovendien was de uitrijping van CD8 enkel-positieve cellen in de thymus ernstig versoord in de *GATA-3* transgene muizen. Ongeveer de helft van de *GATA-3* transgene muizen ontwikkelde een T cel tumor (lymfoom) binnen 9 maanden na de geboorte. Metastasen van de lymfomen werden in de milt, de lymfeklieren, de lever en de nieren aangetroffen. De observatie, dat het manipuleren van de niveau's van *GATA-3* gen-transcriptie, kan leiden tot het ontstaan T cel tumoren, is zeer interessant en behoeft nader onderzoek.

Aangezien GATA-3 ook van groot belang is voor de uitrijping van naive helper T cellen tot Th2 cellen, is het effect van de gemanipuleerde *GATA-3* gentranscriptie op helper T cel differentiatie bestudeerd. Hierbij blijkt de geforceerde expressie van GATA-3 de Th1-afhankelijke afweerreacties te remmen. Analyse van gezuiverde Th cellen toonde aan, dat deze cellen veel van de Th2 cytokines IL-5 en IL-10 maakten, maar slechts weinig van het Th1 cytokine IFNγ. Ook werd aangetoond dat het aantal geheugen T cellen in de *GATA-3* transgene dieren was verhoogd.

Amenvattend tonen we aan, dat GATA-3 betrokken is bij een aantal belangrijke ontwikkelingsstadia van de T cel. Ten eerste is GATA-3 essentieel voor de ontwikkeling van T cellen vanaf het vroegste stadium in de thymus. Vervolgens reguleert GATA-3 de CD4/CD8 ontwikkeling door de maturatie van CD8enkel-positieve T cellen te verminderen. In rijpe Th cellen is GATA-3 van belang voor de Th1/Th2 differentiatie en tenslotte reguleert GATA-3 ontwikkeling van geheugen T cellen. In ons model reguleert GATA-3 deze beslissingen in de T cel differentiatie door de inductie van de ene differentiatie-keuze (CD4/Th2/memory) en de inhibitie van de andere differentiatie-keuze (CD8/Th1/celdood).

Daarnaast is er ook onderzoek verricht naar de regulatie van helper T cel differentiatie door cytokine-gemedieerde signalen. Allereerst is de Th cel differentiatie in de SJA/9 muis onderzocht. Deze muis heeft een defect in de T cel populatie, dat leidt tot een sterk verminderde IgE respons, welke normaliter Th2 afhankelijk is. In de beschreven experimenten wordt aangetoond dat de Th cellen van de SJA/9 muis tijdens een immuunrespons nauwelijks IL-4 aanmaken. Nadere analyse toont echter aan, dat SJA/9 Th cellen op kunstmatige wijze wel tot IL-4 productie kunnen worden aangezet. Na een afweerreactie maken de SJA/9 Th cellen wel grote hoeveelheden van het cytokine IL-10. Neutralizatie van dit IL-10 tijdens de afweerreactie leidt echter niet tot herstel van de IgE respons. SJA/9 Th cellen hebben dus een

verstoorde IL-4 productie tijdens de afweerreactie.

Tenslotte is er onderzoek gedaan naar de rol van SOCS-3 in de Th cel activatie en differentiatie. SOCS-eiwitten kunnen cytokine-signalen sterk dempen, en spelen daardoor mogelijk een rol by Th1/Th2 differentiatie. Na Th cel activatie wordt *SOCS-3* gen-transcriptie geinduceerd. De rol van SOCS-3 in T cel activatie is daarom geanalyseerd door T cellen kunstmatig extra SOCS-3 eiwit te geven. Dit SOCS-3 verhindert de inductie van cytokine gen-transcriptie na TCR-afhankelijke T cel activatie. Er is dus geen rol aangetoond voor SOCS-3 in de Th1/Th2 differentiatie. Daarentegen lijkt SOCS3 een negatieve terugkoppeling te geven op T cel activatie via de TCR.

Samenvattend heeft het onderzoek aangetoond dat GATA-3 een rol speelt in het nemen van belangrijke differentiatie-beslissingen tijdens de T cel ontwikkeling. Dit doet GATA-3 door de ontwikkeling in de ene richting te vergemakkelijken (CD4 enkel-positieve T cel, Th2 cel, geheugen T cel), terwijl de ontwikkeling in de andere richting wordt bemoeilijkt (CD8 enkel-positieve T cel, Th1 cel, celdood). Daarnaast is aangetoond dat een dergelijke regulerende rol niet wordt uitgevoerd door het SOCS-3 eiwit of door de genetische factoren die het defect van de SJA/9 Th cellen veroorzaken.

ABBREVIATIONS

Ag	antigen
AICD	activation-induced cell death
APC	antigen-presenting cell(s)
BCR	B cell receptor
CIS	cytokine-induced SH-2-containing (protein)
CpG	cytosine-guanine (dinucleotides)
DN	double negative
DP	double positive
ds	double-stranded
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
ES	embryonic stem (cell)
FDG	flourescein-di-β-D-galactopyranoside
FISH	fluorescence in situ hybridization
FITC	fluoresceine isothiocyanaat
FSC	forward scatter
FTOC	fetal thymic organ culture
γc	common gamma chain
GAS	IFNγ-activated sequence
GEF	guanosine exchange factor
GM-CSF	granulocyte/macrophage colony stimulating factor
HLH	helix-loop-helix
HMG	high mobility group
HSA	heat-stable antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-4R	interleukin-4 receptor
IRS	insulin-receptor substrate
ITAM	immunoreceptor tyrosine-based activation motif
JAB	Jak-binding protein
Jak	Janus kinase
KLH	Keyhole Limpet hematocyanin
LAT	linker for activation of T cells
LCR	locus control region
LEF	lymphoid enhacer factor
LT	lymphotoxin
mAb	monoclonal antibody

MAP	mitogen-activated protein
MARE	c-Maf responsive element
MHC	major histocompatibility antigens
NF-AT	nuclear factor of activated T cells
NK	natural killer
nls	nuclear localization signal
PBS	phosphate-buffered saline
PE	phyco-erythrine
PGP-1	phagocyte glycoprotein-1
PI_3-K	phosphatidyl-inositol 3-kinase
PIP_3	phosphatidyl-inositol 3,4,5-tri-phosphate
PKC	protein-kinase C
PLC-γ1	phospholipase C-γ1
PSGL-1	P-selectin glycoprotein ligand-1
PTB	phosphotyrosine binding
PTK	phosphotyrosine kinase
PTP	phosphotyrosine phosphatase
Rag	recombination-activating genes
RDA	representational difference analysis
RE	responsive element
RE	responsive element
RIBP	Rlk- and Itk-interacting protein
RSS	recombination signal sequence
SH-2	Src-homology-2
SHIP	SH-2 containing inositol-5 phosphatase
SHP	SH-2 containing phosphatase
SOCS	suppressor of cytokine signaling
SP	single positive
SSC	sideward scatter
SSI	Stat-induced Stat-inhibitor
Stat	signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCF	T cell factor
TCR	T cell receptor
TGF	transforming growth factor
Th	helper T (cell)
Th1/2	type-1/2 helper T (cell)
TLP	thymic lymphoid precursor
TNF	tumor necrosis factor
TNP	tri-nitro phenol
Tr-1	regulatory T cell-1

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