Table 1 | List of primers for the T7 antisense RNA amplification and single-cell analysis

Primer name	Sequence (5′→3′)
T7polyT	TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGAGATTTTTTTTTTTTTTTTTTT
T7Cα30	TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGAGAGTCCAGCACAGTTTTG
T7Cβ30	TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGAGACCTGGCCAAGCACACGAG
AV2-1st	TGACAGTCTGGGAAGGAG
AV2-2nd	AAAAGGAAGATGGACGAT
AV2-3rd	ATAAAAGGGAGAAAAAGC
CA-1st	ATGAACGTTCCAGATT
CA-2nd	CACATTGATTTGGGAGTC
CA-3rd	GTCGGTGAACAGGCAGAG
CB-1st	GGTAGCCTTTTGTTTGT
CB-2nd	TCTCTGCTTTTGATGG
CB-3rd	GGCTCAAACAAGGAGACCTTG

Table 2 | List of primers for two-step nested PCR

Primer name		FW primer sequence (5′→3′)	RV primer Sequence (5′→3′)
IFN-γ	1st	CTGTGATTGCGGGGTTGTAT	ATATGCAATTAGACAGTGAT
IFN-γ	2nd	CCAAGCGGCTGACTGAACTC	GAGCGAGTTATTTGTCATTC
IL-4	1st	CACGGATGCGACAAAAAT	TGCATGATGCTCTTTAGG
IL-4	2nd	AAACGTCCTCACAGCAAC	GTGATGTGGACTTGGACT
IL-17	1st	TTGTTTTTCTGTGGACTTTA	TTTTTAAAACAGAGTAGG
IL-17	2nd	AGACAAAATTCAAGACTCAG	TCACCCCATTCAGAGGAGAG
IL-17F	1st	GACTCTCAGTAAGGTGTGC	GCTTCTTCCTTGCCAGTCCAG
IL-17F	2nd	GAACTTTCTGGCTTGCTTTAG	ATTCTGATTATGTTTGTTTTC
IL-10	1st	TGTTTAAGCTGTTTCCATTG	GCTACAAAGGCAGACAAACA
IL-10	2nd	TATATGATGGGAGGGGTTCT	GGGATGACAGTAGGGGAACC
Foxp3	1st	CTAGGCCCCAAGCAACAGTG	TCAGGCACACTCCAACACAT
Foxp3	2nd	TCACAGAGGGGCAGGCAACA	CACAGGGACTTTATGCTCAC

parameters were 95 °C for 15 min, followed by 50 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 60 s.

Nested PCR amplification of TCRα chain sequences

Messenger RNA extraction from the mouse organs and cDNA preparation was performed as previously described.[39] The CDR3 sequences of the MLK2 and MLK3 TCRα chains in the mouse organs were determined by two-step semi-nested PCR.[39] In the first-step PCR, the nucleotide sequence of the section of RNA from the pMX-vector sequence upstream of the multiple cloning site to the TCR Cα sequence was amplified with the 5′ Dan-FW primer (5′-CACCGCCCTCAAAGTAGACG-3′) and 3′ CA-RV primer (5′-CACATTGATTTGGGAGTC-3′). In the second-step PCR, the nucleotide sequence of the RNA section from the pMX-vector sequence upstream of the multiple cloning site to the TCR CDR3 sequence of MLK2 or MLK3 was amplified with the 5′ Dan-FW primer (5′-CACCGCCCTCAAAGTAGACG-3′) and 3′-MLK2AV2 CDR3-RV primer (5′-ATCTTGTTTGCATAGCCCCC-3′) or the 3′-MLK3AV2CDR3-RV primer (5′-TTGCTGAAGGAGGAGGTTTC-3′), respectively.

ELISA

IgG anti-DNA antibodies were measured as previously described.[39]

Evaluation of nephritis

Kidney sections were stained with hematoxylin and eosin and periodic acid–Schiff reagents. Glomerular lesions and renal vascular lesions were graded on a scale of 0–3.[40,44,45] Semiquantitative scoring of IgG and C3 deposits from 0 to 3 was performed in 15 cortical glomerular sections after immunofluorescent staining, as described.[30,46] To quantify the number of infiltrating macrophages, cryostat-sectioned kidneys were stained with PE-conjugated anti-F4/80 mAb (Caltag Laboratories).[39] Glomerular lesions were evaluated by counting F4/80-positive cells in 40 random glomeruli per kidney. Perivascular lesions were evaluated by counting F4/80-positive cells in all vessels per section. Interstitial lesions were evaluated by counting F4/80-positive cells in 10 randomly selected high-power fields as described.[29] Proteinuria was measured with Albstics (Siemens, Erlangen, Germany) according to the manufacturer's instructions.

Statistics

For comparisons between two groups, the Student's *t*-test or Mann–Whitney *U*-test was used. For comparisons among three groups, one-way analysis of variance or two-way repeated-measures analysis of variance with Bonferroni's *post-hoc* test was used. The proteinuria and survival data were analyzed using Kaplan–Meier curves and the log-rank test. GraphPad Prism (GraphPad Software, San Diego, CA) was used to analyze the data. Differences were considered significant when $P<0.05$.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENT

Regulatory cell subsets in the control of autoantibody production related to systemic autoimmunity

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ABSTRACT

Dysregulated autoantibody production is responsible for the severe organ damage that occurs in systemic autoimmune diseases. Immune complexes play important roles in the pathogenesis of these diseases as they initiate and maintain the inflammatory cascade, which leads to tissue destruction. Conventional therapy for autoimmune diseases suppresses immunological accelerator in the absence of knowledge of the immunological brake. Application of a physiological regulatory system could be a rational strategy to treat autoimmune diseases. Accumulating evidence has suggested that specialised subsets of B cells and T cells could control autoantibody production. A significant decrease and impaired function of regulatory B cells (Breg) was recently reported in patients with systemic lupus erythematosus and a mice model of lupus. In T cells, follicular regulatory T cells and Qa-1 restricted CD8 regulatory T cells (Treg) were identified as the populations that control follicular T helper cell-mediated antibody production. Moreover, other Treg populations might also be involved in the control of autoantibody production. Elucidating the roles of Breg and Treg in the control of antibody production might lead to the development of a new therapeutic approach to antibody-mediated autoimmune disease.

INTRODUCTION

Autoimmune diseases are fairly common disorders affecting approximately 5% of the population, predominantly women. Conventional treatment of autoimmune diseases is based mainly on the use of immunosuppressive drugs such as corticosteroids and cytotoxic reagents. These treatments reduce mortality and significantly lengthen the life expectations of patients with some diseases. However, the titres of autoantibodies are not abrogated during the course of treatment, and discontinuation of treatment often results in the relapse of disease. In systemic lupus erythematosus (SLE), anti-DNA and anti-U1-RNP antibodies often persist in spite of the immunosuppressive therapy, and the presence of these autoantibodies is associated with a poor prognosis.[1]

Autoantibodies play a pivotal role in triggering the inflammation responsible for organ damage. Immune complex deposition is readily detectable in the glomeruli of SLE. The classic hypothesis of circulating immune complex deposits inside the glomerulus was a matter of debate.[2 3] It was proposed that DNA immune complex forms in situ by binding to nucleosomes from renal cells[4] or mesangial annexin II.[5] Immune complex activates glomerular cells by the ligation of Fc receptor and the nucleic acid component of immune complex activates the renal macrophages and dendritic cells through Toll-like receptors.[3] The pathogenecity of SLE immune complex was proved by the observation that passive transfer of human SLE sera into mice expressing human FcγRIIA and FcγRIIB on neutrophils induces lupus nephritis when the mice additionally lack Mac-1.[6]

A previous study in which a proteasome inhibitor was used to deplete the plasma cell population demonstrated the importance of a continuous supply of autoantibodies for systemic autoimmunity. Bortezomib, a highly selective inhibitor of the 26S proteasome, was recently approved for use in the treatment of relapsed multiple myeloma. The pro-apoptotic effects of bortezomib in multiple myeloma are mainly due to its ability to induce the accumulation of unfolded proteins. In two lupus-prone mouse strains, NZB/W F1 and MRL/*lpr*, treatment with bortezomib depleted the number of plasma cells that are producing antibodies against double-stranded DNA; eliminated autoantibody production; ameliorated glomerulonephritis and prolonged survival. Among five bortezomib-treated mice that displayed proteinuria of greater than 100 mg/dl before treatment, four showed proteinuria of less than 100 mg/dl after treatment. These findings suggest the therapeutic potential of suppressing autoantibody production as a means of preventing organ damage in lupus.[7]

Conventional therapy for autoimmune diseases suppresses immunological accelerator in the absence of knowledge of the immunological brake. Accumulating evidence has recently shown that regulatory B cells (Breg) and regulatory T cells (Treg) control antibody production. Application of a physiological regulatory system could be a rational strategy to treat autoimmune diseases. This article will discuss the Breg and Treg-mediated suppression of autoantibody-mediated autoimmunity (figure 1).

PATHWAYS FOR AUTOANTIBODY PRODUCTION

In the steady state, most naive B cells recirculate through the lymphoid organs. On entry to the lymph nodes and spleen, B cells promptly migrate to the B-cell follicle. When B cells bind to an antigen, they become activated and express the chemokine receptor CCR7, which causes them to migrate to the interface of the T-cell zone and

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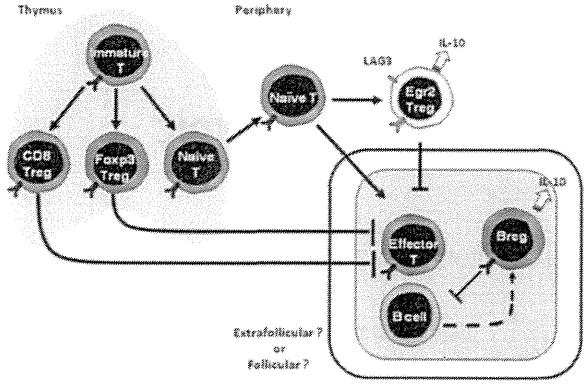

Figure 1 Schematic view of regulatory cell subsets in the control of antibody production. Breg, Foxp3$^+$Treg, CD8$^+$Treg and Egr2$^+$Treg have the capacity to control antibody production. Elucidation of the precise targets and regulatory molecules in these regulatory cell subsets would facilitate the development of a new therapeutic strategy.

B-cell follicle. These activated B cells then differentiate along either the follicular or extrafollicular pathway.[8 9] In the follicular pathway, activated B cells migrate towards the centre of the follicle due to the downregulation of EBI2. These B cells subsequently form germinal centres (GC) and undergo somatic hypermutation and selection by interacting with CD4$^+$CXCR5$^+$PD1$^+$ follicular helper T (T_{FH}) cells. Subsequently, they exit the GC as high affinity long-lived plasma cells or memory B cells. In the extrafollicular pathway, B cells migrate to splenic bridging channels or junction zones and the borders between T-cell zones and the red pulp or extramedullary lymph node cords. This is achieved through the partial downregulation of CXCR5 and CCR7 and the upregulation of CXCR4. These migrating B cells form clusters of short-lived plasmablasts associated with dendritic cells, which aid their survival.[10] Although it is not known what kind of mechanism decides between extrafollicular plasma cell differentiation and GC migration, Paus *et al*[11] reported that antigen recognition strength regulates the choice between extrafollicular plasma cell and GC B-cell differentiation. When B cells expressing a defined B cell receptor (BCR) specificity for hen egg lysozyme were challenged with recombinant mutant hen egg lysozyme proteins engineered to bind this BCR over a 10 000-fold affinity range, B cells reactive against either high affinity or abundant epitopes were skewed towards extrafollicular plasma cell differentiation. Conversely, responding clones with weaker antigen reactivity are primarily directed to GC where they undergo affinity maturation. A number of studies suggested that both the follicular and extrafollicular pathways contribute to antibody production in murine disease models.

RESPONSE OF AUTOANTIBODY TITRES TO B-CELL DEPLETION THERAPY

Dysregulation of the follicular or extrafollicular pathway could lead to systemic autoimmune disease in mice.[12] However, it is hard to evaluate the contributions of the follicular and extrafollicular pathways to the production of disease-associated autoantibodies in humans. It is well known that the serum concentrations of some autoantibodies are correlated with disease activity, while the titres of other autoantibodies remain stable irrespective of disease status. In patients with SLE, the levels of autoantibodies that correlate with disease activity (eg, antibodies to dsDNA and nucleosomes) decrease under effective treatment, whereas those of others (such as anti-Ro and La antibodies) do not. B-cell depletion with the anti-CD20 antibody rituximab helps to distinguish between the antibodies secreted by short-lived and long-lived plasma cells because CD20 is not expressed on plasma cells.[13–15] In lupus patients, the levels of anti-nucleosome and anti-double stranded DNA antibodies are significantly decreased at 6–8 months after the administration of anti-CD20 monoclonal antibody. In contrast, the same treatment does not significantly alter their levels of anti-Ro, Sm, or RNP antibodies, or their total immunoglobulin or protective antibody titres.[14 16] This suggests that anti-nucleosome and anti-double stranded DNA antibodies are produced through extrafollicular responses, which usually generate short-lived plasma cells, while the antibodies to nucleic acid-associated antigens (Ro, Sm and RNP) that remain after rituximab therapy are derived from follicular responses, which generate long-lived plasma cells.

CONTROL OF ANTIBODY-MEDIATED AUTOIMMUNITY BY REGULATORY B CELLS

Although B cells are responsible for positive immune response, a specific and functionally unique subset of B cells, Breg, are able to regulate immune response negatively.[17] B cells are associated with the control of experimental autoimmune encephalomyelitis, chronic colitis, collagen-induced arthritis and non-obese diabetic mouse models. B cells exert their suppression via the production of interleukin (IL)-10. T2-like Breg have been identified in MRL/lpr mice.[18] Watanabe *et al*[19] reported that the transfer of splenic CD1d^{hi}CD5$^+$ B cells from wild-type NZB/W F1 mice into CD19$^{-/-}$ NZB/W F1 mice significantly prolonged their survival. Moreover, CD4$^+$CD25$^+$ Treg were significantly decreased in CD19$^{-/-}$ NZB/W F1 mice, but the transfer of wild-type CD1d^{hi}CD5$^+$ B cells induced expansion of CD4$^+$CD25$^+$ Treg in CD19$^{-/-}$ NZB/W F1 mice. Therefore, Breg may play a crucial role in the induction of Treg. Blair *et al*[20] reported that human CD19$^+$ CD24^{hi}CD38^{hi} B cells exhibit regulatory capacity. After CD40 stimulation, CD19$^+$CD24^{hi}CD38^{hi} B cells suppressed the differentiation of T-helper type 1 cells, partly via IL-10, but not transforming growth factor β (TGFβ). Interestingly, their suppressive capacity was reversed by the addition of CD80 and CD86 monoclonal antibodies. Furthermore, CD19$^+$CD24^{hi}CD38^{hi} SLE B cells isolated from the peripheral blood of SLE patients were refractory to additional CD40 stimulation, produced less IL-10 and lacked the suppressive capacity. This deficit correlated with lower levels of STAT3 phosphorylation. Altered cellular function of this population may affect effector immune responses in SLE and other autoimmune disorders. Despite phenotypic similarities, there are several differences between murine and human Breg. The suppression with murine Breg is primarily IL-10 dependent. On the other hand, although neutralisation of IL-10 in human B-cell and T-cell co-cultures completely restored tumour necrosis factor α production by T cells, IL-10 inhibition alone did not totally restore IFN-γ secretion. Moreover, while mouse Breg exhibit suppressive activity via TGFβ in experimental diabetes, suppressive activity of human CD19$^+$ CD24^{hi}CD38^{hi} B cells is not dependent on TGFβ secretion. Therefore, regulatory machinery other than IL-10 and TGFβ may be operative in human Breg.

Tim-1-mutant mice in which the mucin domain (Tim-1$^{\Delta mucin}$) was deleted showed a profound defect in IL-10 production from Breg.[21] Although Tim-1$^{\Delta mucin}$ mice do not develop frank systemic autoimmune disease, Fas-mutated Tim-1$^{\Delta mucin}$ lpr mice developed accelerated and fulminant systemic autoimmunity

with accumulation of autoantibodies to a number of lupus-associated autoantigens. When these results are taken together, expression of Tim-1 appears to be crucial in maintaining effector functions in IL-10-producing B cells. Breg functions are compromised in the presence of mutant Tim-1 that does not contain the mucin domain, and this compromise is associated with the development of autoimmunity.

Therefore, B cells themselves may control antibody-mediated autoimmunity, although the precise mechanism for suppression remains to be clarified. B-cell depletion with rituximab may affect Breg fraction and this may be one reason for the inefficiency of rituximab in SLE.

TREG SUPPRESSION OF ANTIBODY-MEDIATED AUTOIMMUNITY

Antibody suppression with $CD4^+CD25^+$ $Foxp3^+$ Treg

T cells are the primary sources of the help signals that promote B-cell antibody production. Therefore, suppressing antibody production at the T-cell level could be a rational therapeutic approach. Actually, several T-cell populations are able to suppress B-cell antibody production. Bystry *et al*[22] demonstrated that $CD4^+CD25^+$ Treg inhibit lipopolysaccharide-mediated B-cell proliferation. Although $CD4^+CD25^+$ Treg from human tonsil can effectively suppress T cells, they can also directly suppress B-cell response without the need to first suppress T helper cells. The direct suppression of B-cell Ig production by $CD4^+CD25^+$ Treg is accompanied by the inhibition of Ig class switch recombination.[23] $CD4^+CD25^+$ Treg from C57BL/6 mice kill antigen-presenting B cells in a cell contact-dependent manner.[24] The induction of B-cell death is not mediated by the Fas–Fas ligand pathway, but depends on the upregulation of perforin and granzymes in the $CD4^+CD25^+$ Treg. The $CD4^+CD25^+$ Treg-mediated direct suppression of B cells has also been reported in chronic systemic autoimmunity.[25] $CD4^+CD25^+$ Treg inhibit B-cell antibody production in in-vitro models of murine and human lupus. $CD4^+CD25^+$ Treg utilise granule perforin and granzyme to induce contact-dependent apoptosis in B cells. However, despite the the ability of $CD4^+CD25^+$ Treg from both young and old NZB/W F1 mice to suppress IgG production in B cells, autoantibody accumulation continues in these mice. In lupus-prone MRL/lpr mice with Fas mutation, $CD4^+CD25^+$ Treg and $CD4^+CD25^+CCR2^+$ Treg showed no therapeutic effect against glomerulonephritis and renal vasculitis, although they showed significant suppressive activity in vitro.[26] Moreover, analyses of the function and phenotypic properties of $CD4^+CD25^+$ Treg in SLE patients have led to conflicting results.[27] Therefore, it is not clear whether $CD4^+CD25^+$ Treg could be used to control autoantibody production efficiently in systemic autoimmunity.

It was recently demonstrated that the GC suppressing function of $CD4^+CD25^+$ Treg is limited to a subset of $CD4^+CD25^+$ Treg found within GC that share some characteristic of T_{FH} cells in mice and humans.[28–30] These specialised $CD4^+CD25^+$ Treg are termed as follicular regulatory T (T_{FR}) cells on account of their localisation and function. Acquisition of a $CXCR5^{hi}PD\text{-}1^{hi}$ phenotype depends on interaction with B cells and SAP expression, and CXCR5 expression on Treg depends on Bcl6. $CXCR5^+Bcl6^+$ Treg are not detected in the thymus but can develop from $CXCR5^-Foxp3^+$ natural Treg precursors. T_{FR} cells can be found in GC and coexpress markers characteristic for Treg, GITR and CTLA-4 and high amounts of IL-10 messenger RNA. A deficiency of $CXCR5^+$ Treg results in enhanced GC reactions involving B cells, the affinity maturation of antibodies and plasma cell differentiation. These results indicate that the Bcl6-CXCR5 axis in $CD4^+CD25^+$ Treg is one regulatory mechanism by which GC responses are controlled. In addition, Foxp3-mutated scurfy mice display moderate increases in their T_{FH} populations, but markedly increased numbers of $GL7^+CD95^+$ GC B cells. Therefore, T_{FR} cells are more specialised for controlling the generation of GC B cells. Linterman *et al*[29] also described a population of $Foxp3^+Blimp\text{-}1^+CD4^+$ T cells that accounted for 10–25% of the $CXCR5^{high}PD\text{-}1^{high}CD4^+$ T cells found in immunised GC. In the absence of these T_{FR} cells, they observed the accumulation of non-antigen-specific B cells in GC and a decreased number of antigen-specific B cells, while the production of antigen-specific antibodies was not altered. Therefore, T_{FR} cells control GC reactions by inhibiting the selection of B cells and might suppress GC-mediated autoantibody production. However, the IL-2–STAT5 axis was recently reported to inhibit GC formation by limiting T_{FH} cell differentiation.[31] As $CD4^+CD25^+$ Treg consume IL-2 and cause cytokine deprivation in co-existing T cells,[32] there remains a possibility that T_{FR} cells potentially promote T_{FH} cell differentiation via IL-2 deprivation. Whether T_{FR} cells actually suppress autoantibody production remains to be evaluated and the disease induced by T_{FR} deficiency should be examined.

Lessons from IPEX syndrome

Immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) is a rare X-linked recessive disorder of immune regulation due to overwhelming systemic autoimmunity.[33 34] IPEX is associated with mutations of Foxp3, which is essential for the development and functions of $CD4^+CD25^+$ Treg. Inflammatory enteropathy, insulin-dependent diabetes mellitus, thyroiditis, haemolytic anaemia and thrombocytopenia are frequent. However, SLE has not been diagnosed either in 'classic' IPEX babies nor in the few older children with less severe disease.[34] Notably, the production of anti-DNA antibody is rarely observed in IPEX. Although IPEX patients might develop SLE if they survive long enough, regulatory cell subsets other than $Foxp3^+$ Treg may be associated with the tolerance defect in SLE and other systemic autoimmune disease.

Antibody suppression with CD8 Treg

Recent advances in analyses of the CD8 lineage of Treg (CD8 Treg) have underscored the contribution of Qa-1-restricted CD8 Treg to the inhibition of $Qa\text{-}1^+$ T_{FH} cells. Qa-1 is a murine homologue of human leucocyte antigen E, a non-classic major histocompatibility complex (MHC) class Ib molecule encoded by the H2-T23 gene. Qa-1 binds to two receptors with opposing functions. The binding of Qa-1-peptide complexes to T-cell receptors (TCR) leads to the activation of CD8 T cells, while the binding of the Qa-1-Qdm peptide to the CD94/NKG2A receptor attenuates the activities of CD8 T cells and natural killer cells.[35] Qa-1 presents a dominant set of peptides derived from the signal sequence of classic MHC class I proteins, termed Qa-1 determinant modifiers (Qdm), as well as peptides derived from proteins associated with infectious or inflammatory responses.[36] Qa-1-binding peptides display a conserved motif consisting of a nonamer, whose termini become embedded in the antigen-binding groove, two dominant anchors and additional subdominant anchors. More than a decade ago, a subpopulation of CD8 T cells was reported to suppress T-cell help to B cells,[37] and more recent reports have demonstrated that enhanced responses to proteolipid protein self-peptide are associated with resistance of Qa-1-deficient CD4 T cells to Qa-1-restricted CD8 T suppressor activity and increased

susceptibility to experimental autoimmune encephalomyelitis.[38] Qa-1 is able to interact with both the TCR on CD8 T cells and the CD94/NKG2A receptors expressed by activated CD4 T cells. Analysis of Qa-1-restricted suppression by CD8 T cells in perforin-deficient hosts indicated a primary function for TCR-dependent rather than NKG2-dependent recognition. Moreover, expression of TCRαβ was necessary for suppressive activity, as constraints placed on TCRαβ repertoire expression resulted in loss of CD8-dependent suppressive activity. Because spontaneous autoimmunity is not observed in Qa-1-deficient mice, studies were needed to delineate fully the potential contribution of NKG2-containing receptors to suppressive activity expressed by CD8 T cells.

Kim *et al*[39] reported Qa-1 knock-in mice, B6 Qa-1 (D227K) mice, that have a Qa-1 amino acid mutation that disrupts Qa-1 binding to the TCR/CD8 complex, but the binding of Qa-1 to the inhibitory NKG2A receptor is not affected. Interestingly, B6 Qa-1 (D227K) mice displayed lupus-like systemic autoimmune disease associated with increased T_{FH} cells at approximately 6 months of age. GC formation and antibody production in response to antigen challenge are enhanced in B6 Qa-1 (D227K) mice. These phenotypes are attributed to the defect in the function of CD8 Treg, and surface phenotype analysis of CD8 Treg revealed that they express CD44, ICOSL and CXCR5. Actually, $CD44^+ICOSL^+CD8^+$ T cells have the capacity to inhibit the generation of high-affinity antibodies and $Qa\text{-}1^+$ T_{FH} cells. Moreover, the finding that IL-15 is required for the development and functioning of CD8 Treg facilitated the identification of three surface markers (CD44, CD122 and the class I MHC inhibitory receptor Ly49) that can be used to identify this specialised CD8 T-cell subset.[35] $Ly49^+CD8^+$ T cells efficiently suppressed CD4 T cells from wild-type mice, but not those from Qa-1 D227K mice, indicating that $Ly49^+CD8^+$ T cells display Qa-1-dependent suppressive activity. $CD44^+CD122^+Ly49^+$ CD8 T cells represent 3–5% of all CD8 T cells and account for virtually all Qa-1-restricted suppressive activity by CD8 T cells. On the other hand, the antigen specificity of CD8 Treg in T_{FH} cell suppression is not clarified because the repertoire of peptides presented by Qa-1 is substantially smaller than the repertoire of classic MHC molecules.[36] The dominant Qdm as well as peptides from HSP60, insulin, *Salmonella* GroEL and TCR Vβ chains have been identified as a limited repertoire of Qa-1 binding peptides. Therefore, CD8 Treg might control T_{FH} cells irrespective of the antigen specificity of their TCR. As CD8 Treg express CXCR5 and migrate to lymphoid follicles,[39] CD8 Treg might specifically suppress GC-mediated autoantibody production. Interestingly, the development of lupus-like disease in B6.*Yaa* mutant mice was reported to be associated with a pronounced defect in CD8 Treg activity.[40] B6.*Yaa* mice have increased numbers of T_{FH} and GC B cells at an early age, and CD8 Treg from these mice are unable to suppress WT CD4 T cells in adoptive hosts.

Possibility of antibody suppression with other Treg subsets

T_{FR} and CD8 Treg are regarded as important regulators of antibody production. Nevertheless, no Treg populations have been reported to be able to control autoantibody production and autoimmunity in an antigen-specific manner. A number of reports have described the prominent role of IL-10-producing CD4 type I Treg (Tr1 cells) cells in controlling immune responses. However, the role of Tr1 cells in the regulation of antibody production still remains unclear. In NZB/W F1 mice, high-dose tolerance to SmD_{83-119} (from D1 protein of the Smith small nuclear ribonucleoprotein) delays the production of autoantibodies and the onset of lupus, by inducing $IFN\text{-}\gamma^+$/$IL\text{-}10^+$ Tr1 cells.[41] On the other hand, anti-CD46-induced IL-10-secreting T cells adversely enhance antibody production by B cells.[42] These discrepancies may be due to the definition of Tr1 cells that are mainly characterised by their unique pattern of cytokine.[43 44] Several novel CD4 T-cell populations that exhibit regulatory functions have recently been reported.[45] $CD4^+CD25^-LAP^+$ T cells and $CD4^+NKG2D^+$ T cells secrete both IL-10 and TGFβ,[46 47] and $CD4^+CD25^-IL\text{-}7R^-$ T cells and $CD4^+CD25^-LAG3^+$ T cells produce large amounts of IL-10.[48 49] The capacity of these recently identified Treg populations to suppress autoantibody production should be investigated. In particular, $CD4^+CD25^-LAG3^+$ Treg, which characteristically express the anergy-associated transcription factor Egr2, might be associated with autoantibody suppression, as CD2-driven Egr2-deficient mice displayed lupus-like disease.[50] In addition, polymorphisms in the EGR2 gene are associated with human SLE susceptibility.[51] Interestingly, the adoptive transfer of $CD4^+CD25^-LAG3^+$ Treg from Fas-sufficient MRL/+ mice suppressed the progression of nephritis and autoantibody production in MRL/*lpr* lupus-prone mice (Okamura *et al*., manuscript under preparation). Therefore, $CD4^+CD25^-LAG3^+$ Treg might be useful for treating autoantibody-mediated autoimmune diseases, including SLE.[52] Although both T_{FR} cells and CD8 Treg express CXCR5 and might be associated with the suppression of GC-mediated autoantibody production, the identification of Treg populations that suppress extrafollicular responses is required for a more detailed understanding of autoantibody regulation.

CONCLUSION

The current standard treatment for autoimmune disease is non-specific immunosuppression with steroids and immunosuppressants, which frequently leads to opportunistic infections. As autoantibodies are key components in the development of autoimmune inflammation, targeting autoantibody production is a rational approach to the treatment of autoimmune diseases. Enhancing Breg and Treg functions may be one possible physiological approach to suppress autoantibody-mediated autoimmunity. Further examination of Breg, $CD4^+CD25^+$ Treg, CD8 Treg and other Treg subsets is necessary to aid the development of such treatments.

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Egr-2 transcription factor is required for Blimp-1 mediated IL-10 production in IL-27 stimulated CD4$^+$ T cells

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Summary

Interleukin-27 (IL-27) suppresses immune responses through inhibition of the development of IL-17 producing T-helper (Th) 17 cells and induction of IL-10 production. We previously showed that forced expression of early growth response gene 2 (Egr-2), a transcription factor required for T-cell anergy induction, induces IL-10 and lymphocyte activation gene 3 (LAG-3) expression and confers regulatory activity on $CD4^+$ T cells in vivo. Here we evaluated the role of Egr-2 in IL-27-induced IL-10 production. Among various IL-10-inducing factors, only IL-27 induced high levels of Egr-2 and LAG-3 expression. Intriguingly, IL-27 failed to induce IL-10 in Egr-2-deficient T cells. IL-27-mediated induction of *Prdm1* that codes Blimp-1, a transcriptional regulator important for IL-10 production in $CD4^+$ T cells, was also impaired in the absence of Egr-2. Although IL-27-mediated IL-10 induction was dependent on both STAT1 and STAT3, only STAT3 was required for IL-27-mediated Egr-2 induction. These results suggest that IL-27 signal transduction through Egr-2 and Blimp-1 plays an important role in IL-10 production. Furthermore, Egr-2-deficient $CD4^+$ T cells showed dysregulated production of IFN-γ and IL-17 in response to IL-27 stimulation. Therefore, Egr-2 may play key roles in controlling the balance between regulatory and effector cytokines.

Introduction

Naïve $CD4^+$ T cells play central roles in immune regulation by differentiating into effector as well as regulatory T (Treg)-cell subsets. Recently, a number of Treg-cell subsets, which are important for suppressing effector T cells, tissue inflammation, and autoimmunity, have also been identified. On one hand, $CD4^+CD25^+$ Treg cells, which express the transcription factor Foxp3, have a dominant function in immune suppression and the maintenance of immune homeostasis [1,2]. On the other hand, other Treg cells, which arise in the periphery, such as Treg type I (Tr1) cells and Th3 cells produce the suppressive cytokines IL-10 and transforming growth factor-β1 (TGF-β1), and contribute to the suppression of immune responses in a Foxp3-independent manner [3,4]. IL-10 is an anti-inflammatory cytokine which was initially described as a cytokine associated with Th2 cells that inhibits the production of IFN-γ by Th1 cells [5,6]. A number of reports have revealed that IL-10 suppresses cytokine production and proliferation of T cells [7,8] and inhibits the T cell-stimulating capacity of antigen-presenting cells (APCs) [9]. IL-10-deficient mice die with spontaneously developed inflammatory bowel disease [10].

IL-27, a member of the IL-12/IL-23 heterodimeric family of cytokines produced by APCs, is composed of two chains, p28 and Epstein-Barr virus-induced gene 3 (EBI3) [11]. IL-27 induces the expansion of Th1 cells by activating the signal transducer and activator of transcription (STAT) 1-mediated T-bet pathway [12], but IL-27Rα-deficient mice developed severe experimental autoimmune encephalomyelitis (EAE) with enhanced Th17-cell responses [13]. The immunosuppressive effects of IL-27 depend on inhibition of the development of Th17 cells and induction of IL-10 production [14]. Recently, IL-27 has been identified as a differentiation factor for IL-10-producing Tr1 cells [15-17]. On the other hand, B lymphocyte induced maturation protein-1 (Blimp-1) (coded by *Prdm1* gene), a zinc finger-containing transcriptional regulator that is well known to be a regulator of plasma cell differentiation, is also important for IL-10 production in naïve

$CD4^+$ T cells. Martins et al. [18, 19] reported that Blimp-1-deficient $CD4^+$ T cells proliferated more and produced excess IL-2 and IFN-γ, but reduced IL-10 after T-cell receptor (TCR) stimulation.

Early growth response gene 2 (Egr-2) and Egr-3 have been reported to be transcription factors for TCR-induced negative regulatory program controlling Cbl-b expression [20]. We previously identified a Treg population expressing lymphocyte activation gene 3 (LAG-3) in a fraction of $CD4^+CD25^-CD45RB^{low}$ T cells and showed that forced expression of Egr-2 induces IL-10, LAG-3, and Blimp-1 expressions and confers regulatory activity in vivo on $CD4^+$ T cells [21]. We here describe that IL-27 induces Egr-2 and LAG-3 as well as IL-10 in $CD4^+$ T cells. Moreover, Egr-2-deficient $CD4^+$ T cells exhibited reduced expression of IL-10 and Blimp-1 and reciprocally enhanced secretion of IFN-γ and IL-17 in response to IL-27. Results from a luciferase assay and chromatin immunoprecipitation (ChIP) assay show that Egr-2 binds to the promoter lesion of *Prdm1* to activate its transcription. These results indicate that IL-27 signal transduction through Egr-2 and Blimp-1 is required for IL-10 production in $CD4^+$ T cells and controls the balance between regulatory and inflammatory cytokines.

Results

IL-27 induces Egr-2, IL-10, and LAG-3 expression in naïve $CD4^+$ T cells

We previously reported that the forced expression of Egr-2 induces IL-10 production in $CD4^+$ T cells and confers the phenotype of $CD4^+CD25^-LAG3^+$ Treg cells [21].Firstly, we confirmed the moderate induction of intracellular Egr-2 in TCR-stiumulated $CD4^+$ T cells and observed that IL-10 production was restricted to cells expressing intracellular Egr-2 (Fig. 1A). Then, we explored the factor inducing Egr-2, which confers the phenotype of $CD4^+CD25^-LAG3^+$ Treg cells. Various IL-10-inducible cytokines, such as IL-27, TGF-β [22], IL-21 [23], and IL-10, were added to a co-culture of splenic $CD4^+$ T cells from TEα TCR transgenic mice expressing I-Eα-specific TCR [24] and B cells from B6 wild type (WT) mice in the presence of $E\alpha_{52\text{-}68}$ peptides. In addition, the effect of the IL-10-inducible chemical substance zymosan was examined because it induces DCs to secrete abundant IL-10 in a Toll-like receptor (TLR) 2- and dectin-1-mediated activation of ERK/MAPK dependent manner [25]. Notably, IL-27 predominantly induced both Egr-2 and LAG-3 mRNA expressions relative to the other cytokines and zymosan. IL-27 did not induce Foxp3 mRNA expression (Fig. 1B), which is compatible with previous reports [15] and the fact that $CD4^+CD25^-LAG3^+$ Treg cells hardly expressed Foxp3 protein [21]. When we added IL-27 to naïve $CD4^+$ T cells stimulated with plate-coated anti-CD3ε and anti-CD28 monoclonal antibodies (mAb), Egr-2 protein was clearly detected by intracellular staining. This induction was abolished in Egr-2-deficient $CD4^+$ T cells cultured with IL-27 and also in IL-27Rα (WSX1)-deficient $CD4^+$ T cells (Fig. 1C). Interestingly, LAG-3 was predominantly induced in B6 WT $CD4^+$ T cells expressing Egr-2, and IL-27 alone did not induce Egr-2 in the absence of TCR stimulation. IL-27 more efficiently induced $Egr2^+LAG3^+$ cells than the other IL-12 family cytokines, IL-12 and IL-23 (Fig. 1D). Although IL-2 is required for IL-27-induced IL-10 expression through Blimp-1 in $CD8^+$ T cells [26], IL-2 by itself could not induce $Egr2^+LAG3^+$ cells and showed no additive effect on IL-27-induced Egr-2 and LAG-3 expressions (Fig. 1D). No significant association was seen between

the extent of cell division and the amount of Egr-2 expression, while Egr-2 induction was limited to proliferating cells (Fig. 1E).

IL-27-mediated induction of IL-10 and Blimp-1 is impaired in Egr-2 conditional knockout mice

Multiple observations support the idea that Blimp-1 regulates T-cell responsiveness by attenuating IL-2 production. IL-2 production in Blimp-1-deficient $CD4^+$ T cells is elevated by stimulation via TCR [18]. As IL-2 signaling induces Blimp-1 transcription, Blimp-1 makes a negative feed-back loop for *Il2* transcription in T cells [19]. Recently, it was shown that Blimp-1 positively regulates IL-10 production in $CD4^+$ T cells [18,27]. Blimp-1 is required for IL-10 production and high ICOS expression in $CD4^+CD25^+Foxp3^+$ Treg cells [28]. Therefore, the role of Egr-2 and Blimp-1 in IL-27-induced IL-10 production was examined using naïve $CD4^+$ T cells from Egr-2 conditional knockout (CKO) ($Egr2^{fl/fl}$-CD4-Cre^+) and Blimp-1 CKO ($Prdm1^{fl/fl}$-CD4Cre^+) mice. Consistent with our previous observation that the forced expression of Egr-2 induced the high mRNA expression levels of Blimp-1 in $CD4^+$ T cells [21], Egr-2-induction by IL-27 was not affected in the absence of Blimp-1 (Fig. 1C). In $CD4^+$ T cells both from Egr-2 CKO mice and Blimp-1 CKO mice, the induction of *Il10* transcription and IL-10 protein expression by IL-27 was impaired (Fig. 2A and B), and these inductions were not observed in $CD4^+$ T cells from WSX-1 knockout (KO) mice (Fig. 2A and B). Moreover, Blimp-1 mRNA induction by IL-27 was also impaired in Egr-2-deficient $CD4^+$ T cells (Fig. 2A). This result suggested that Egr-2 is essential for IL-10 production via Blimp-1 expression in IL-27-stimulated $CD4^+$ T cells. When we analyzed the induction of IL-10 and Blimp-1 mRNA expressions by other IL-12 family cytokines, IL-12 showed only marginal induction of IL-10 and Blimp-1 mRNA expressions and IL-23 induced no up-regulation of IL-10 and Blimp-1 mRNA expressions (Fig. 2C). We also found that IL-2 had no additive effect on IL-27-induced IL-10 and Blimp-1 mRNA expressions in $CD4^+$ T cells (Fig. 2C).

Egr-2 directly binds to the promoter region of *Prdm1* and enhances its activity

Next, we investigated whether Egr-2 regulates Blimp-1 transcription. To address this possibility, we performed a luciferase reporter assay. A pGL3-Luc vector subcloned with the promoter region from -1500 bp to the *Prdm1* transcription start site [29] was co-transfected with a pMIG-Egr-2 vector to 293T cells. As shown in Figure 3A, Egr-2 significantly enhanced the activity of the *Prdm1* promoter. Next, a ChIP assay was performed with antibodies against Egr-2 to investigate whether Egr-2 directly binds to the promoter region of Blimp-1 in $CD4^+$ T cells. Among four promoter regions examined (-3000bp, -2000bp, -1000bp and +1000bp from its transcription site) of Blimp-1, only one region (-1000bp) showed significant enrichment compared with control, indicating that Egr-2 specially binds to the Blimp-1 promoter, but not to *Lag3* and *Il10* promoters (Fig. 3B and Supporting Information Fig. 2A). Cretney *et al.* reported that Blimp-1 binds to intron 1 of the *Il10* locus and, together with interferon regulatory factor (IRF)-4, directly regulates IL-10 expression in $CD4^+CD25^+$ Treg cells by the remodeling of active chromatin at the *Il10* locus [28]. Our observation suggested that IL-10 regulation with Blimp-1 was controlled by Egr-2.

IL-27 induced Egr-2 expression is dependent on STAT3

STAT1 and STAT3 have been shown to be crucial for IL-10 production from IL-27 stimulated naïve $CD4^+$ T cells [17]. We investigated the effect of STAT1- and STAT3- deficiencies on IL-27 induced Egr-2 expression. As shown in Figure 4A and B, Egr-2 induction by IL-27 in $CD4^+$ T cells was impaired by a STAT3- deficiency, but not by a STAT1- deficiency. When we analyzed the induction of *Il10* transcription and IL-10 protein expression by IL-27 in STAT1- and STAT3-deficient $CD4^+$ T cells, IL-10 protein induction by IL-27 was abolished both in STAT1 KO and in STAT3 CKO $CD4^+$ T cells, although IL-10 mRNA expression levels were slightly up-regulated by IL-27 in STAT1 KO $CD4^+$ T cells (Fig. 4C and D). These results suggest that IL-27-induced Egr-2 expression in $CD4^+$ T cells is mostly dependent on STAT3, although both STAT1 and STAT3 are important for IL-10

production by IL-27. Next, we investigated the effect of other STAT1 or STAT3 activating cytokines for Egr-2 induction. IL-6 and IFN-γ were selected as the representatives of cytokines activating STAT3- and STAT1- mediated pathways, respectively. As shown in Figure 4E, IL-6 induced Egr-2 expression as effectively as IL-27 in $CD4^+$ T cells, but IFN-γ did not. Interestingly, both IL-10 and Blimp-1 mRNA expressions were also elevated by IL-6, but expression levels seemed to be lower than those by IL-27 (Fig. 4F). IL-6 is a type I cytokine that shares structural homology and a receptor subunit, gp130, with IL-27 and has already been shown to induce IL-10 in $CD4^+$ T cells [17]. These results suggest that Egr-2 is important for IL-10 production mediated both by IL-27 and by IL-6 through the STAT3-dependent pathway.

Enhanced production of IFN-γ and IL-17 by IL-27 stimulation in Egr-2 CKO mice

To examine the role of Egr-2 in inflammatory cytokine production, we investigated the production of IFN-γ and IL-17 in response to IL-27 stimulation. It has already been shown that Egr-2-deficient $CD4^+$ T cells produce high amounts of IFN-γ and IL-17 after TCR stimulation [30]. As shown in Figure 5, IFN-γ and IL-17 production from IL-27-stiumulated $CD4^+$ T cells was enhanced by an Egr-2 deficiency, which suggests that Egr-2 may also play an important role in controlling effector cytokine production.

Discussion

Recently, Tr1 cells, characterized by their high secretion of IL-10 and lack of Foxp3 expression, were induced by IL-27 [15-17, 31]. STAT1 and STAT3 have been shown to play an important role in the molecular mechanism of IL-10 production by IL-27 in $CD4^+$ T cells [17]. Although it is clear that STAT1 driven IL-10 production is independent of T-bet, the precise mechanism still remains unclear [17]. The underlying mechanism of IL-10 production through the activation of STAT3 is that the activation of STAT3 leads to the induction of transcription factor c-Maf [32], which is essential for IL-10 production induced by IL-27 [33]. Motomura et al. [34] have reported that transcription factor E4 promoter-binding protein 4 (E4BP4) is important for IL-10 production from IL-27-stimulated $CD4^+$ T cells cultured under a Th1 skewing condition. E4BP4-deficient Th1 cells failed to produce IL-10 by IL-27 stimulation. It seems that IL-10 production from T cells is controlled by a complex pathway, depending on each subset or surrounding cytokine condition. In this study, we found that another transcription factor Egr-2 mediates IL-10 expression in IL-27-stimulated $CD4^+$ T cells via direct binding to the Blimp-1 promoter. Furthermore, we have shown that IL-27 induced Egr-2 expression in $CD4^+$ T cells is dependent on STAT3, but not on STAT1. Although Egr-2 may be less involved in STAT1- and T-bet-mediated pathways, which are required for IL-10 production, Egr-2 is associated with STAT3-mediated IL-10 production.

IL-27- induced IL-10 production has been considered to be important for gut immunity. In IL-27 receptor (WSX1)-deficient mice, higher steady-state levels of Th17 cells were observed in the lamina propria and these mice were susceptible to high-dose dextran sulfate (DSS), a model of acute intestinal inflammation-induced colitis [35]. Similar to IL-10-deficient mice [36], WSX1-deficcient mice infected with *T. gondii* develop a lethal $CD4^+$ T-cell-mediated response characterized by excessive production of proinflammatory cytokines and massive lymphocytic infiltrates in multiple organs [37]. WSX1-deficient $CD4^+$ T cells have been shown to be impaired in

IL-10 production in $CD4^+$ T cells [17]. Although the $Foxp3^+$ Treg cell is one of the IL-10 producers, it has been shown that there are IL-10-producing T cells other than $Foxp3^+$ Treg cells in the intestine [38]. Moreover, CD4-specific IL-10-deficient mice have been shown to develop more severe colitis than $Foxp3^+$ Treg-specific IL-10-deficient mice [39], suggesting that Foxp3-negative, IL-10-producing T cells may be important for the maintenance of homeostasis in gut immunity. Egr-2-expressing $CD4^+CD25^-LAG3^+$ Treg cells are Foxp3-negative IL-10-producing T cells and are enriched in Peyer's patch [21]. Our observation that IL-27 induces $CD4^+Egr2^+LAG3^+$ T cells may be associated with IL-27-mediated control of gut homeostasis; however, a more detailed investigation is required to elucidate the role of IL-27 in keeping intestinal homeostasis.

It has been well documented that stimulation of T cells through TCR in the absence of co-stimulation can result in long-term hyporesponsiveness to subsequent stimulation, which is termed anergy. It has been also reported that Egr-2 is required for the full induction of T-cell anergy [20,40]. Egr-2 expression is rapidly induced within 6 h after TCR stimulation [41] and our results indicated that although IL-27-mediated Egr-2 induction was dependent on TCR stimulation, the TCR signal was not sufficient to support sustained Egr-2 expression. In addition to IL-27, another STAT3 activating cytokine, IL-6, also induced expressions of Egr-2, Blimp-1 and IL-10. This result was consistent with a previous report in which IL-6 induced STAT3-mediated production of IL-10 in $CD4^+$ T cells [17] and suggested that not only STAT1-STAT3 heterodimers in response to IL-27 stimulation but also STAT3 homodimers in response to IL-6 stimulation could induce Egr-2 expression. However, IL-27 induces Blimp-1 and IL-10 more efficiently than IL-6 and the involvement of STAT1 should be addressed further.

It is well known that IL-2 has paradoxical functions in T-cell homeostasis, acting as a T-cell growth factor and having a crucial function in the maintenance of self-tolerance. Sun et al. [26] reported that the effective induction of IL-10-producing $CD8^+$ cytotoxic T lymphocytes (CTLs) by IL-27 requires the presence of IL-2, and that the IL-2-IL-27-mediated induction of IL-10 as well as the

IL-27- mediated induction of IL-10 was Blimp-1 dependent. However, we observed that the addition of IL-2 did not up-regulate IL-10 and Blimp-1 mRNA induction levels by IL-27 in $CD4^+$ T cells. In addition, IL-2 showed no synergistic effect on IL-27-induced Egr-2 and LAG-3 expressions in our experiments. This result is consistent with the fact that increased Egr-2 level by antigen activation was not affected by the addition of IL-2 in peptide treatment-induced $CD4^+$ Treg cells [42]. These observations suggest that Blimp-1 is important for IL-27-induced IL-10 production both in $CD4^+$ and $CD8^+$ T cells, but the pathway leading to the activation of Blimp-1 is differently regulated between these cells.

Egr-2-expressing $CD4^+CD25^-LAG3^+$ Treg cells are anergic and have regulatory activities at least in part via IL-10 production. Because our results showed that Egr-2 is indispensable for the full production of IL-10 in $CD4^+$ T cells after IL-27 stimulation, Egr-2 could be one of the molecular links between anergy and IL-10 production in $CD4^+$ T cells. Further studies will be required to elucidate the relationship between the Egr-2-Blimp-1 pathway and other pathways, which have already been reported to contribute to IL-10 production by IL-27 stimulation.

We also found enhanced production of IFN-γ and IL-17 in Egr-2 CKO mice after IL-27 stimulation. Egr-2 CKO mice develop autoimmune disease characterized by the accumulation of IFN-γ and IL-17-producing $CD4^+$ T cells, and massive infiltration of T cells into multiple organs. The expressions of T-bet, a Th1 transcription factor, IL-6, IL-21, and IL-23, which can induce Th17 differentiation in $CD4^+$ T cells, were not altered in aged Egr-2 CKO mice [30]. Blimp-1 CKO mice develop severe colitis with age and Blimp-1-deficient $CD4^+$ T cells have been shown to produce more IFN-γ than WT after stimulation with PMA plus ionomycin or with TCR plus IL-2 [18]. Recently, Lin et al. [43] reported that NOD-background Blimp-1-deficient $CD4^+$ T cells exhibit significantly enhanced IL-17 production in a steady-state as well as in a Th17-polarizing condition. These observations indicate that increased IFN-γ and IL-17 production in IL-27-stimulated Egr-2-deficient $CD4^+$ T cells may be a direct consequence of reduced Egr-2-Blimp-1 signaling.

Although Egr-2 CKO mice did not exhibit colitis, a single nucleotide polymorphism (SNP) in a locus at chromosome 10q21, which was identified by genome-wide analysis to have a strong relationship with Crohn's disease susceptibility, exists in a strong linkage disequilibrium region of Egr-2 [44, 45].

In summary, we have shown that Egr-2 mediates IL-27-induced IL-10 production through Blimp-1 transcription in $CD4^+$ T cells. Additionally, IFN-γ and IL-17 production by IL-27 was reciprocally regulated by Egr-2. Egr-2 may play a crucial role in maintaining the balance between regulatory and inflammatory cytokines. Our observation could contribute to the elucidation of the molecular regulation of IL-10 production in $CD4^+$ T cells.

Materials and Methods

Mice.

C57BL/6 mice and Prdm1-floxd mice were purchased from Japan SLC and The Jackson Laboratory, respectively. Blimp-1 conditional knockout (Blimp-1 CKO) mice were generated by crossing Prdm1-floxd mice with CD4-Cre transgenic mice in which Cre-induced recombination was detected only in $CD4^+$ T cells. Egr-2 conditional knockout (Egr-2 CKO) mice were generated by crossing Egr-2-floxed mice [46] with CD4-Cre transgenic mice. TEα TCR transgenic mice were purchased from The Jackson Laboratory. WSX-1 deficient (WSX-1 KO) mice were prepared as described previously [47]. STAT1 knockout (STAT1 KO) mice were purchased from Taconic. STAT3 conditional knockout (STAT3 CKO) mice ($STAT3^{fl/fl}$-$CD4\text{-}Cre^+$) were generated by crossing STAT3-floxed mice with CD4-Cre transgenic mice. CD4-Cre transgenic mice (line 4196), originally generated by C.B. Wilson and colleagues [48], were purchased from Taconic. All mice were used at 7-10 weeks of age. All animal experiments were conducted in accordance with Institutional and National Guidelines.

Reagents, antibodies, and media

The following reagents were purchased from BD Pharmingen: purified mAbs for CD3ε (145-2C11) and CD28 (37.51), Fc block (anti-CD16/32), FITC anti-CD45RB (16A), phycoerythrin (PE) anti-LAG3 (C9B7W), PE anti-IgG2a (R35-95), PE anti-CD62L (MEL-14), allophycyanin-anti-CD25 (PC61), allophycyanin-Cy7 anti-CD25 (PC61), allophycyanin anti-CD4 (RM4-5), allophycyanin-Cy7 anti-CD4 (RM4-5), allophycyanin anti-LAG3 (C9B7W), allophycyanin anti-IL-10 (JES5-16E3), allophycyanin anti-IgG2b (MPC-11), biotinylated mAb for CD8α (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD25 (PC61), CD62L (MEL-14), Ter119 (TER119), and streptavidin (SA)- allophycyanin, SA- allophycyanin Cy7, SA-FITC. Qdot605 anti-CD4