Roles of LAG3 and EGR2 in regulatory T cells

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ABSTRACT

Regulatory T cells (Tregs) participate in the maintenance of tolerance to self-antigens and suppressive control of excessive immune responses to exogenous antigens. A lack or dysfunction of these cells is responsible for the pathogenesis and development of many autoimmune diseases. It is well known that CD4 Tregs play a major role in controlling immune responses and can be classified into two main populations: thymus-derived naturally occurring Tregs (nTregs) and induced Tregs (iTregs) generated from $CD4^+CD25^-$ precursors in the peripheral lymphoid organs. The most extensively studied Tregs are the nTregs, which express the interleukin 2 (IL-2) receptor CD25 and the transcription factor Forkhead box P3 (Foxp3). On the other hand, iTregs contain multiple heterogeneous subsets, including interleukin (IL)-10-producing CD4 type I Tregs (Tr1 cells) and transforming growth factor -β-producing Th3 cells, and so on. However, the extent of the contribution of iTregs to immunoregulation in normal animals has been difficult to evaluate because of the lack of suitable cell surface markers. It has been found recently that IL-10-secreting iTregs can be delineated as $CD4^+CD25^-Foxp3^-$ T cells that characteristically express both the lymphocyte activation gene-3 (*LAG3*) and the early growth response gene-2 (*EGR2*). In this review, opinions about the roles of *LAG3* and *EGR2* in Tregs are presented.

INTRODUCTION

Regulatory T cells (Tregs) play crucial roles in preventing autoimmune diseases and maintaining immune homoeostasis. These Tregs predominantly comprise two groups: naturally occurring CD4+CD25- Tregs (nTregs) and induced Tregs (iTregs), generated in the thymus and periphery, respectively. The nTregs, which characteristically express the transcription factor Forkhead box P3 (Foxp3),[1] have been intensively studied because their deficiency abrogates self-tolerance and causes autoimmune disease.[2] Scurfy mice, which have a frame shift mutation in the *Foxp3* gene, display extensive lymphoproliferation and severe inflammatory infiltration in some organs such as the lung, skin and liver.[3] The autoimmune regulator (*Aire*) gene, which affects the central induction of tolerance by regulating the clonal deletion of self-reactive thymocytes, is responsible for autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).[4] *Aire* regulates the ectopic expression of a battery of peripheral-tissue antigens—for example, insulin, fat acid-binding protein and salivary protein-1.[5] An additional defect in central tolerance induction in scurfy mice, which are generated by crossing mice that carry a null mutation in the *Aire* gene, did not noticeably extend the range of the affected sites, and many organs remained unaffected.[6] This result suggests that additional important mechanisms other than central tolerance and the Foxp3 system are required to enforce immunological self-tolerance in the periphery.

Although Foxp3-independent iTregs have been a focus of active investigation, it is difficult to assess the in vivo physiological function of iTregs because of the lack of specific markers that can reliably differentiate them from the other T cell subsets. Recently, we reported the interleukin 10 (IL-10) secreting $CD4^+CD25^-Foxp3^-$ iTregs that express both the lymphocyte activation gene-3 (*LAG3*) and early growth response gene-2 (*EGR2*), and ectopic expression of Egr2 conferred suppressive function on naïve CD4 T cells.[7] This review provides an overview of our knowledge about the molecular features of *LAG3* and *EGR2* in Tregs.

MOLECULAR PROPERTIES OF LAG3

LAG3 (CD223), a type I membrane glycoprotein of the immunoglobulin (Ig) superfamily, was first reported as a cell surface protein that is expressed on activated human natural killer cells and T cells.[8] LAG3 expression has also been detected in many different cell types, such as plasmacytoid dendritic cells (DCs),[9] B cells,[10] natural killer T cells,[11] γδ T cells,[12] tumour-infiltrating lymphocytes,[13 14] exhausted $CD8^+$ T cells[15] and Tregs.[7 16]

The gene coding for LAG3 protein lies adjacent to the gene coding for CD4 on human chromosome 12p13 and shares approximately 20% homology with the *CD4* gene. These two molecules have four extracellular Ig-like domains with conserved structural motifs throughout D1 to D4 domains.[8] Both human and mouse LAG3 bind to major histocompatibility complex (MHC) class II molecules with higher affinity than CD4.[17 18] Although LAG3 is closely related to CD4, CD4 molecules are mainly expressed at the cell surface. In contrast, almost half of LAG3 molecules are retained intracellularly.[19] LAG3 co-localises with Rab11b, which is a marker of the endosomal recycling compartment. This observation suggests that LAG3 is continuously recycled and/or rapidly translocated to the plasma membrane in response to antigenic stimulation.

In CD4 T cells, LAG3 dimerisation on the cell surface is required for the formation of stable MHC binding sites.[20] After in vivo T cell activation in mice, significant amounts of soluble LAG3 (sLAG3) accumulated in their serum (~200 ng/ml). Li *et al*[21] reported that LAG3 was cleaved within the short C peptide located between the membrane proximal D4 domain and the transmembrane

domain, resulting in the release of sLAG3. The cell surface expression of LAG3 is regulated by cleavage of the extracellular domains by two transmembrane metalloproteases, ADAM10 and ADAM17.[22] ADAM10 is responsible for constitutive and activation-induced cleavage, while ADAM17 mediates protein kinase C-Θ-dependent cleavage. Additionally, ADAM10 siRNA suppressed T cell proliferation in an LAG3-dependent manner. Thus, the cell surface expression of LAG3 is strictly regulated by several mechanisms.

ROLE OF LAG3 IN CD4 T CELLS

LAG3 is well known as an activation-induced cell surface molecule. In naïve T cells, LAG3 expression is upregulated after antigen-specific activation.[12] Although an initial analysis of LAG3 knockout mice did not show any defects in T cell function,[23] a subsequent analysis demonstrated that LAG3 regulates both expansion of activated primary T cells and development of the memory T cell pool.[24] The cross-linking of the T cell receptor (TCR) and LAG3 on activated CD4 T cells induced less calcium release than TCR stimulation alone.[25] Consistent with these observations, it has been demonstrated that non-cleavable LAG3 mutant vector transduced-CD4 T cells exhibited a more potent inhibitory effect on their activation than wild-type LAG3 vector.[22] Other reports have also suggested that mouse LAG3 negatively regulates CD4 T cell activation[26–28] and that signalling is mediated by the KIEELE motif, which is conserved between humans and mice, in the cytoplasmic domain of LAG3.[29] It was also proposed that the interaction of MHC class-II-bearing antigen presenting cells and $CD4^+LAG3^+$ T cells induces a T cell-intrinsic inhibitory signalling pathway.[30] These observations clearly indicate that LAG3 is a 'cell-intrinsic' inhibitory molecule.

EXPRESSION OF LAG3 IDENTIFIES INTERLEUKIN-10 (IL-10)-PRODUCING $CD4^+CD25^-FOXP3^-$ TREGS

LAG3 has recently been shown to be a new extrinsic and intrinsic inhibitory molecule that is required for the maximal regulatory function of $CD4^+CD25^+Foxp3^+$ Tregs[16] and controls effector T cell expansion and homoeostasis.[28] Ectopic LAG3 expression also confers regulatory activity on naïve T cells.[16] The LAG3 on $CD4^+CD25^+$ Tregs interacts with MHC class II molecules on dendritic cells (DCs), and the binding of LAG3 to the MHC class II molecules expressed by immature DCs induces ITAM-mediated inhibitory signalling, which suppresses DC maturation and immunostimulatory capacity.[31] These observations indicate that LAG3 possesses 'cell-intrinsic' and also 'cell-extrinsic' regulatory activity. However, in naïve mice, LAG3 protein is hardly detected on the cell surface of $CD4^+CD25^+Foxp3^+$ Tregs[16] and is mainly restricted to a population of $CD4^+CD25^-CD45RB^{low}$ memory T cells,[7] which are assumed to include IL-10-secreting $CD4^+Foxp3^-$ T cells, resembling type 1 Tregs (Tr1 cells).[32] Although it is known that LAG3 is expressed in $CD4^+CD25^-$ T cells, its role in $CD4^+CD25^-$ T cells has remained elusive. In our analysis, approximately 2% of the $CD4^+CD25^-$ T cell population in the spleen consisted of $CD4^+CD25^-LAG3^+$ T cells.[7] These $CD4^+CD25^-LAG3^+$ T cells express higher levels of LAG3, IL-10 and Blimp-1 mRNA than other CD4 T cell subsets. The transcription factor Blimp-1 is required for IL-10 production by CD4 T cells[33] and is also indispensable for the formation of IL-10-producing effector Tregs.[34] Indeed, $CD4^+CD25^-LAG3^+$ T cells secreted high amounts of IL-10 upon in vitro antigenic stimulation.[7] In addition, $CD4^+CD25^-LAG3^+$ T cells were hypoproliferative in response to anti-CD3 and anti-CD28 monoclonal antibody stimulation, and suppressed the in vivo development of colitis induced in $RAG\text{-}1^{-/-}$ recipients by the transfer of naïve T cells in an IL-10-dependent manner. These $CD4^+CD25^-LAG3^+$ Tregs did not express Foxp3 protein, and scurfy mice still had functional $CD4^+CD25^-LAG3^+$ Tregs, which expressed IL-10 mRNA and retained regulatory activity in vitro. Unlike $CD4^+CD25^+Foxp3^+$ nTregs, high-affinity interactions with selecting peptide/MHC ligands expressed in the thymus did not induce the development of $CD4^+CD25^-LAG3^+$ Tregs. These observations indicate that LAG3 is a phenotypic marker of IL-10-producing Foxp3-independent CD4 iTregs that plays a role in their regulatory activity in the normal immune system.

ANERGY-ASSOCIATED EGR2 AS A REGULATORY GENE OF CD4+CD25−LAG3+ TREGS

In order to address the molecular mechanisms responsible for the functions of $CD4^+CD25^-LAG3^+$ Tregs, we compared the differential gene expression profiles of $CD4^+CD25^-LAG3^+$ Tregs, $CD4^+CD25^+$ Tregs, $CD4^+CD25^-LAG3^-$ T cells and naïve $CD4^+CD25^-CD45RB^{high}$ T cells by gene array analysis. Interestingly, the transcription factor *Egr2* gene was preferentially expressed in $CD4^+CD25^-LAG3^+$ Tregs.[7] Intracellular staining of Egr2 revealed a strong correlation between Egr2 and LAG3 expression in CD4 T cells (unpublished data).

Egr2 is a member of a family of Cys_2His_2-tpye zinc finger transcription factors, which consists of four members, Egr-1, -2, -3 and -4. Egr2 plays an essential role in hind-brain development and myelination of the peripheral nervous system, and the null mutation of Egr2 resulted in perinatal or neonatal death due to respiratory and/or feeding deficits.[35] In CD4 T cells, it is reported that Egr2 is closely associated with anergy induction.[36 37] Egr2 inhibits interferon -γ (IFN-γ) and IL-2 secretion by T cells and enhances the expression of the E3 ligase Cbl-b, which is critical for the regulation of T cell tolerance and anergy.[37] Egr2 binds directly to the promoter of the cell cycle inhibitor $p21^{cip1}$ in T cells.[38] Consistent with these observations, Egr2-associated $CD4^+CD25^-LAG3^+$ Tregs were fully anergic upon in vitro TCR stimulation, similarly to $CD4^+CD25^+$ Tregs. Intriguingly, in Egr2 conditional knockout mice, in which the *Egr2* gene was deleted in CD2 T cells, the T cells did not show altered primary activation but became hyperproliferative in response to prolonged stimulation, leading to the development of a lupus-like syndrome.[38] Although these observations indicate that Egr2 controls the self-tolerance of T cells, the extrinsic regulatory function of Egr2 has not been investigated. We therefore employed a retroviral gene transfer system. The forced expression of Egr2 converted naïve CD4 T cells into IL-10-secreting and LAG3-expressing Tregs.[7] Moreover, these Egr2-transduced CD4 T cells exhibited antigen-specific immunosuppressive effects on the delayed-type hypersensitivity response. Thus, Egr2 confers suppressive activity on naïve CD4 T cells in vivo. These results suggest that Egr2, a 'cell-extrinsic' regulatory molecule, might be a critical regulator of IL-10-producing $CD4^+CD25^-LAG3^+$ Tregs. Further analysis of Egr2 regulation and elucidation of the precise mechanisms by which Egr2 exerts its regulatory activity will provide important insights into autoimmunity and might result in new therapeutic modalities.

CD4+CD25−LAG3+ TREGS AND OTHER IL-10-PRODUCING CD4+FOXP3− TREGS

IL-10, an anti-inflammatory cytokine, can both directly and indirectly inhibit effector T cell responses during infection, autoimmunity and cancer.[39–41] Published data have suggested major roles for IL-10-producing CD4+CD25−Foxp3− Tregs that are distinct from those of nTregs in the maintenance of the immune regulatory system,[42] and Tr1 cells have also been a focus of active investigation. Tr1 cells are generated by repeated antigen stimulation in the presence of either excess IL-10 in vitro[43] or by culture in the presence of vitamin D3 and dexamethasone.[44] Interestingly, Tr1 cells obtained in vitro in the presence of vitamin D3 and dexamethasone also co-expressed high levels of LAG-3 and IL-10 (unpublished data). We have also confirmed that Egr2-transduced CD4 T cells expressed Egr2, Blimp-1 and IL-10 mRNA. These findings are in keeping with that of previous reports, which found that the phenotype of Blimp-1-expressing Tregs resembled that of IL-10-producing Tregs in humans and mice.[34]

However, it remains unclear whether Tr1 cells are naturally present in the normal immune system because Tr1 cells are mainly characterised by their unique pattern of cytokine production after in vitro stimulation. Tr1 cells produce high levels of IL-10, with or without transforming growth factor-β (TGF-β and IL-5; low levels of IFN-γ; and little or no IL-2 or IL-4.[43] Their immunological properties, including their strong IL-10 production, peripheral development, anergic phenotype and lack of Foxp3 expression, suggest that CD4+CD25−LAG3+ Tregs are analogous to Tr1 cells. However, CD4+CD25−LAG3+ Tregs produce IL-10 and also IFN-γ but do not produce IL-5 or TGF-β.[7] Although CD4+CD25−LAG3+ Tregs partially fulfil the criteria for Tr1 cells,[32 43 45] further studies will be required to elucidate the inconsistency between these Tregs in more detail. Recently, some candidate cell surface markers for IL-10-producing CD4 Tregs have been reported. Ochi and colleagues reported that latency-associated peptide (LAP)-expressing CD4+CD25− Tregs produce both IL-10 and TGF-β.[46] The CD4+CD25−LAG3+ Tregs hardly expressed LAP protein on their cell surface, indicating that they differ from CD4+CD25−LAP+ Tregs.[7] Although it was also reported that a subpopulation of IL-10-producing CD4 Tregs express CD103 (integrin αEβ7),[47] the CD4+CD25−LAG3+ Tregs did not express CD103 on their cell surface.[7]

Several IL-10-secreting T cell subpopulations with regulatory activities, such as Th1, Th2 or Th17 cells, have been identified.[48–50] It is now evident that Tregs are composed of several phenotypically distinct subsets, and they appear to have plasticity.[51] The Egr2-associated CD4+CD25−LAG3+ Tregs, which produce a large amount of IL-10, might be useful for investigating the induction mechanisms of IL-10-producing CD4 Tregs.

EGR2 AND HUMAN DISEASES

Egr2 plays a critical role in the nervous system. Mutations in the *Egr2* gene prevent Schwann cell development and peripheral nerve myelination in mice and lead to the development of demyelinating neuropathy.[35] In humans, *EGR2* mutations are associated with Charcot–Marie–Tooth disease type 1, Dejerine–Sottas syndrome and congenital hypomyelination neuropathy.[52] Interestingly, several recent genome-wide association studies (GWAS) have found new genetic links between *EGR2* and human autoimmune diseases. Two independent GWAS identified strong association signals which were located on chromosomes10q21 in Crohn's disease, the most common form of chronic inflammatory bowel disease.[53 54] The associated intergenic region was flanked by *EGR2*, suggesting that this genetic variation regulates *EGR2* expression. In line with these observations, Egr2-associated CD4+CD25−LAG3+ Tregs effectively prevented intestinal inflammation in a murine T cell transfer model of colitis.[7]

Furthermore, a candidate gene analysis indicated that polymorphisms in EGR2 influence systemic lupus erythematosus (SLE) susceptibility in humans.[55] SLE is a systemic autoimmune disease characterised by autoantibody production and is associated with a wide range of clinical manifestations. Although the cause of SLE is unknown, overwhelming evidence points to a combination of underlying genetic susceptibility and environmental factors.[56] Interestingly, T cell-specific Egr2 conditional knockout mice develop progressive lupus-like autoimmunity.[38] We have found a human counterpart of murine CD4+CD25−LAG3+ Tregs in the tonsils (manuscript under preparation). The gene expression profile of human CD4+CD25−LAG3+ T cells showed a marked similarity to that of murine CD4+CD25−LAG3+ Tregs. Thus, it is conceivable that, like mouse Egr2, human EGR2 may be a key molecule for nerve systems and also for immune regulatory systems controlling both T cell and B cell responses.

FUTURE PROSPECTS AND CONCLUSIONS

As mentioned above, CD4 Treg subsets can be classified into two main populations: thymus-derived CD4+CD25+Foxp3+ nTregs and iTregs, such as CD4+CD25−LAG3+ Tregs, which are generated in the periphery. However, the relative contribution of each Treg subset to the regulation of immune responses is poorly understood. A recent study by Huber and colleagues showed that pathogenic Th17 cells were controlled by both Foxp3+ and LAG3+Foxp3− CD4 Tregs in an IL-10-dependent manner.[57] Accumulating evidence suggests that Th17 cells may play a significant role in the pathogenesis of multiple inflammatory and autoimmune disorders including SLE.[58] Given that CD4+CD25−LAG3+ Tregs express high levels of Egr2, Blimp-1 and IL-10, CD4+CD25−LAG3+ Tregs might play a distinct role in regulating Th17 cells.

IL-10 has an antiapoptotic effect on B cells. IL-10 is also involved in B cell isotype switching, and plays a role in autoimmune diseases associated with B cell dysregulation such as SLE.[59 60] However, nasal anti-CD3 administration induced IL-10-producing CD4+CD25−LAP+ Tregs which suppress lupus pathogenesis through the downregulation of IL-17+CD4+ICOS+CXCR5+ follicular helper T cells in an IL-10-dependent manner.[61] These findings indicate that, in some settings, IL-10 can suppress the pathogenesis of lupus. The MRL/*lpr* mouse, possessing a single gene mutation in the *Fas* gene, is a prototypical model of human SLE.[62] Deficits in the apoptosis-promoting Fas/FasL receptor–ligand pair of cell surface molecules in MRL/*lpr* mice are associated with hypergammaglobulinaemia, autoantibody production and a spectrum of autoimmune manifestations that resemble lupus. Interestingly, the adoptive transfer of CD4+CD25−LAG3+ Tregs from control MRL/+ mice suppressed the progression of nephritis and autoantibody production in MRL/*lpr* lupus-prone mice (manuscript under preparation). Inconsistent with the previous report, CD4+CD25+ Tregs from MRL/+ mice exhibited no significant therapeutic effect after being transferred to MRL/*lpr* mice.[63] Thus, CD4+CD25−LAG3+ Tregs might be useful for treating autoantibody-mediated autoimmune diseases, including SLE.

Immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome is a rare disease caused by a mutation in the *FOXP3* gene that results in the absence or a severe deficiency of CD4+CD25+ Tregs.[64] Patients with the condition are characterised by autoimmune enteritis, type 1 diabetes mellitus, eczema,

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hypothyroidism, autoimmune haemolytic anaemia, membranous nephropathy and recurrent infections. Enteropathy, endocrinopathy and dermatitis, which are not common symptom in patients with SLE, are seen in most IPEX patients.[65] Although a variety of autoantibodies to multiple organs were observed in IPEX patients, antinuclear antibodies are either absent or present at low titres, and anti-dsDNA antibodies are rarely detected. Glomerulonephritis, which is the most common form in patients with SLE, is rare in patients with IPEX syndrome.[66] On the other hand, SLE is characterised by high serum titres of antinuclear antibodies and dsDNA antibodies.[56] Thus, diagnosis of SLE has not been established in patients with IPEX syndrome.[67] These facts suggest that Foxp3 might not have direct association with the physiopathology of lupus in humans. Indeed, analyses of the function and phenotypic properties of $CD4^+CD25^+Foxp3^+$ Tregs in patients with SLE have led to conflicting results.[68] The data for the role of $CD4^+CD25^+Foxp3^+$ Tregs in the pathogeneses of lupus mouse models are also a matter of debate.[63 69–71] On the other hand, mice in which the *Egr2* gene was conditionally knocked out in T cells developed progressive lupus-like autoimmunity with antibodies to histone and dsDNA and were characterised by the accumulation of IL-17-producing CD4 T cells.[38] These observations suggest that Egr2-associated $CD4^+CD25^-$ $LAG3^+$ Tregs are the major subset of circulating IL-10-producing Tregs and that their functions are complementary to those of $CD4^+CD25^+Foxp3^+$ nTregs. Thus, fine tuning the balance of $CD4^+CD25^+Foxp3^+$ nTregs and $CD4^+CD25^-LAG3^+$ Tregs might provide new therapeutic methods for autoimmune diseases. Although additional studies are required to elucidate the relationship between $CD4^+CD25^+Foxp3^+$ nTregs and $CD4^+CD25^-$ $LAG3^+$ iTregs, distinct markers of IL-10-producing CD4 Tregs, such as LAG3 and Egr2, will substantially contribute to resolving this issue.

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Regulatory T Cell-Mediated Control of Autoantibody-Induced Inflammation

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Abstract

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Autoimmune inflammation including autoantibody-induced inflammation is responsible for the lethal organ damage. Autoantibody-induced inflammation can be separated in two components, autoantibody production, and local inflammatory responses. Accumulating evidence has suggested that regulatory T cells (Treg) control both antibody production and the numbers and functions of effector cells such as innate cells and T helper cells. Autoantibodies are produced by both the follicular and extrafollicular pathways. Recently, follicular regulatory T cells (T_{FR}) and Qa-1 restricted $CD8^+$ Treg were identified as populations that are capable of suppressing follicular T helper cell (T_{FH})-mediated antibody production. In local inflammation, $CD4^+CD25^+Foxp3^+$ Treg have the capacity to control inflammation by suppressing cytokine production in T helper cells. Although complement proteins contribute to autoantibody-induced local inflammation by activating innate cells, Treg including $CD4^+CD25^+Foxp3^+$ Treg are able to suppress innate cells, chiefly via IL-10 production. IL-10-secreting T cells such as T regulatory type I (Tr1) and Tr1-like cells might also play roles in the control of Th17 and innate cells. Therefore, several kinds of Tregs have the potential to control autoimmune inflammation by suppressing both autoantibody production and the local inflammatory responses induced by autoantibodies.

Keywords: chronic inflammation, autoantibody, regulatory T cells, IL-10, Tr1 cells

Introduction

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Autoimmune inflammation is responsible for the lethal organ damage, and autoantibodies play a pivotal role in triggering inflammation. Immune complexes are readily detectable in the articular tissues of rheumatoid arthritis (RA) patients and the glomeruli of systemic lupus erythematosus (SLE) patients. These immune complexes are regarded as important players in the pathogeneses of these diseases as they initiate and maintain the inflammatory cascade and tissue destruction. In addition, immune complex deposition in articular tissue has been reported to have harmful effects in many experimental models of arthritis. The passive transfer of antibodies to an autoantigen that is found in the joints is an established method for inducing arthritis. For example, antibodies to glucose-6-phosphate isomerase (GPI), a ubiquitous cytoplasmic enzyme, induce spontaneous arthritis upon injection into susceptible mice (Matsumoto et al., 2002), and the administration of a cocktail of anti-type II collagen antibodies to DBA/1 mice also invokes severe arthritis (Terato et al., 1992). However, it is particularly notable that these antibody-induced arthritis models are transient and fail to achieve chronicity (Myers et al., 1997), which suggests that a continuous supply of autoantibodies is required for the development of chronic joint inflammation.

In contrast to arthritis models, there are no lupus models that are invoked by the passive transfer of autoantibodies. However, plasma cell-depletion by a proteasome inhibitor clearly demonstrated the importance of a continuous supply of autoantibodies for systemic autoimmunity; i.e., treatment with bortezomib, a proteasome inhibitor, depleted the number of plasma cells producing antibodies to double stranded DNA; eliminated autoantibody production; ameliorated glomerulonephritis; and prolonged the survival of two lupus-prone mice strains, NZB/W F1, and MRL/*lpr* mice. Among five bortezomib-treated mice that displayed proteinuria of >100 mg/dl before treatment, four showed proteinuria of <100 mg/dl after treatment. These findings suggest that the suppression of autoantibody production leads to reduced organ inflammation in lupus (Neubert et al., 2008).

In addition to the production of autoantibodies by B cells, antibody-induced inflammation by itself is another target of therapeutic intervention. In mouse models of arthritis, the synthesized immune complexes bind to "inflammatory" Fc-receptors on intra-articular cells and then activate complement protein (Rowley et al., 2008). Complement fragments bound to immune complexes induce tissue injury, and FcR stimulation cumulatively activates mononuclear cells *in situ*, causing the activated cells to release pro-inflammatory cytokines. In turn, these responses attract neutrophils and macrophages, which can damage synoviocytes and chondrocytes. As mentioned above, immune complex-induced arthritis is a prototypic inflammatory process that is characterized by the release of pro-inflammatory cytokines and the activation of degradative enzymes. In a GPI-induced arthritis model, it was found that anti-GPI autoantibodies act through FcγRIII receptors and C5a (Ji et al., 2002). Micro-positron emission tomography studies revealed that the localization of anti-GPI antibodies is dependent on mast cells, neutrophils, Fc-receptors, and immune complexes (Mandik-Nayak and Allen, 2005). In anti-collagen antibody-induced arthritis (CAIA), complement activation, and innate cells are also critical for the effector phase of arthritis (Hietala et al., 2004; Daha et al., 2011). With regard to systemic autoimmunity, MRL/*lpr* mice lacking factor B or factor D developed less severe nephritis than the control mice (Watanabe et al., 2000; Elliott et al., 2004). Factor B is cleaved by factor D and resulting catalytic subunit Bb forms C_3 convertase. In addition, the anti-double stranded DNA antibody titer is not altered by factor D deficiency, indicating that complement activation is not required for the production of autoantibodies in MRL/*lpr* mice. Activated complement interacts with Fcγ receptors and complement receptors on innate effector cells (such as macrophages and monocytes) to induce local inflammation (Wasowska, 2010).

Therefore, autoantibody-induced inflammation can be separated into two components, autoantibody production and the local inflammatory response. Recently, accumulating evidence has shown that regulatory T cells (Treg) control both antibody production and the numbers and functions of effector cells such as innate cells and T helper cells. This article will discuss the Treg-mediated suppression of these two components during inflammation (Figure 1).

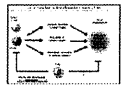

Figure 1

Control of autoantibody-induced inflammation by regulatory T cells.

Treg-Mediated Suppression of Autoantibody Production

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Two mechanisms for autoantibody production

In the course of thymus-dependent responses, B cells interact with T cells in the outer T cell zones of the lymphoid organs and differentiate along either the follicular or extrafollicular pathway (Lee et al., 2011). In the follicular pathway, activated B cells form germinal centers (GC) and undergo somatic hypermutation and selection. Subsequently, they exit 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. These migrated B cells form clusters of short-lived plasmablasts. Thus both the follicular and extrafollicular pathways contribute to autoantibody production in murine disease models.

Extrafollicular B cell response-mediated autoantibody production William et al. (2002) observed that the splenic autoreactive B cells of autoimmune MRL/*lpr* mice proliferated and undergo active somatic hypermutation at the T zone-red pulp border rather than in GC. They examined the extrafollicular generation of plasmablasts in AM14 VH transgenic (Tg) mice, which possess rheumatoid factor (RF)-producing B cells with moderate affinity for IgG2a. Intriguingly, AM14 B cells on the MRL/*lpr* background spontaneously differentiate into extrafollicular plasmablasts and undergo somatic hypermutation at the T zone/red pulp border. In addition, they reported that the extrafollicular plasmablast response is induced by the administration of IgG2a anti-chromatin antibodies, which presumably form immune complexes *in vivo* with endogenous chromatin (Herlands et al., 2007). This response was found to be T cell independent, although it was totally dependent on MyD88 signaling downstream of Toll-like receptor 7 (TLR7) and TLR9 (Herlands et al., 2008). However, another study revealed that although AM14 B cells can be activated, differentiate, and undergo isotype-switching independent of antigen-specific T helper cells, T cells dramatically enhance the AM14 B cell response via CD40L and IL-21 signaling (Sweet et al., 2011).

GC-mediated autoantibody production Because affinity-enhancing somatic hypermutations are prevalent in autoantibodies, it has long been hypothesized that these autoantibodies are derived from GC. Mouse strains that frequently develop autoimmune diseases (NZB/W F1, BXSB, MRL/*lpr*, sanroque, and NOD mice) spontaneously form GC-like structures in their spleens, and the onset of autoantibody production correlates with GC formation. Recently, several pieces of evidence have suggested that dysregulated T follicular helper (T_{FH}) cells significantly contribute to autoimmunity by inducing the aberrant selection of autoreactive B cells. The lupus-like disease that occurs in sanroque mice is caused by Roquin$^{san/san}$-induced accumulation of T_{FH} cells that maintain spontaneously formed GC (Vinuesa et al., 2005). The glomerulonephritis and pathogenic autoantibody production displayed by sanroque mice are ameliorated by *Bcl6* haploinsufficiency (Linterman et al., 2009). Moreover, SLAM-associated protein (SAP) deficiency experiments have highlighted the important roles played by T_{FH} cells in the conditions suffered by sanroque mice. SAP interacts with a conserved tyrosine-based motif that is found in the cytoplasmic tail of SLAM family members, and *Sh2d1a* (the gene for SAP) deficiency abrogates T_{FH} formation and GC responses, but not extrafollicular antibody responses. Since SAP deficiency ameliorates the lupus-like phenotype of sanroque mice, it can be assumed that aberrant T_{FH} cell activation is responsible for the autoimmunity that they display. BXSB mice develop a severe form of lupus caused by the *yaa* locus, which induces the overexpression of a cluster of X-linked genes that includes *Tlr7* gene. Although B6.*yaa* mice are not overtly autoimmune, the introduction of *Sle1*, which contains the autoimmune-predisposing *Slam/Cd2* haplotype, into their genome causes them to develop fetal lupus (Subramanian et al., 2006). Intriguingly, CD4$^+$ T cells from B6.*Sle1.yaa* mice develop the molecular signature of T_{FH} cells and also show altered expression levels of various cytokines and chemokines.

The source of human autoantibodies revealed by B cell-depletion therapy As discussed above, dysregulation of the follicular or extrafollicular pathway can cause systemic autoimmune disease in mice. However, the contributions of follicular and extrafollicular checkpoints to the production of disease -associated autoantibodies are more difficult to evaluate in humans than in mice. Levels of autoantibodies do not always correlate with disease activity and response to treatment. For example, the serum concentrations of some autoantibodies correlate with disease activity (i.e., anti-double stranded DNA antibodies and anti-PR3 antibodies), while the titers of other autoantibodies [i.e., anti-ribonucleoprotein (RNP) antibodies and anti-Ro and La antibodies] remain stable irrespective of disease status. The heterogeneous autoantibody effects have also been observed in patients treated with anti-CD20 monoclonal antibody, which depletes B cells and plasmablasts but not long-lived plasma cells (Cambridge et al., 2003, 2006; Lu et al., 2009). 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 the levels of anti-Ro, Sm, or RNP antibodies (Cambridge et al., 2006). This suggests that anti-nucleosome and anti-double stranded DNA antibodies are produced through extrafollicular responses, which usually generate short-lived plasma cells, while antibodies to nucleic acid-associated antigens (Ro, Sm, and RNP) are derived from follicular responses, which generate long-lived plasma cells. In RA patients, the levels of IgA-RF, IgG-RF, and IgG anti-cyclic citrullinated peptide (CCP) antibodies are decreased at 6 months after the administration of anti-CD20 monoclonal antibody, and the decreases are proportionately greater than the decreases in their respective total immunoglobulin classes (Cambridge et al., 2003). Plasmablasts and short-lived plasma cells originating from the extrafollicular response might be the major source of RF and anti-CCP antibodies (Looney et al., 2008). Therefore, both extrafollicular- and follicular-mediated antibody productions should be controlled in the treatment of human autoimmune inflammation.

Approaches to autoantibody suppression

Antibody suppression with CD4$^+$CD25$^+$ Foxp3$^+$ Treg In general, T cells are indispensable sources of help signals, which promote B cell antibody production. Therefore, control of antibody production at the level of T cells is a rational approach to autoantibody suppression. Indeed, several T cell populations are able to suppress B cell antibody production. In humans, CD4$^+$CD25$^+$CD69$^-$ Treg that suppress antibody production *in vitro* have been found in GC. The fact that these CD4$^+$CD25$^+$CD69$^-$ Treg hardly express CXCR5 suggests that they mainly reside in the T cell-rich zones of secondary lymphoid tissues (Lim et al., 2004). However, T cell activation switches their chemokine receptor expression pattern from CCR7 to CXCR5 and switches their chemotactic responses from CCL19 to CXCL13. Thus, activation might change the migratory behavior of CD4$^+$CD25$^+$CD69$^-$ Treg so that they can migrate to GC. After migrating to GC, CD4$^+$CD25$^+$CD69$^-$ Treg negatively regulate T cell-dependent B cell responses through their suppressive activity toward T cells.

The preferential killing of antigen-presenting B cells by CD4$^+$CD25$^+$ Treg was reported in C57BL/6 mice (Zhao et al., 2006). B cell death is not mediated by the Fas–Fas ligand pathway, but instead is mediated by a granzyme-dependent, partially perforin-dependent pathway. Direct suppression of B cells by Treg was also reported in chronic systemic autoimmunity (Iikuni et al., 2009). For example, CD4$^+$CD25$^+$ Treg have been demonstrated to inhibit B cell antibody production in *in vitro* models of murine and human lupus. Treg use granule exocytosis pathways involving perforin and granzyme to induce contact-dependent apoptosis in B cells. However, in spite of the fact that CD4$^+$CD25$^+$ Treg from both young and old NZB/W F1 mice retain a capacity to suppress IgG production in B cells, autoantibodies continuously accumulate in these mice. Therefore, whether CD4$^+$CD25$^+$ Treg could be used to efficiently control autoantibody production in systemic autoimmunity needs to be examined further.

Recently, several groups simultaneously identified mice CD4$^+$CD25$^+$Foxp3$^+$ Treg subpopulations that are able to suppress B cell antibody production (Chung et al., 2011; Linterman et al., 2011;Wollenberg et al., 2011). Chung et al. (2011) identified a subset of Treg cells that express CXCR5 and Bcl6 and localize to GC in mice and humans. The expression of CXCR5 on Treg depends on Bcl6, and CXCR5$^+$Bcl6$^+$ Treg are absent from the thymus but can be generated from CXCR5$^-$Foxp3$^+$ natural Treg precursors. A deficiency of CXCR5$^+$ Treg results in enhanced GC reactions involving B cells, affinity maturation of antibodies, and plasma cell differentiation. These results demonstrated that the Bcl6–CXCR5 axis of Treg is one mechanism by which GC responses are controlled. In addition, they observed that Foxp3-mutated scurfy mice display a moderate increase in their T_{FH} population but a markedly increased number of GL7$^+$CD95$^+$ B cells. Collectively, these observations suggest that Foxp3$^+$ follicular regulatory (T_{FR}) cells are more specialized for controlling the generation of GC B cells. Linterman et al. (2011) also described a population of Foxp3$^+$Blimp-1$^+$CD4$^+$ T cells that accounted for 10–25% of the CXCR5^{high}PD-1^{high}CD4$^+$ T cells found in immunized GC. In the absence of these T_{FR} cells, they noted outgrowths of non-antigen-specific B cells in GC and a decreased number of antigen-specific B cells. Therefore, both groups revealed that T_{FR} play a role in controlling GC reactions by inhibiting the selection of antigen-specific- and non-specific B cells. Because CXCR5-expressiong T_{FR} localize to GC, T_{FR} may suppress GC-mediated autoantibody production. However, whether T_{FR} actually suppress autoantibody production and whether T_{FR} deficiency results in autoimmunity remain to be addressed.

Antibody suppression with Qa-1 restricted CD8$^+$ Treg and other Treg subsets A recent study reported that Qa-1 restricted CD8$^+$ Treg cells directly inhibit Qa-1$^+$ T_{FH} cells. Qa-1 is a non-classical MHC class Ib molecule presenting a peptide derived from the signal sequence of classical MHC class I proteins, named Qa-1 determinant modifier (Qdm), as well as peptides derived from proteins associated with infectious or inflammatory responses (Lu et al., 2006). Previously, a subpopulation of CD8$^+$ T cells was reported to suppress T cell help to B cells (Noble et al., 1998), and subsequent studies have shown that Qa-1 restricted CD8$^+$ T cells inhibit experimental autoimmune encephalomyelitis (EAE) by targeting autoreactive CD4$^+$ cells (Hu et al., 2004). Nevertheless, although Qa-1 deficient mice showed dysregulated immune responses to immunization with self and foreign antigens, Qa-1$^{-/-}$ mice do not develop spontaneous autoimmunity. Since Qa-1 interacts with both the T cell receptor (TCR) on CD8$^+$ T cells and the CD94/NKG2A receptor expressed by activated CD4$^+$ T cells, Qa-1 knock-in mice, B6 Qa-1(D227K) mice, were generated. B6 Qa-1 (D227K) mice

harbor a Qa-1 amino acid exchange mutation that disrupts the binding of Qa-1 to the TCR/CD8 complex, but has no effect on its binding to the inhibitory NKG2A receptor. Intriguingly, the B6 Qa-1 (D227K) mice exhibit lupus-like systemic autoimmune disease and a fivefold to sixfold increase in their numbers of T_{FH} cells (Kim et al., 2010).

Analysis of the surface phenotype of Qa-1 restricted $CD8^+$ Treg indicated that they express CD44, ICOSL, and $CXCR5$ and the $CD44^+ICOSL^+CD8^+$ T cells inhibit the generation of high-affinity antibodies and Qa-1^+ T_{FH} cells. This observation provides a clue that might greatly increase our understanding of autoantibody production. However, the antigen-specificity of Qa-1 restricted $CD8^+$ Treg during T_{FH} cell suppression remains unclear because the repertoire of peptides presented by Qa-1 is substantially smaller than the repertoire of classical MHC molecules (Lu et al., 2006). Only a small number of peptides have been identified that bind to Qa-1 and stimulate $CD8^+$ T cells, including dominant Qdm as well as peptides from HSP60, insulin, *Salmonella* GroEL, and TCR Vβ chains. Thus, Qa-1 restricted $CD8^+$ Treg might suppress T_{FH} cells irrespective of the antigen-specificity of the TCR on T_{FH} cells. Because Qa-1 restricted $CD8^+$ Treg express $CXCR5$ and migrate to lymphoid follicles (Kim et al., 2010), Qa-1 restricted $CD8^+$ Treg may suppress GC-mediated autoantibody production.

T_{FR} and Qa-1 restricted $CD8^+$ Treg appear to be important checking mechanisms for antibody production. However, no Treg populations that control autoantibody production and autoimmunity in an antigen-specific manner have yet been identified. Although the importance of T regulatory type I (Tr1) cells for controlling immune responses has been described in a number of reports, anti-CD46-induced IL-10-secreting T cells even enhance antibody production by B cells (Fuchs et al., 2009). Recently, several $CD4^+$ T cell populations that possess regulatory activity have been identified (Fujio et al., 2010). $CD4^+CD25^-LAP^+$ T cells and $CD4^+NKG2D^+$ T cells produce both IL-10 and TGF-β (Oida et al., 2003; Dai et al., 2009), and $CD4^+CD25^-IL\text{-}7R^-$ T cells and $CD4^+CD25^-LAG3^+$ T cells produce large amounts of IL-10 (Haringer et al., 2009; Okamura et al., 2009). The association between these recently identified Treg and antigen-specific autoantibody suppression should be investigated. In particular, $CD4^+CD25^-LAG3^+$ T cells, which characteristically express the anergy-linked transcription factor Egr2, might be associated with autoantibody suppression, because T cell-specific Egr2-deficient mice exhibit lupus-like disease (Zhu et al., 2008) and polymorphisms in the EGR2 gene are associated with human SLE susceptibility (Myouzen et al., 2010). Although both T_{FR} cells and Qa-1 restricted $CD8^+$ Treg express $CXCR5$ and may suppress GC-mediated autoantibody production, Treg populations which suppress extrafollicular response are yet to be identified.

Treg-Mediated Suppression of Local Inflammation

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IL-10-mediated suppression of inflammation

Nguyen et al. (2007) reported a role of $CD4^+CD25^+Foxp3^+$ Treg in antibody-induced arthritis at several levels. They examined the effect of the *scurfy* loss of function mutation of the *Foxp3* gene in K/BxN mouse model. These mice carry the KRN transgene, which encodes a TCR reactive against a peptide from GPI and the autoreactive T cells promote the production of vast quantities of anti-GPI antibodies, which are sufficient to induce arthritis after transfer into normal recipients (Korganow et al., 1999). The absence of $CD4^+CD25^+Foxp3^+$ Treg led to more accelerated aggressive arthritis with significantly earlier autoantibody production. However, the broadened spectrum of affected joints in *Foxp3*-mutated K/BxN mice was not due to the earlier appearance of autoantibodies and could not be reproduced by increasing anti-GPI antibody load. Therefore, $CD4^+CD25^+Foxp3^+$ Treg are supposed to play a role in effector phase manifestations. Their another observation that $Foxp3^+$ Treg accumulated in inflamed joint of K/BxN serum-transferred B6 mice suggested that $Foxp3^+$ Treg actively migrate to the site of antibody-induced inflammation and control the local inflammatory process. Although the mechanism of this $Foxp3^+$ Treg-mediated suppression was not clarified, IL-10 was mentioned as a candidate mediator.

Furthermore, a single transfer of $CD4^+CD25^+$ Treg markedly slowed the progression of collagen-induced arthritis (CIA), which could not be attributed to the loss of systemic type II collagen-specific T and B cell responses (Morgan et al., 2005). The transferred $CD4^+CD25^+$ Treg were found in the inflamed synovium soon after the transfer, indicating that regulation occurs locally in the joints. It is unlikely that the transferred $CD4^+CD25^+$ Treg acted against CIA solely via the suppression of T cell immunity involving the Th17 cell response since the effector phase of CIA depends on T cell-independent immune responses (Ehinger et al., 2001). Thus, the transferred $CD4^+CD25^+$ Treg might have interacted with local innate cells as well as effector T cells.

$CD4^+CD25^+$ Treg-mediated control of innate cells was found to be IL-10 and TGF-β dependent in a colitis model (Maloy et al., 2003). Indeed, IL-10 production might be a key factor controlling local inflammation. Human IL-10 suppresses the expression of MHC class II, co-stimulatory, and adhesion molecules (De Waal Malefyt et al., 1991; Willems et al., 1994). IL-10 also inhibits the production of inflammatory cytokines and the T cell stimulating capacity of antigen-presenting cells (APC; Fiorentino et al., 1991; Allavena et al., 1998), and local IL-10 production has been shown to suppress TNF-α and IL-1α production (Lubberts et al., 2000). $CD4^+CD25^+$ Treg downregulate the expression of co-stimulatory molecules on APC (Cederbom et al., 2000) and restrain the maturation and antigen-presenting function of dendritic cells in an IL-10-dependent manner (Misra et al., 2004; Houot et al., 2006). Furthermore, IL-10 was recently reported to suppress Th17 cells (Huber et al., 2011). Interestingly, both $CD4^+Foxp3^+$ Treg and $CD4^+Foxp3^-$IL-10-producing cells (Tr1) are able to control Th17 cell numbers in an IL-10-dependent manner. Therefore, it was suggested that Tr1 cells can compensate for a paucity of $Foxp3^+$ Treg and vice versa during the suppression of innate and Th17 cells.

Tr1 cells are considered to be different from Th1, Th2, and Th17 cells based on their cytokine production profile; i.e., they secrete high levels of IL-10. Tr1 cells are inducible *in vitro* and *in vivo*, and they can also be isolated from humans and mice in steady state conditions (Roncarolo et al., 2011). Tr1 cells are able to suppress Th1-mediated colitis induced by the transfer of naïve $CD4^+CD45RB^{hi}$ cells into SCID mice as well as EAE (Roncarolo et al., 2001). Although few reports have directly compared IL-10 production between $CD4^+CD25^+$ Treg and Tr1-like cells, $CD4^+CD25^-LAG3^+$ Treg secrete significantly higher amounts of IL-10 than $CD4^+CD25^+$ Treg (Okamura et al., 2009). Thus, Tr1 cells and Tr1-like cells might have the ability to control innate immune cells.

Suppression of T cell cytokine production

In several antibody-induced autoimmune inflammations such as RA-synovitis and lupus nephritis, co-existence of antibody deposition and T cell infiltration is frequently observed. RA is a prototypic autoimmune disease characterized by chronic joint inflammation and the production of cytokines, including TNF-α, IL-6, IL-15, IL-17, and IL-1β. These cytokines are thought to be derived from both innate cells and effector T cells. In the K/BxN arthritis model, T cells can augment antibody-induced arthritis independently of their influence on antibody production (Jacobs et al., 2009). This enhancement was mediated by IL-17 producing $CD4^+$ T cells preferentially recruited to the environment of the arthritic joint. Therefore, Treg-mediated suppression of effector T cells may be also beneficial in controlling autoantibody-induced inflammation accompanied with T cell infiltration. In the past, IFN-γ producing Th1 cells were thought to be the principal mediators of autoimmune inflammation such as that observed in RA. However, IL-17 has emerged as a key driver of inflammation and is detectable in the RA synovium. IL-17 and IL-17F promote inflammation on several levels, as their receptors IL-17RA and IL-17RC are expressed on both hematopoietic and non-hematopoietic cells. IL-17 and IL-17F induce the production of pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α, and pro-inflammatory chemokines such as CXCL1, GCP-2, and IL-8 and thus promote tissue inflammation and neutrophil recruitment at sites of inflammation (Bettelli et al., 2008).

$CD4^+CD25^+$ Treg not only suppress the proliferation of conventional T cells, but also their production of inflammatory cytokines, such as TNF-α and IFN-γ. In contrast, IL-17 production is not suppressed when human $CD4^+CD25^+$ Treg are added to responder T cells *in vitro* (Annunziato et al., 2008; Flores-Borja et al., 2008), and murine $CD4^+CD25^+$ Treg promote Th17 cell development both *in vitro* and *in vivo* (Chen et al., 2011; Pandiyan et al., 2011). As IL-17 is important for infection control, the resistance of Th17 cells to suppression by $CD4^+CD25^+$ Treg cells makes sense. However, in a previous study $CD4^+CD25^+Foxp3^+$ Treg-specific ablation of STAT3 resulted in the development of fetal intestinal inflammation due to the loss of Th17 cell suppression in mice (Chaudhry et al., 2009). Moreover, other studies have suggested that some subpopulations of $CD4^+CD25^+$ Treg cells are capable of regulating Th17 cell responses. For example, $CD4^+CD25^+$ Treg expressing CD39 (an ectonucleotidase that hydrolyzes ATP) were reported to be able to suppress Th17 cell responses (Fletcher et al., 2009). In addition, $CD4^+CD25^+CD39^+$ Treg numbers are reduced in patients

with multiple sclerosis (MS), suggesting that an association exists between this Treg population and the suppression of pathogenic Th17 cells. Therefore, at least some $CD4^+CD25^+$ Treg are suspected to suppress the production of inflammatory cytokines in inflamed organs.

EAE is an animal model of MS that is induced by the injection of myelin components. Until recently, the pathogeneses of MS and EAE were thought to be initiated by myelin-specific Th1 cells. However, a number of lines of evidence have indicated that Th17 cells induce central nervous system (CNS) inflammation (Oukka, 2007). For example, it was reported that the Th17:Th1 ratio of infiltrating T cells in EAE determines where inflammation occurs in the CNS (Stromnes et al., 2008), and T cell infiltration and inflammation in the brain parenchyma only occur when Th17 cells outnumber Th1 cells and trigger a disproportionate increase in IL-17 expression in the brain. In contrast, T cells showing a wide range of Th17:Th1 ratios induce spinal cord parenchymal inflammation. Tg mice bearing a TCR against the myelin basic protein (MBP) that had been crossed with recombination-activating gene 1 (*Rag1*)-deficient mice (Tg MBP/$Rag^{-/-}$) developed spontaneous EAE, whereas Tg MBP/$Rag^{+/+}$ mice did not (Lafaille et al., 1994). This discrepancy can be explained by the existence of Treg in the $Rag^{+/+}$ mice but not the $Rag^{-/-}$ mice because the adoptive transfer of $CD4^+CD25^+$ Treg from wild-type mice to Tg MBP/$Rag^{-/-}$ mice prevented the development of spontaneous EAE (Hori et al., 2002). Moreover, adoptive transfer experiments have revealed that transferring large numbers of $CD4^+CD25^+$ Treg purified from the peripheral lymph nodes of naive mice reduced the incidence and severity of EAE (Kohm et al., 2003). In a study conducted by Matsumoto et al. (2007), peripheral $CD4^+CD25^+$ Treg from mice with EAE suppressed the development of chronic EAE in the recipient rats. Therefore, $CD4^+CD25^+Foxp3^+$ Treg apparently have the capability to suppress T helper cell-mediated organ inflammation, and this effect may be beneficial in the control of the antibody-induced inflammation accompanied with effector T cell infiltration.

Conclusion

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The current standard treatment for autoimmune disease is non-specific immunosuppression with steroids and immunosup-pressants, which inevitably leads to opportunistic infections. As autoantibodies are key components in the development of autoimmune inflammation, targeting autoantibody-induced immunity is a rational approach to the treatment of autoimmune diseases. The modulation of Treg function is a promising physiological approach to suppressing both autoantibody production and autoantibody-induced local inflammation. Further examinations of $CD4^+CD25^+$ Treg and other Treg subsets are necessary in future.

Conflict of Interest Statement

Go to:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Kidney-infiltrating CD4$^+$ T-cell clones promote nephritis in lupus-prone mice

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In systemic lupus erythematosus, CD4$^+$ T cells play key roles in the initiation and promotion of autoantigen-specific humoral immunity, and indirect evidence suggests that T cells are pathogenic effectors in lupus nephritis. The contribution of kidney-infiltrating T cells to nephritis, however, has not been verified because of the difficulty in directly analyzing organ-infiltrating T cells. Here, we examined the pathogenic roles of autoreactive cytokine-expressing CD4$^+$ T cells from the kidneys of early nephritic MRL/lpr mice. Interferon (IFN)-γ-secreting cells were enriched among CD5^{high}CD4$^+$ T cells found in the inflamed kidneys. Using single-cell analysis of the T-cell receptor (TCR)^{high}CD5^{high}CD4$^+$ T cells from the kidneys of early nephritic MRL/lpr mice, two IFN-γ-expressing CD4$^+$ T cell clones, MLK2 and MLK3, were identified. CD4$^+$ T cells transduced with the T-cell receptor genes from each clone responded to splenic dendritic cells in an MHC class II -dependent manner, but not to B cells or macrophages. MLK3-transduced CD4$^+$ T cells proliferated in the spleens of prenephritic mice, promoted nephritis progression upon adoptive transfer, and enhanced the deposition of C3 without promoting anti-double-stranded DNA antibody production. Thus, CD4$^+$ T cells in the inflamed kidneys of MRL/lpr mice contribute to nephritis progression.

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KEYWORDS: glomerulonephritis; immunology and pathology; lupus nephritis

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Systemic lupus erythematosus is an autoimmune disease characterized by autoantibody production and multiorgan damage. As many autoantibodies in systemic lupus erythematosus exhibit high-affinity and somatic mutations,[1,2] it is supposed that the activation of autoreactive B cells is preceded by the activation of autoreactive T cells, which supply help signals to B cells. Nucleosomes, ribonucleoprotein, and Sm are candidate targets for autoreactive T cells in systemic lupus erythematosus.[3–5] In a previous study, nucleosome-specific T-cell clones induced the development of lupus nephritis in lupus-prone mice.[6] Although these autoantibody-associated T cells are considered to exacerbate lupus nephritis by enhancing autoantibody production, it is unclear whether organ-infiltrating T cells directly contribute to kidney inflammation.

T cells are pathogenic effectors in lupus glomerulonephritis.[7] J_HD-MRL/lpr mouse strain, a strain that lacks circulating immunoglobulins but possess B cells expressing a B-cell receptor transgene, suffer from renal disease.[8] Treating (NZB × NZW) F_1 mice with cytotoxic T-lymphocyte antigen-4Ig and a suboptimal dose of cyclophosphamide resulted in a significant delay in mortality without a reduction in glomerular immune complex deposits.[9] In NZM2328 mice, early immune complex deposition and acute cellular glomerulonephritis were found to be associated with glomerular and periglomerular T-cell infiltration.[10] However, the pathogenicity of kidney-infiltrating T cells has not been verified because of the difficulty in directly analyzing organ-infiltrating T cells.

In this study, we aimed to identify pathogenic autoreactive T-cell receptors (TCRs) in the kidneys of early nephritic MRL/lpr mice via single-cell analysis. We focused on interferon (IFN)-γ as a pathogenic cytokine because the deletion or inhibition of IFN-γ and its receptor ameliorated nephritis progression in MRL/lpr mice.[11–13] IFN-γ also contributes to crescent formation and cell-mediated immune injury.[12–15] In human lupus, some researchers have observed a skew toward T-helper (Th) 1 predominance in proliferative glomerulonephritis.[16,17] Moreover, polymorphisms in IFN-γ or its receptor are associated with lupus in some patients.[18,19]

We identified two TCRα- and β-chain pairs in IFN-γ-expressing CD4$^+$ T cells from inflamed kidneys, and CD4$^+$

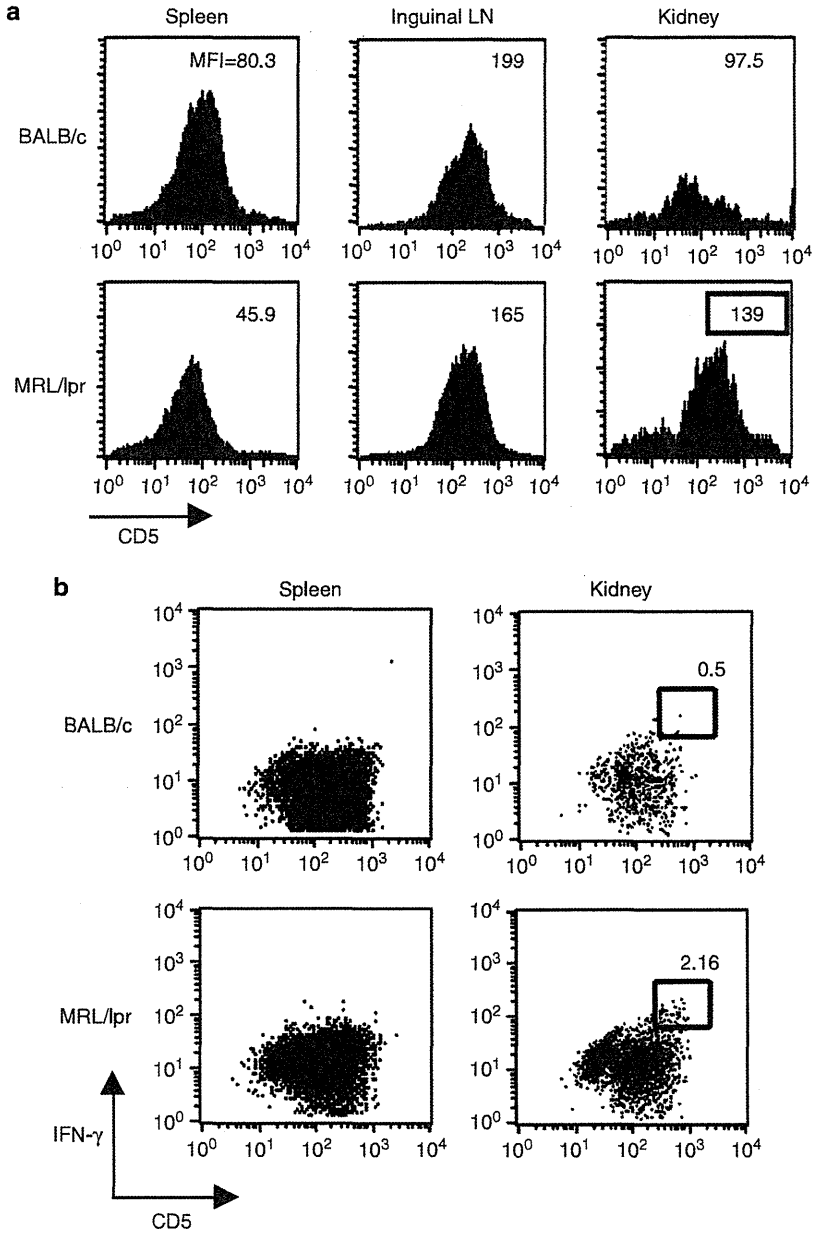


Figure 1 | Identification of interferon (IFN)-γ^+ CD5^{high} CD4$^+$ T cells in the early inflamed kidneys of prenephritic MRL/lpr mice. (**a**) Increased expression of CD5 in CD4$^+$ T cells from the kidneys of prenephritic MRL/lpr mice. Histograms of CD5 expression in CD4$^+$ T cells were produced for the spleen, inguinal lymph nodes (LN), and kidneys of BALB/c and MRL/lpr mice. The mean fluorescence intensities (MFIs) of the CD5$^+$ population are indicated. Representative results of three independent experiments are shown. (**b**) IFN-γ-producing CD4$^+$ T cells express high levels of CD5 in the kidneys of prenephritic MRL/lpr mice. CD4$^+$ T cells stimulated with immobilized anti-CD3 and anti-CD28 for 16 h were stained for IFN-γ secretion. The plots indicate the IFN-γ and CD5 expression of CD4$^+$ T cells from the indicated organs of BALB/c or MRL/lpr mice. Representative results of three independent experiments are shown.

T cells that had been transduced with either TCR pair displayed autoreactivity to splenic dendritic cells (DCs). Upon adoptive transfer to prenephritic mice, CD4$^+$ T cells that had been transduced with one of the identified TCR, MLK3, promoted nephritis without enhancing autoantibody titers. Our results suggest that CD4$^+$ T cells in the kidneys of MRL/lpr mice contribute to nephritis progression.

RESULTS

CD5 levels in the CD4$^+$ T cells of the early inflamed kidney

First, we selected the T-cell surface molecules that are associated with autoreactivity and IFN-γ expression. Previous studies of TCR transgenic mice reported that high CD5 and TCR expression are reflective of high-avidity interactions with self-peptide:major histocompatibility complex (MHC)

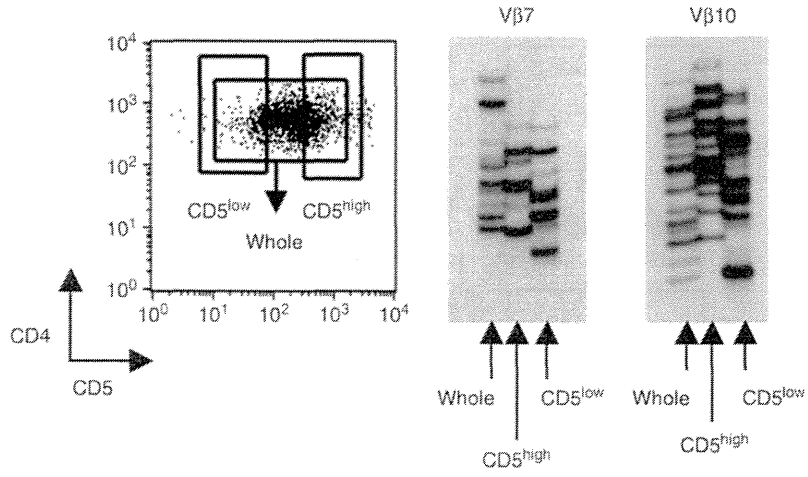


Figure 2 | The dominant clonotype of the $CD5^{high}CD4^+$ T cells was different from those of the total $CD4^+$ T-cell and $CD5^{low}CD4^+$ T-cell populations. Total $CD4^+$ T cells, $CD5^{low}CD4^+$ T cells, and $CD5^{high}CD4^+$ T cells were sorted from the kidneys of nephritic MRL/lpr mice. The dot plot in the left panel shows the gates used for the sorting. The reverse transcription (RT) PCR was performed with Vβ- and Cβ-specific primers for the complementary DNA of sorted cells, and the T-cell clonality of each group was analyzed by T-cell receptor RT-PCR/single-strand conformational polymorphism analysis. Electrophoresis bands for Vβ7 and Vβ10 are depicted.

and are able to predict the survival/homeostatic expansion capacity of $CD4^+$ T cells.[20,21] In mice expressing influenza hemagglutinin and hemagglutinin determinant S1-specific TCR, CD5 upregulation was a marker of their interaction with the S1 self-peptide.[22] Therefore, we hypothesized that the autoreactive T cells in the early inflamed kidney would exhibit increased CD5 expression levels.

We then compared the CD5 levels of $CD4^+$ T cells from the spleens and kidneys of control BALB/c and early nephritic MRL/lpr mice. In the early nephritic MRL/lpr mice, the kidney $CD4^+$ T cells exhibited increased CD5 levels (Figure 1a). This was consistent with previous studies that detected CD5 upregulation in activated T cells[23,24] and suggested that autoreactive T cells respond to self-antigens in the early inflamed kidney. When the association between CD5 expression and IFN-γ production was examined in kidney $CD4^+$ T cells, an IFN-γ-positive subset was detected among the T cells with high CD5 levels in the kidneys of early nephritic MRL/lpr mice (Figure 1b).

T-cell clonality was then compared according to the CD5 expression level in order to examine whether $CD5^{high}CD4^+$ T cells are clonally different from total $CD4^+$ T cells and $CD5^{low}CD4^+$ T cells. The reverse transcription PCR/single-strand conformational polymorphism (SSCP) method for TCR (TCR-SSCP) efficiently detects subtle nucleotide changes in the complementarity determining region (CDR) 3 sequences of clonally expanded T cells *in vivo*.[25,26] When total $CD4^+$ T cells, $CD5^{low}CD4^+$ T cells, and $CD5^{high}CD4^+$ T cells from the nephritic kidneys of MRL/lpr mice were sorted and had their T-cell clonality compared using TCR-SSCP analysis, most of the dominant $CD5^{high}CD4^+$ T cells clonotypes were different from those of the total $CD4^+$ T cells and $CD5^{low}CD4^+$ T-cell populations (Figure 2). This indicated that $CD5^{high}CD4^+$ T cells constitute a distinct clonal population that is different from those of total $CD4^+$ T cells and $CD5^{low}CD4^+$ T cells.

Identification of $V\alpha2^{high}CD5^{high}CD4^+$ T-cell clones that expand in the early inflamed kidney using single-cell sorting

Although the $CD5^{high}CD4^+$ T-cell population contained IFN-γ-secreting cells, not all of the $CD5^{high}CD4^+$ T cells secreted IFN-γ. We used the TCR expression level as an additional indicator of autoreactivity because TCR expression levels are also associated with avidity for the self-peptide:MHC complex.[21] To efficiently identify the TCRα chain gene in single-cell analysis, we focused on $CD4^+$ T cells that express Vα2, which is one of the major subfamilies of the murine TCR Vα chain. Interestingly, the frequency of $V\alpha2^{high}CD5^{high}CD4^+$ T cells was increased in the kidneys of the early nephritic mice, but not in the spleens or kidneys of the prenephritic mice (Figure 3a). Single-cell sorting of $V\alpha2^{high}CD5^{high}CD4^+$ T cells was performed to obtain pairs of TCRα and β chains from single cells that had expanded in the early inflamed kidney. In parallel, the total kidney $V\alpha2^+CD5^+CD4^+$ T cell population was also sorted as a reference group (Figure 3a).

The α-chain sequences of 85% of the sorted cells were successfully determined in our single-cell analysis (Figure 3b). The dominant $V\alpha2^{high}CD5^{high}CD4^+$ T-cell clones were distinct from those of the total $CD4^+$ T cells in the early inflamed kidney. Although most of the $V\alpha2^{high}CD5^{high}CD4^+$ T-cell clones were identified among the $V\alpha2^+CD5^+CD4^+$ T cells, they were not the dominant clones in this population, suggesting that the $V\alpha2^{high}CD5^{high}CD4^+$ T cells found in the kidneys of MRL/lpr mice are a distinct T-cell population.

MLK2- and MLK3-transduced cells displayed strong autoreactive responses to $CD11c^+$ DCs from prenephritic mice

The cytokine and Foxp3 gene expression levels of identified single-cell clones were examined by two-step nested PCR. Among the identified single cell clones obtained from

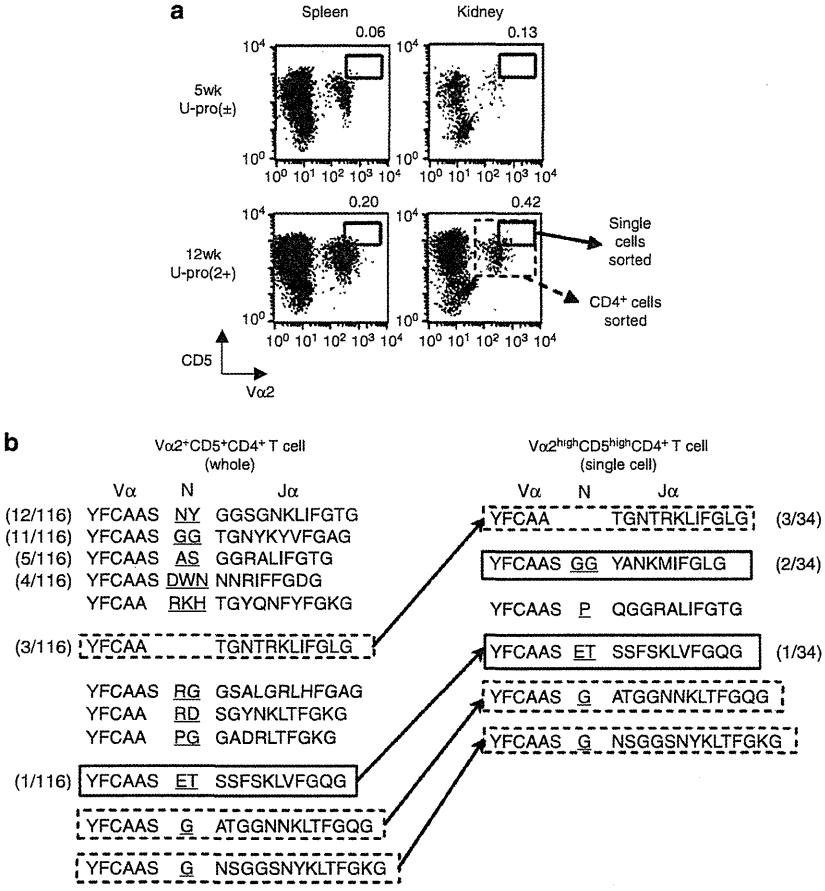


Figure 3 | The dominant clones of Vα2^{high} CD5^{high} CD4$^+$ single T cells were distinct from those of the total Vα2$^+$CD5$^+$CD4$^+$ T-cell population in the early inflamed kidney. (**a**) Flow cytometry profiles of CD5 and Vα2 staining gated on CD4$^+$ cells in the spleen and kidneys. To obtain the T-cell receptor α- and β-chain pairs of the clones that had expanded in the early inflamed kidneys from 12-week (wk)-old MRL/lpr mice, single-cell sorting of Vα2^{high}CD5^{high}CD4$^+$ cells was performed (solid gate). The total Vα2$^+$CD5$^+$CD4$^+$ cell population was sorted as a reference population (hatched gate). (**b**) The complementarity determining region (CDR) 3 sequences of the Vα2^{high}CD5^{high} CD4$^+$ single T-cell clones and total Vα2$^+$CD5$^+$CD4$^+$ T cells from the early inflamed kidneys. The Vα, N, and Jα sequences are indicated. As a result, 116 sequences were identified for the total Vα2$^+$CD5$^+$CD4$^+$ T cells, and 34 clones were identified for the single Vα2^{high} CD5^{high} CD4$^+$ T-cell clones. The number of identical sequences is indicated beside the sequences. For the total Vα2$^+$CD5$^+$CD4$^+$ T cells, sequences that were repeated more than three times and nonrepetitive sequences that were identical to those found in the single Vα2$^+$CD5$^+$CD4$^+$ T-cell clones are shown. c.p.m., counts per minute. Solid line boxed sequences are MLK2 and MLK3.

Vα2^{high}CD5^{high}CD4$^+$ T cells, two clones exclusively expressed IFN-γ, and these clones were designated MLK2 and MLK3 (Figure 4a and b). The Vα2-CDR3 sequence of MLK2 was identified in two single cell–sorted Vα2^{high}CD5-^{high}CD4$^+$ T cell clones, and the MLK3 Vα2-CDR3 sequence was identified in one single cell–sorted Vα2^{high} CD5^{high}CD4$^+$ T-cell clone and the total kidney Vα2$^+$ CD5$^+$CD4$^+$ T-cell population. The CDR3 sequences of the TCRβ chains of MLK2 and MLK3 were determined by three-step semi-nested PCR using a series of Vβ1–19 primers (Figure 4a). The same TCRβ CDR3 sequence was identified in the two MLK2 clones. Although the TCRβ CDR3 sequence that paired with the MLK3 Vα2-CDR3 sequence from the total kidney Vα2$^+$CD5$^+$CD4$^+$ T-cell population was not identified, the identity of the Vα-CDR3 sequence in different sources strongly suggested the *in vivo* clonal expansion of MLK3.[27] The full-length sequences of the MLK2 and MLK3 TCRα and β chains were synthesized from a combination of V, CDR3, and C region sequences using heteroduplex PCR, as described previously.[28] Furthermore, the phenotypes of MLK2 and MLK3 were consistent with Th1 because they did not express interleukin (IL)-4, IL-17, IL-17F, IL-10, or the Foxp3 gene (Figure 4b). On the other hand, there were differences in the distributions of these clones. Although the Vα2 CDR3 sequence of MLK2 was only identified in Vα2^{high}CD5^{high}CD4$^+$ T cells, the Vα2 CDR3 sequence of MLK3 was identified in both Vα2$^+$CD5$^+$CD4$^+$ T cells and Vα2^{high}CD5^{high}CD4$^+$ T cells.

The complementary DNA (cDNA) of the MLK2α- and β-chain, or the MLK3α- and β-chain, were subcloned into a bicistronic retrovirus vector. After retroviral infection, the transduction efficiency of the MLK3 clonotype was

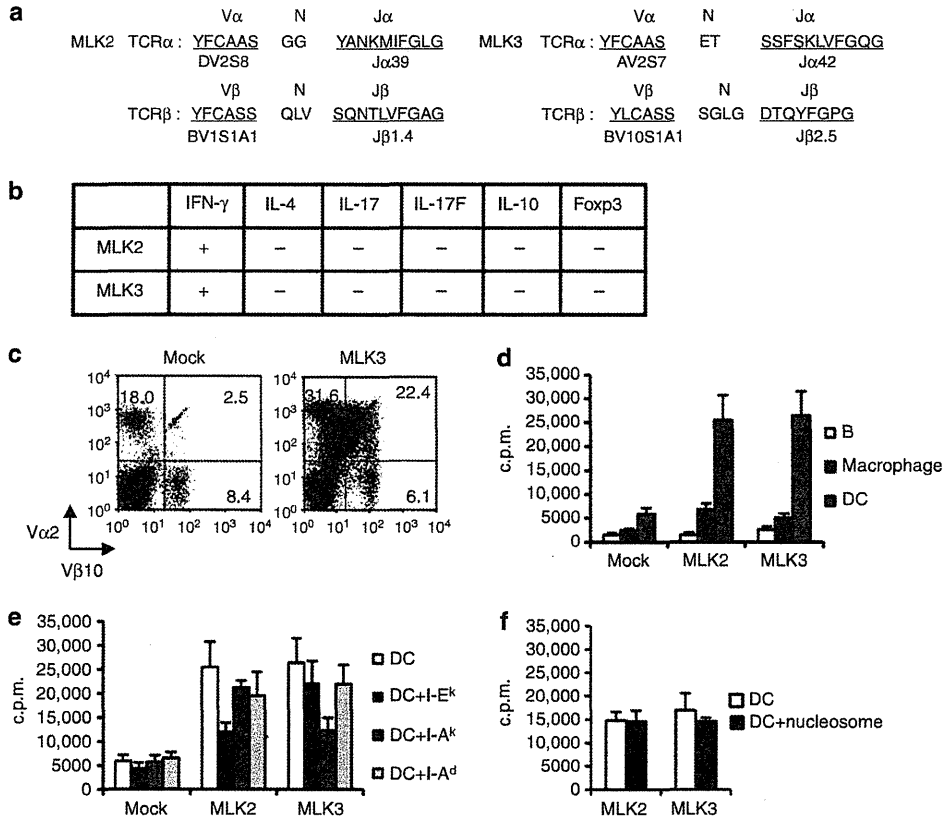


Figure 4 | MLK2- and MLK3-transduced CD4$^+$ T cells displayed major histocompatibility complex class II-restricted autoreactivity to dendritic cells (DCs). (**a**) Complementarity determining region 3 amino-acid sequences of MLK2 and MLK3 TCRα/β chains identified from Vα2^{high}CD5^{high}CD4$^+$ single T-cell clones in the early inflamed kidney. (**b**) Expression levels of interferon (IFN)-γ, interleukin (IL)-4, IL-17, IL-17F, IL-10, and the Foxp3 gene in MLK2- and MLK3-tranduced cells were determined by two-step nested PCR. (**c**) Representative results for the retroviral transduction of MLK3 into MRL/lpr splenocytes. pMXW (mock)- or MLK3-transduced CD4$^+$ T cells were stained for Vα2, Vβ10, and CD4, and CD4-gated dot plots are shown. (**d**) Representative results for the autoreactivity of MLK2- and MLK3-transduced CD4$^+$ T cells. TCR-transduced CD4$^+$ T cells were cultured with CD19$^+$ B cells (white bar), F4/80$^+$ macrophages (black bar), or CD11c$^+$ DC (dark gray bar) in 96-well plates. Proliferation was assessed by measuring ^{3}H-thymidine incorporation. (**e**) Blocking effects of anti-I-E^k and anti-I-A^k antibodies on the proliferative responses of MLK2 and MLK3 to splenic CD11c$^+$ DC. CD11c$^+$DC (white bar), CD11c$^+$DC plus anti-I-E^k (black bar), CD11c$^+$DC plus anti-I-A^k (dark gray bar), and CD11c$^+$DC plus anti-I-A^d (light gray bar). (**f**) Representative results for the proliferation of MLK2- and MLK3-transduced CD4$^+$ T cells in response to the addition of nucleosomes in the presence of DCs. TCR-transduced CD4$^+$ T cells were cultured with CD11c$^+$ DC alone (white bar) or with CD11c$^+$ DCs and nucleosomes (black bar) in 96-well plates. Their proliferation was assessed by measuring their ^{3}H-thymidine incorporation. The addition of nucleosomes did not result in a significant increase in proliferation in the MLK2- or MLK3-transduced CD4$^+$ T cells. (**d–f**) Results represent means ± s.e.m. of triplicate wells. Data shown are representative of three independent experiments with similar results.

determined using anti-Vα2 and Vβ10 antibodies (mean clonotypic transduction efficiency: 20–30%; Figure 4c). Although we could not determine the transduction efficiency of the MLK2 clonotype owing to a lack of anti-mouse Vβ1 antibody, the transduction efficiency of Vα2 in MLK2 was similar to that in MLK3 (data not shown). The MLK2- and MLK3-transduced CD4$^+$ T cells displayed a proliferative response to the splenic DCs of prenephritic MRL/lpr mice, whereas neither the splenic B cells nor macrophages induced the proliferation of MLK2- or MLK3-transduced CD4$^+$ T cells (Figure 4d). When the MHC class II restriction of autoreactivity was examined, it was found that anti-I-E^k monoclonal antibody (mAb) blocked the proliferative response of the MLK2-transduced CD4$^+$ T cells to splenic DCs. In contrast, the proliferative response of the MLK3-transduced CD4$^+$ T cells to splenic DCs was inhibited by anti I-A^k mAb (Figure 4e). Therefore, the I-E^k molecule presents the cognate antigen to MLK2, and the I-A^k molecule presents the cognate antigen to MLK3. Although systemic autoantigens were candidates for the antigen recognized by inflammatory T cells in MRL/lpr mice, both the MLK2- and MLK3-transduced CD4$^+$ T cells failed to display enhanced proliferation in response to the addition of nucleosomes in the presence of DCs (Figure 4f).

Adoptive transfer of MLK3-transduced cells exacerbated nephritis progression without increasing anti-double-stranded (ds) DNA antibody production

We then examined the *in vivo* dynamics of MLK3-transduced CD4$^+$ T cells in prenephritic mice, as we were able to track

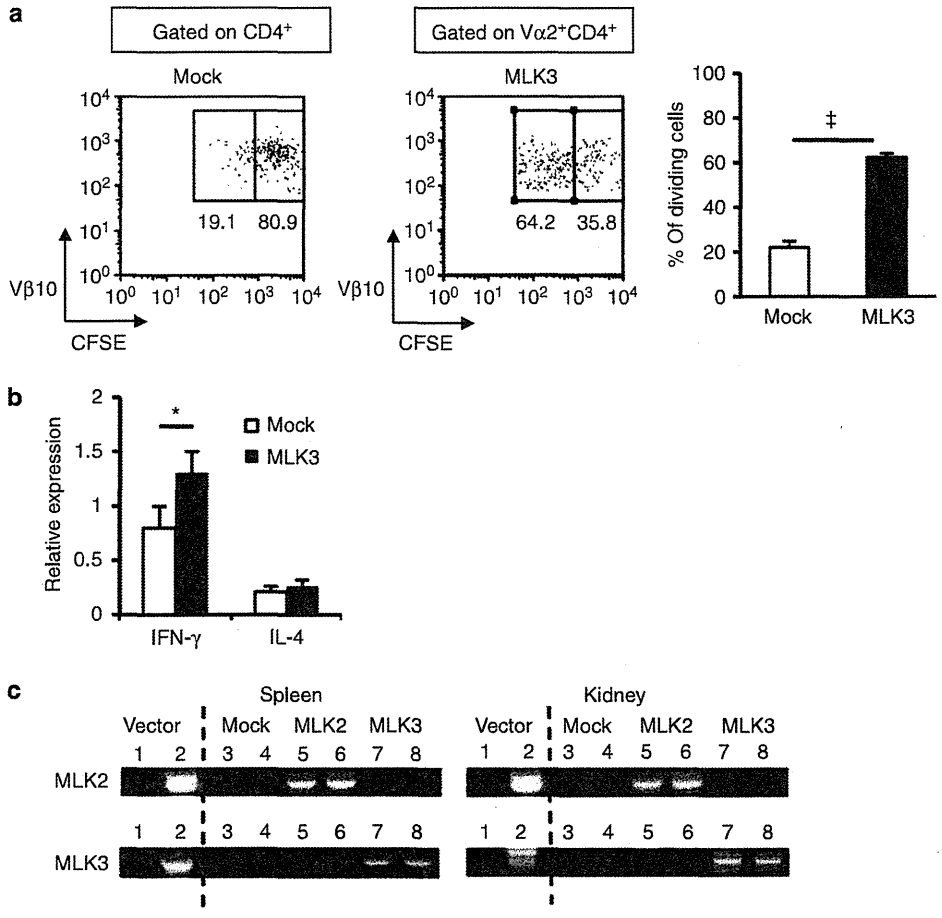


Figure 5 | Proliferation of MLK3-transduced CD4$^+$ T cells in the spleens of prenephritic mice. (**a**) Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled mock- or MLK3-transduced CD4$^+$ T cells were transferred into prenephritic MRL/lpr mice. Seven days later, the CFSE$^+$Vβ10$^+$CD4$^+$-gated (mock) or CFSE$^+$Vα2$^+$Vβ10$^+$CD4$^+$-gated (MLK3) cells in the spleen were analyzed. Representative dot plots are shown in the upper panel. The mean percentages of dividing cells among the CFSE$^+$Vβ10$^+$CD4$^+$ cells from the mock-transferred mice and the CFSE$^+$Vα2$^+$Vβ10$^+$CD4$^+$ cells from the MLK3-transferred mice are shown on the graph. The results represent the means ± s.d. of four mice. ‡A significant difference ($P<0.001$) compared with the mock-transferred mice. (**b**) CFSE$^+$Vα2$^+$CD4$^+$ cells from the mock-transferred mice and CFSE$^+$Vα2$^+$Vβ10$^+$CD4$^+$ cells from the MLK3-transferred mice were sorted, and their complementary DNA was synthesized. Cytokine expression was examined by quantitative PCR. Results represent the means ± s.e.m. of four mice. *A significant difference ($P<0.05$) compared with the mock-transferred mice. (**c**) Nested PCR amplification of T-cell receptor (TCR) α-chain sequences from the spleens and kidneys of mock-, MLK2-, and MLK3-transferred mice 1 week after the adoptive transfer ($n=5$). Lane 1: pMXW; lane 2: pMX-MLK2-TCR or pMX-MLK3-TCR vector as a positive control; lanes 3 and 4: mock-transferred mice; lanes 5 and 6: MLK2-transferred mice; lanes 7 and 8: MLK3-transferred mice. The size of the expected bands was 493 bp for both MLK2 and MLK3. Results from two representative mice are shown. IFN, interferon; IL, interleukin.

the MLK3-transduced cells using anti-Vα2 and Vβ10 antibodies. Mock- or MLK3-transduced CD4$^+$ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and transferred to prenephritic mice via the tail vein. Seven days after the transfer, the Vα2$^+$Vβ10$^+$CFSE$^+$CD4$^+$ T cells in the spleens of the MLK3-transferred mice displayed lower CFSE fluorescence than the Vα2$^+$CFSE$^+$CD4$^+$ T cells in the spleens of the mock-transferred mice (Figure 5a). The Vα2$^+$Vβ10$^+$CFSE$^+$CD4$^+$ T cells in the spleens of the MLK3 group displayed significantly higher IFN-γ expression than the Vα2$^+$CFSE$^+$CD4$^+$ T cells in the spleens of the mock-transferred mice (Figure 5b). Although the difference in IFN-γ expression between the groups was relatively small, the strong T-cell activation upon retroviral gene transfer might have been associated with elevated background IFN-γ expression in the mock-transduced cells. Furthermore, the migration of MLK2- or MLK3-transduced CD4$^+$ T cells to the kidney was confirmed by the detection of the TCRα chain sequence of MLK2 or MLK3 by PCR in the spleens and kidneys of the MLK2- or MLK3-transferred mice (Figure 5c).

Next, the pathogenicity of the MLK2- and MLK3-transduced cells was investigated in an *in vivo* transfer experiment. Two million mock-, MLK2-, or MLK3-transduced CD4$^+$ T cells were intravenously transferred to 12-week-old prenephritic MRL/lpr mice. Intriguingly, the MLK3-transferred mice developed nephritis earliest and

displayed the shortest survival among the three groups (Figure 6a). The MLK3-transferred mice displayed severe glomerulonephritis, mesangial proliferation, and thickening of the capillary walls at 26 weeks of age (Figure 6b). C3 deposition was significantly enhanced in the kidneys of the MLK3-transferred mice (Figure 6c). Although the difference in IgG deposition between the mock- and MLK3-transferred mice was not significant, there was a tendency toward increased IgG deposition in the MLK3-transferred mice. In accordance with the previous findings that Th1 cells activate macrophages[29] and macrophages have pathogenic roles in murine lupus nephritis,[30,31] macrophage infiltration was enhanced in the kidneys of the MLK3-transferred mice (Figure 6d). Although it was difficult to identify the TCRα chain of MLK3 by flow cytometry or immunofluorescent staining, the TCRα chain sequence of MLK3 was detected by PCR in the spleens and kidneys of MLK3-transferred mice at 26 weeks of age (Figure 6e). The TCRα chain sequence of MLK3 was not detected in the lungs or inguinal lymph nodes of the MLK3-transferred mice, which was suggestive of the kidney-specific accumulation of MLK3-transduced cells. In contrast to the progression of nephritis, the IgG anti-dsDNA antibody titers of the three groups were not significantly different (Figure 6f). Moreover, the total IgG, IgG2a, and IgG3 levels of the three groups were not significantly different (Figure 6g). These results suggest that MLK3-expressing $CD4^+$ T cells significantly enhance nephritis progression without affecting anti-dsDNA antibody production.

DISCUSSION

We demonstrated the nephritis-promoting effect of a kidney-infiltrating $CD4^+$ T-cell clone in lupus-prone mice. Although it is difficult to isolate and culture cytokine-expressing clones that have infiltrated a parenchymatous organ, a combination of single-cell sorting and TCR reconstitution could verify the pathological role of kidney-infiltrating $CD4^+$ T cells in lupus-prone mice.

In experimental autoimmune encephalomyelitis, different levels of autoreactivity were produced by activating autoreactive T cells in the central nervous system.[32] In the aforementioned study, highly pathogenic T cells were significantly activated within the central nervous system to express IFN-γ, and our result is consistent with this observation. However, autoreactivity and IFN-γ expression are not sufficient for pathogenicity, because only MLK3 was found to be pathogenic in our analysis. The reasons for the differential effects of MLK2 and MLK3 are not clear. Both the MLK2- and MLK3-transduced $CD4^+$ T cells failed to recognize nucleosomes and displayed autoreactivity that was not related to autoimmune inflammation because they exhibited similar degrees of proliferation to DCs from MRL/lpr, MRL/+, and haplotype-matched non-lupus-prone C3H mice (data not shown). MLK2 and MLK3 might recognize autoantigens that are irrelevant to humoral autoimmunity, because the transfer of MLK2- and MLK3-expressing T cells did not increase anti-dsDNA antibody titers or the amount of total IgG.

Our observation that MLK3-transduced $CD4^+$ T cells enhanced the deposition of C3 without enhancing humoral immunity is worth noting. IFN-γ was suggested to contribute to enhanced immune complex deposition in previous studies, which found that the deposition of IgG and C3 was decreased in the glomeruli of MRL/lpr mice lacking the IFN-γ receptor[11] or IL-12p40.[30] The more marked decrease in C3 deposition than in IgG deposition observed in these mice might be in accordance with our results. It was reported that IFN-γ induces the expression of the high-affinity receptor for IgG.[33] Therefore, we speculate that IFN-γ produced by MLK3-transduced cells might promote kidney inflammation and Fc-receptor expression to augment immune complex deposition. However, the precise mechanism responsible for the MLK3-induced augmentation of C3 deposition should be investigated further.

We selected CD5 and TCR expression levels as indicators of autoreactive cytokine-secreting T cells. However, several major T-cell clones from the MRL/lpr kidney were not detected in the single cell–sorted $V\alpha2^{high}CD5^{high}CD4^+$ T-cell populations. It is not clear whether the major clones in the MRL/lpr kidney are stimulated *in situ* and express pathogenic cytokines. The high expression levels of CD5 and TCRs did not correlate with strong clonal expansion in the kidney (Figure 3b), and there is a possibility that another combination of markers could be used to identify the T-cell clones that expand most and display the highest cytokine expression. Nevertheless, our results indicate that CD5 and TCRs are useful markers for identifying autoreactive pathogenic clones.

In previous studies, several antigen-specific TCRs were obtained from hybridomas stimulated with antigen *in vitro*, such as myelin basic protein-specific TCR,[34] nucleosome-specific TCR,[3] and Type II collagen-specific TCR.[35] Although T cells with these TCR accumulate at inflammatory sites upon adoptive transfer,[36,37] it is not known whether they reflect the true nature of autoantigen-specific TCRs at *in vivo* inflammatory sites. Using our method, we were able to address the pathological contribution of organ-infiltrating T cells to inflammatory diseases.

In summary, we have identified pathogenic autoreactive TCRs from the kidneys of early nephritic MRL/lpr mice via single-cell analysis. Our results suggest that IFN-γ-expressing $CD4^+$ T cells in the inflamed kidneys of MRL/lpr mice contribute to nephritis progression. Our methods will enable us to analyze the characteristics of inflammatory T cells that have infiltrated parenchymatous organs.

MATERIALS AND METHODS

Animals

MRL/lpr and MRL/+ mice were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were conducted in accordance with the relevant institutional and national guidelines.

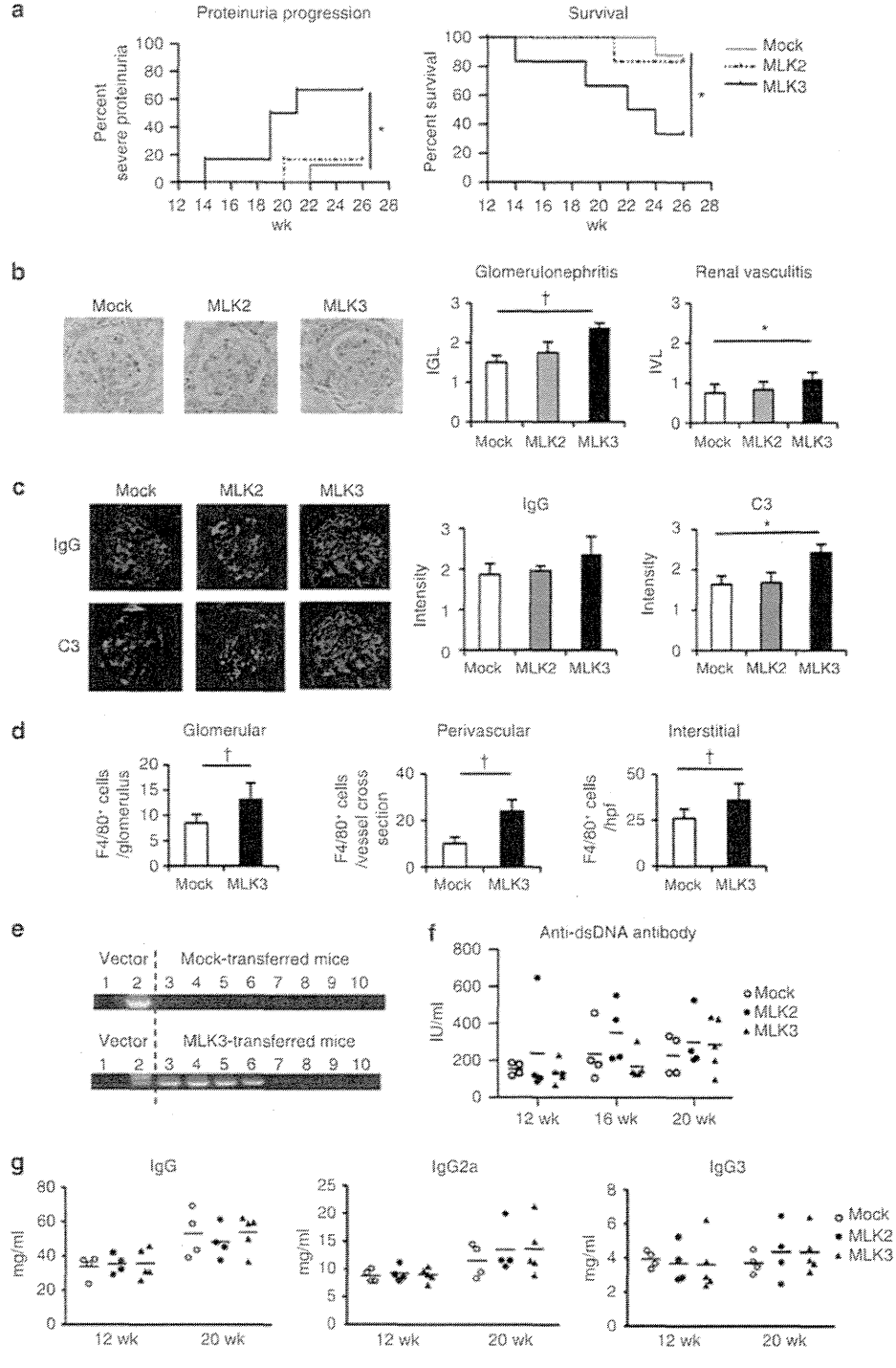
Reagents, antibodies, and media

Fc block (anti-CD16/CD32) and the following purified mAbs were purchased from BD Bioscience (San Jose, CA): FITC-conjugated

anti-CD4 (L3T4), allophycocyanin-conjugated anti-CD4, PE-conjugated anti-Vα2 (B20.1), FITC-conjugated anti-Vβ10(B21.5), FITC-conjugated anti-CD5 (53-7.3), PE-conjugated anti-CD5, biotin-conjugated anti-CD11c (HL3), biotin-conjugated anti-CD19 (1D3), and biotin-conjugated anti-CD8a (53-6.7). Recombinant murine IL-2 was obtained from R&D Systems (Minneapolis, MN). The T cells and the packaging cell line Plat-E[38] were cultured as described previously.[28,39,40]

Cell purification

A $CD4^+$ T-cell population was prepared by negative selection with MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) using biotin-conjugated anti-CD19 mAb, biotin-conjugated anti-CD11c mAb, and biotin-conjugated anti-CD8a mAb, followed by streptavidin-conjugated MicroBeads (Miltenyi Biotec). $CD11c^+$ DC and $F4/80^+$ macrophages were prepared as described previously.[28,40] Briefly, minced spleen cells were digested with collagenase type IV (Sigma-



Aldrich, St Louis, MO), and single-cell suspensions were incubated with anti-F4/80-biotin mAb (Caltag Laboratories) and anti-CD11c-conjugated MicroBeads. After positive selection for CD11c$^+$ cells, a mean purity of 85% was achieved. The negative fraction was additionally incubated with streptavidin-conjugated MicroBeads, and F4/80$^+$ macrophages were isolated with two rounds of positive selection (>90% purity). For CFSE labeling, the cells were incubated with 5 μmol/l CFSE (Molecular Probes, Eugene, OR) for 5 min at 37 °C. For the single kidney cell preparations, anesthetized mice were killed by cardiac perfusion with phosphate-buffered saline, and their minced tissues were digested in medium containing type IV collagenase (Sigma-Aldrich), strained through nylon mesh, and washed with phosphate-buffered saline. IFN-γ secretion by CD4$^+$ T cells was evaluated using the mouse IFN-γ secretion assay detection kit (Miltenyi Biotec).

Single-cell sorting and cDNA synthesis

The CD4$^+$ T cells from the MRL/lpr mouse kidneys were stained with FITC-conjugated anti-Vα2, PE-conjugated anti-CD5, and APC-conjugated anti-CD4. The Vα2^{high}CD5^{high}CD4$^+$ T cells were sorted at a ratio of one cell per well using an automatic cell-dispensing unit driven by a FACS Vantage cell counter and the Clone-Cyt software (BD Biosciences). RNA was extracted using an RNeasy Micro kit (Qiagen, Valencia, CA). A modified version of the T7 antisense RNA amplification method was used.[41] Briefly, a ds cDNA library containing a T7 RNA polymerase promoter site at its 5′ end was produced from the input messenger RNA and transcribed using T7 RNA polymerase. The process was repeated in a second round. For the first round, the T7polyT primer, T7Cα30 primer, and T7Cβ30 primer were used (primer sequences are listed in Table 1). Superscript III (200 U per reaction; Invitrogen, Carlsbad, CA) was then used to synthesize cDNA in the presence of RNasin (Promega, Madison, WI)-containing buffer for 60 min at 50 °C. The second strand was synthesized, and the cDNA was purified as described previously.[41] The ds cDNA carrying the T7 RNA polymerase promoter was transcribed using an Ampliscribe transcription kit (Epicentre, Madison, WI), and the resultant RNA was purified using an RNeasy kit (Qiagen). cDNA was synthesized using random hexamers (Invitrogen) and Superscript III followed by second-strand synthesis.

Identification of TCR gene sequences

The TCRα and β genes were identified by three-step nested or semi-nested PCR, as described.[39] The primers used for the TCRα amplification are listed in Table 1. The following primers were used for the TCRβ amplification: the BV1-19 primers used for the TCR-SSCP analysis, and the CB-1st primer, CB-2nd primer, and CB-3rd primer. Foxp3 and cytokine (IFN-γ, IL-4, IL-17, IL-17F, and IL-10) gene expression levels were assessed by two-step nested PCR. The primer pair sequences are listed in Tables 1 and 2. The CDR3 sequences of the MLK2 and MLK3 TCRα chains were combined with the Vα2 and Jα39-Cα sequences and Vα2 and Jα42-Cα sequences, respectively, using heteroduplex PCR, as described.[28] The CDR3 sequences of the MLK2 and MLK3 TCRβ chains were combined with the Vβ1 and Jβ1.4-Cβ sequences and Vβ10 and Jβ2.5-Cβ sequences, respectively.

Single-strand conformational polymorphisms

The SSCP study was conducted as described previously.[25,42]

Vector construction and retroviral gene transfer

The vectors pMX-MLK2-TCR (pMX-MLK2α-internal ribosome entry site (IRES)-MLK2β) and pMX-MLK3-TCR (pMX-MLK3α-IRES-MLK3β) were constructed and used to transduce the desired TCR clonotype into activated CD4$^+$ T cells. The full-length sequences of the MLK2 and MLK3 TCRα and β chains were synthesized from a combination of the V, CDR3, and C region sequences using heteroduplex PCR, as described previously.[28] Retroviral gene transfer was performed as described previously.[28,40] In brief, total splenocytes were cultured for 48 h in the presence of concanavalin A (10 μg/ml) and mIL-2 (50 ng/ml; R&D Systems), and stimulated splenocytes were cultured in each well of retrovirus-coated 24-well plates for 36 h. The TCR-transduced CD4$^+$ T-cell proliferation assay was performed.[43]

Real-time PCR

Real-time quantitative PCR was performed using the SYBR Green Master Mix (Qiagen) and an iCycler (Bio-Rad, Hercules, CA). Primer pairs were selected as described previously for glyceraldehyde 3-phosphate dehydrogenase, IFN-γ, and IL-4.[39] The PCR

Figure 6 | MLK3-transduced CD4$^+$ T cells promoted nephritis without inducing a significant elevation of the anti-double-stranded (ds) DNA antibody titer upon adoptive transfer to prenephritic MRL/lpr mice. (**a**) Two million mock-, MLK2-, or MLK3-transduced CD4$^+$ T cells were intravenously transferred to 12-week (wk)-old MRL/lpr mice. Cumulative proteinuria progression and survival are shown. *$P<0.05$ compared with the mock-transferred mice according to the Kaplan–Meier method. (**b**) Histological evaluation of glomerulonephritis in mock-, MLK2-, or MLK3-transferred mice at 26 weeks of age. Kidney sections from mock-, MLK2-, or MLK3-transferred mice were stained with hematoxylin and eosin. The degree of tissue damage was graded as described in Materials and Methods. Values are shown as the mean and s.d. index of glomerular lesions (IGL; 40 random glomeruli per kidney) or index of vascular lesions (IVL; all vessels in the section; $n=6$). *A significant difference ($P<0.05$) compared with the mock-transferred mice. †A significant difference ($P<0.01$) compared with the mock-transferred mice. No significant difference was observed between the MLK2- and MLK3-transferred mice. (**c**) Kidney sections from mock-, MLK2-, or MLK3-transferred mice stained with anti-IgG or anti-C3 at 26 weeks of age ($n=5$). Representative immunofluorescent staining of C3 and IgG is shown. The mean ± s.e.m. of semiquantitative scoring of IgG and complement C3 deposits is shown at the right panel. *A significant difference ($P<0.05$) compared with the mock-transferred mice. No significant difference was observed between the MLK2- and MLK3-transferred mice. (**d**) The numbers of F4/80$^+$ macrophages in the glomerular, perivascular, and interstitial regions were assessed in the mock- and MLK3-transferred mice ($n=5$). Values are shown as the mean ± s.e.m. †A significant difference ($P<0.01$) compared with the mock-transferred mice. (**e**) Nested PCR amplification of TCRα chain sequences from the spleen, kidneys, inguinal lymph nodes (LN), and the lungs of mock- or MLK3-transferred mice at 26 weeks of age ($n=5$). Lane 1: pMXW; lane 2: pMX-MLK3-TCR vector; lanes 3 and 4: spleen; lanes 5 and 6: kidneys; lanes 7 and 8: inguinal LN; lanes 9 and 10: lungs. The size of the expected bands was 493 bp. Results for two representative mice are shown. (**f**) Serum anti-ds DNA antibody levels of the three experimental groups at 12, 16, and 20 wk of age. No significant difference was observed among the three groups at any time point. (**g**) Serum IgG, IgG2a, and IgG3 levels of the three experimental groups were measured at 12 and 20 wk of age. No significant difference was observed among the three groups.