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Naive CD4⁺ T Cells from Lupus-Prone Fas-Intact MRL Mice Display TCR-Mediated Hyperproliferation Due to Intrinsic Threshold Defects in Activation¹

Christina E. Zielinski,²* Simon N. Jacob,³‡ Farida Bouzahzah,⁴* Barbara E. Ehrlich, † and Joe Craft⁵**‡

Autoreactive T cell activation is a consistent feature of murine lupus; however, the mechanism of such activation remains unclear. We hypothesized that naive CD4⁺ T cells in lupus have a lower threshold of activation through their TCR-CD3 complex that renders them more susceptible to stimulation with self-Ags. To test this hypothesis, we compared proliferation, IL-2 production, and single cell calcium signaling of naive CD4⁺ T cells isolated from Fas-intact MRL/Fas⁺ mice with H-2k-matched B10.BR and CBA/CaJ controls, following anti-CD3 stimulation in the presence or absence of anti-CD28. We also assessed the responsiveness of naive CD4⁺ T cells isolated from Fas-intact MRL and control mice bearing a rearranged TCR specific for amino acids 88–104 of pigeon cytochrome c to cognate and low affinity peptide Ags presented by bone marrow-matured dendritic cells. TCR transgenic and wild-type CD4⁺ T cells from MRL mice displayed a lower threshold of activation than control cells, a response that was class II MHC dependent. The rise in intracellular calcium in MRL vs controls was enhanced and prolonged following anti-CD3 triggering, suggestive of proximal defects in TCR-engendered signaling as the mechanism for the observed hyperactivity. These findings were observed as early as 1–2 mo postweaning and, based on analysis of F₁ T cells, appeared to be dominantly expressed. This genetically altered threshold for activation of MRL T cells, a consequence of a proximal defect in CD3-mediated signal transduction, may contribute to the abrogation of T cell tolerance to self-Ags in lupus. The Journal of Immunology, 2005, 174: 5100–5109.

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¹ Section of Rheumatology, Department of Medicine, ² Departments of Pharmacology, and Cellular and Molecular Physiology, and ³ Section of Immunobiology, Yale University School of Medicine, New Haven, CT 06520

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The autoimmune disease systemic lupus erythematosus (SLE) is characterized by high titered IgG autoantibodies to certain intracellular components, including chromatin and ribonucleoproteins (1). Several inbred mouse strains also develop spontaneous lupus, with the same spectrum of pathogenic autoantibodies resulting in immune complex glomerulonephritis as in human lupus (2). Autoantibody production in lupus requires αβ CD4⁺ T cell help, a concept first supported by the observation that neonatal thymectomy of lupus-prone mice led to abrogation of anti-dsDNA IgG synthesis, glomerulonephritis, and to increased survival (3). Similar results were obtained after Ab depletion of either T cells (4) or CD4⁺ cells (5), or genetic deletion of αβ or CD4⁺ T cells in lupus-prone mice (6, 7).

Autoantigen-specific CD4⁺ T cells appear to be both sufficient and necessary for disease promotion in murine lupus, as demonstrated by disease induction after adoptive transfer of chromatin-reactive T cells to prenephritic lupus-prone mice (8), and by disease abrogation with replacement of potentially autoreactive T cells in lupus-prone mice with T cells of a single specificity (9, 10). Such autoreactive T cells appear to be specific for ubiquitous self-peptides in murine and human lupus, including those derived from chromatin and ribonucleoproteins (8, 11, 12) and therefore would lead to enhanced B cell autoimmunity, which is critical for pathogenic Ab production. Autoantigen-specific T cells in lupus presumably oligoclonally expand (13) as a consequence of ongoing T cell-B cell collaboration (14). Nevertheless, polyclonal CD4⁺ T cell activation is a hallmark of murine (including Fas-intact) lupus (15). Moreover, initial B cell help and follicular migration in lupus appear to depend upon polyclonal-activated T cells, rather than merely upon monoclonal or oligoclonal populations (16), adding credence to the notion that polyclonal activation is critical for disease initiation.

A body of evidence indicates that mature T cells isolated from humans and mice with lupus are intrinsically abnormal, leading to aberrant activation and effector function in the periphery. In vitro observations have shown that T cells from patients with SLE have abnormalities in TCR signaling (reviewed in Ref. 17) and anergy avoidance (18, 19), as well as in expression of effector molecules, including CD40L (CD154) (20, 21). Genetic studies have also suggested that a locus on chromosome 7 from lupus-prone New Zealand mixed mice contributes to increased T cell activation and a higher threshold for apoptotic death (22), although these latter defects may be secondary to APC abnormalities (23).
Although lupus T cells have defects that convey aberrant activation and effector function, the mechanisms of tolerance escape for T cells responsive to ubiquitous self-peptides in lupus remain unknown. Central tolerance appears intact to conventional peptide Ags (24), including in Fas-intact (CD95) mice (25); thus, it appears that autoreactive T cells of low enough affinity to escape central deletion must bypass normal tolerance mechanisms in the periphery. Support for this notion comes from observations that naive, mature CD4 + T cells from lupus-prone Fas-intact MRL/Mp+Fas-lpr (MRL+/+Fas-lpr) mice appear to be intrinsically hyper-responsive compared with control T cells following engagement with high- and low-affinity MHC-peptide complexes in vitro (26) and avoid tolerance induction by neo-self-Ag in vivo (27). Based upon this logic and these findings, we have hypothesized that defects in lupus T cells would render them more susceptible to activation through their TCR-CD3 complex after contact with self-peptides in the periphery that have a low affinity for TCR engagement (28). The current work provides additional support for this hypothesis with the observation that naive CD4 + T cells from lupus-prone MRL+/+Fas-lpr mice have a lower threshold of TCR-CD3-mediated activation than control cells, an abnormality that appears intrinsic to these cells and independent of the extrinsic stimulatory potential of dendritic cells (DC). Proximal defects in TCR-engendered signaling provide a mechanism for this phenotype. An altered threshold for activation of MRL T cells may contribute to the abrogation of T cell tolerance to ubiquitously displayed self-Ags in lupus with subsequent autoreactive T cell activation and B cell help for autoantibody production.

Materials and Methods

Mice

Inbred Fas-intact MRL+/+Fas-lpr, B10.BR, CBA/CaJ, AKR/J, C57BL/6 (B6), invariant chain-deficient (B6.129-D1Tm1Liz; B6.12/3), and RAG1-deficient (B6.129S-Rag1tm1Mom; RAG1-/-) mice were purchased from The Jackson Laboratory. AND TCR transgenic mice expressing an αβ TCR (Vα11, Vβ3) recognizing amino acids 88–103 of pigeon cytochrome c (PCC) were originally provided on the H-2 k-B10.BR (B10.BR.AND) background by Dr. H. Heinzel, University of California, San Diego, CA (29). The transgenic locus was serially backcrossed to the MRL+/+Fas-lpr (MRLAND) and the H-2 k-matched control backgrounds CBA/Ca (CBA.AND) and AKR/J (AKR.AND) for 20, 10, and 6 generations, respectively. We also backcrossed the RAG1-deficient locus to the MRL and B10.BR backgrounds for >20 and >10 generations, respectively, with a subsequent intercross to MRL and B10.BR. Animals were maintained in IIE-/-, CD11c+/-, RAG1-/-/B10.BR.MRD mice. The test strains lack endogenous (viral) superantigens, products of murine mammary tumor viruses that bind the transgenic Vβ3 chain, with resultant central deletion in the context of I-Eκ (30). Levels of expression of the transgenic Vα11 chain and the CD4 co receptor were equivalent among MRL, B10.BR, and CBA/Ca strains, as determined by flow cytometry. AND TCR transgenic mice were maintained as heterozygotes, with screening performed by PCR of tail DNA with phenotypic confirmation by flow cytometry (FACS-Calibar; BD Biosciences) (10). Animals were identically housed in specific pathogen-free facilities. The Institutional Animal Care and Use Committee at Yale University School of Medicine (New Haven, CT) approved all experiments.

T cell preparation

Pooled axillary, brachial, cervical, and inguinal lymph node and spleens from 1- to 2-month-old wild-type (WT) or AND transgenic mice were dissociated mechanically to obtain single cell suspensions, followed by hypotonic shock for RBC lysis. CD4 + TCR transgenic T cells were enriched by negative selection to avoid activation before in vitro stimulation. All Abs used for negative selection and for flow cytometry were from BD Pharmingen. Briefly, the cells were labeled with biotinylated Ab to CD8 (53-6.7), used for negative selection and for flow cytometry were from BD Pharmingen. Supernatants from T cell/DC coculture specimens were stored at −18°C until used in this assay according to the instructions of the manufacturer. Supernatant from T cell/DC coculture experiments were dislodged and replated with fresh media at 1 × 10^6 cells/ml. Nonadherent cells were harvested and assessed for surface expression of CD11c (HL3), B7-1 (16-10A1), B7-2 (GL1), and I-Eκ using the appropriate isotype controls. Only preparations containing DC that had >60–70% expression of B7 molecules and class II MHC at day 8 were used. DC were treated with mitomycin C and then cocultured for 7 h with T cells (DC to T cell ratio 2:1). Expression of CD80, CD86, CD11c, and I-Eκ on bone marrow-derived cells before and 8 days after culture was equivalent across the three strains. The maturation of DC was enhanced in some experiments by adding LPS (1 μg/ml; Sigma-Aldrich) 24 h before harvesting.

Generation of DC

DC were obtained from bone marrow precursors. In brief, bone marrow suspensions were obtained from the tibias and femurs of 4- to 7-week-old mice and depleted of erythrocytes with RBC lysing buffer and of lymphocytes by complement-mediated lysis using treatment with purified mAbs for the B220 (BD4, and B220). The remaining bone marrow cells were depleted at 1 × 10^6 cells/ml in complete RPMI 1640 supplemented with GM-CSF and cultured for 8 days in 24-well plates. On day 2, nonadherent granulocytes were gently removed and replaced by fresh media (1 ml/well). Subsequently, every 48 h before harvesting loosely adherent DC clusters were dislodged and replated with fresh media at 1 × 10^6 cells/ml. Nonadherent cells were harvested and assayed for surface expression of CD11c (HL3), B7-1 (16-10A1), B7-2 (GL1), and I-Eκ using the appropriate isotype controls. Only preparations containing DC that had >60–70% expression of B7 molecules and class II MHC at day 8 were used. DC were treated with mitomycin C and then cocultured for 7 h with T cells (DC to T cell ratio 2:1). Expression of CD80, CD86, CD11c, and I-Eκ on bone marrow-derived cells before and 8 days after culture was equivalent across the three strains. The maturation of DC was enhanced in some experiments by adding LPS (1 μg/ml; Sigma-Aldrich) 24 h before harvesting.

T cell stimulation assays

T cell proliferation assays as measured by uptake of [H]thymidine were performed in 96-well flat-bottom tissue culture plates. A total of 2 × 10^5 T cells in a final volume of 200 μl of Click’s media were cultured in triplicates for 72 h in anti-CD3-coated (2C11) wells at titrated concentrations in the presence or absence anti-CD28 at 1 μg/ml and pulsed with 1 μCi/ml [H]thymidine (38–40).

For measurement of CD69 and CD154 (CD40L), T cells were cultured under the same conditions as described in 24-well flat-bottom tissue culture plates at 1 × 10^6 cells/well. Surface markers were assessed by flow cytometry in triplicate wells, using isotype controls. Analysis was limited to live CD4 + T cells, as determined by gating and by forward and side scatter.

In coculture studies T cells were cultured for 72 h in the presence of mitomycin C-treated bone marrow-matured DC at the ratios indicated at 1 × 10^6 cells/ml and harvested as described. DC were pulsed either with the dominant agonist peptide PCC (PCC 88–104) KAERADILAYL KQATAK (peptide purity >90% by HPLC analysis; American Peptide Company), with the altered peptide ligand (APL) K9QQ (both 5 μM), or cocultured without any external peptide. K9QQ differs from PCC 88–104 by a glutamine substitution for lysine at position 99 (peptide purity >90%; W.M. Keck Foundation Biomedical Resource Laboratory, Yale University, New Haven, CT). This peptide functions as a weak antagonist of the AND TCR (34, 35), and is of such low affinity for the transgenic TCR that when presented in vitro by APC, it does not lead to CD69 up-regulation, T cell activation, or ZAP70 phosphorylation, along with only minimal phosphorylation of CD3ζ (35–37). This pattern is consistent with a weak partial agonist or antagonist peptide (35), and similar to the interaction of CD4 + T cells and self-peptide MHC ex vivo (38–40).

Cytokine production

IL-2 production was determined by intracellular cytokine staining. Negatively selected naive CD4 + T cells were stimulated for 2 h with anti-CD3 mAb (2C11, 10 μg/ml) with or without anti-CD28 (1 μg/ml) and pulsed with brefeldin A (GolgiPlug; BD Pharmingen) for another 4 h. They were then harvested and fixed with 2% paraformaldehyde for 20 min at room temperature and permeabilized with saponin (0.5% in PBS) for 10 min (Sigma-Aldrich). Cells were incubated with PE-labeled anti-H-2 mAb (BD Pharmingen) and isotype controls at room temperature. After 1 h, they were washed and then incubated with FITC-conjugated anti-CD4 (RM4-5), followed by flow cytometric analysis.

IL-2 production by TCR transgenic T cells that were cocultured with peptide-pulsed DC was measured using the BD cytometric bead array (BD Biosciences). Supernatant from T cell/DC coculture specimens were stored at −18°C until used in this assay according to the instructions of the manufacturer.

Single cell calcium imaging

CD4 + T lymphocytes from MRL.AND, B10.BR.AND, and CBA.AND transgenic mice were isolated as described and used on the same day for experiments. Cells in complete PBS supplemented with 1% FCS, 50 μM penicillin, 50 μg/ml streptomycin, and 1 mM L-glutamine (In Vitro Technologies) were loaded with 4 μM Fluo-4 (Molecular Probes) in 20% Pluronic F-127 in DMEM (Molecular Probes) for 10 min at room temperature. Hamster anti-CD3 (10 μg/ml) provided by A. Badou (Yale University) was added, and cells were transferred to ice for 30 min to minimize

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from MRL mice were hyperproliferative after anti-CD3 engagement as the ratio of fluorescence intensity determined by [3H]thymidine incorporation. The results are representative of at least seven independent experiments. All experiments were performed at 21°C.

**Statistics**

Data were analyzed with the unpaired Student t test with two-tailed p-value calculations. Error bars indicate SD of triplicate measurements. Data shown are representative of at least three separate experiments, if not indicated otherwise. At least two age- and sex-matched animals per strain were used in each experiment.

**Results**

**Naive CD4+ MRL T cells have a lower threshold of activation than control T cells after stimulation with anti-CD3**

We initially addressed the question whether naive CD4+ T cells from MRL mice were hyperproliferative after anti-CD3 engagement, compared with cells from H-2-matched B10.BR and CBA/CaJ mice. For these experiments, we used naive CD4+ TCR transgenic T cells (CD44low, CD62Lhigh) from MRL.AND, B10.BR.AND, and CBA.AND mice to ensure that our starting populations were homogeneous. AND T cells were initially stimulated with varying concentrations of immobilized anti-CD3, with or without anti-CD28. Under these conditions, MRL.AND T cells proliferated significantly more than their control counterparts (Fig. 1, A and B; Table I). These differences became more striking as the intensity of stimulation declined suggesting that MRL T cells have an altered threshold for responsiveness (Fig. 1, A and B, right panels; Table I). Although addition of anti-CD28 augmented proliferation in all the strains tested, differences between responses in MRL vs control T cells remained (Fig. 1A). Moreover, the necessity for costimulation in the lupus-prone strain to achieve similar phenotypic responses was less stringent (Fig. 1B). The same hyperresponsive phenotype of MRL T cells was observed when comparing naive CD4+ T cells from WT (nontransgenic) mice (Fig. 1, C and D; Table I). This suggests that our initial results using T cells from AND mice was not attributable to the presence of the TCR transgenes, although we did observe that proliferation of WT T cells was somewhat greater than that for TCR transgenic cells at low concentrations of anti-CD3; the reasons for this finding are unclear. The proliferation assay was also performed with T cells from RAG1+/− MRL.AND and RAG1−/− B10.BR.AND mice. Again, MRL cells were hyperresponsive compared with controls (Fig. 1E). Thus, the MRL phenotype was not a consequence of altered TCR-α rearrangements in this strain, and presumably not a consequence of any suppressor cell abnormality (41).

To rule out the possibility that altered proliferation in MRL T cells was confounded by differences in T cell survival over the 72 h incubation period, an apoptosis assay was conducted with the same T cell populations that were used for proliferation assays, testing for annexin-5 (early apoptotic marker) and 7-aminoactinomycin D internalization of the TCR. After washing, cells were diluted to 1 × 10^6/ml in complete PBS and attached to poly-L-lysine coated coverslips (BD Biosciences) via gentle centrifugation. Extracellular solution contained (mM): 155 NaCl, 4.5 KCl, 2 CaCl2, 1 MgCl2, 10 d-glucose, and 5 HEPES (pH 7.4 with NaOH). In the calcium-free solution, 2 mM MgCl2 and 1 mM EGTA were substituted for CaCl2. Before imaging, cells were washed with extracellular solution for 5 min to allow for de-esterification of the dye.

The coverslip was used as the bottom of an open superfusion chamber (Warner Instruments). The chamber was mounted onto the stage of a Zeiss Axiovert 135 inverted confocal microscope. Cells were excited with a krypton-argon laser at 488 nm, and the emission signal was detected with a 522/35 nm bandpass filter. Cells were observed using a 63× oil-immersion objective, and images consisting of a complete field of view were captured (right panels; Table I). For data analysis, fluorescence traces were synchronized before averaging so that the shape of the average response was similar to the (asynchronous) single cell recordings. The single exponential decay curve (fitted in Fig. 6B) was y = y_0 + ae^{−bx}.

There was no change in size, shape, or location of cells during the experiments. All experiments were performed at 21°C.

**FIGURE 1.** CD4+ T cells from MRL mice are hyperproliferative after CD3 engagement, compared with T cells from H-2-matched control mice. Negatively selected naive (CD44low, CD62Lhigh) CD4+ T cells were stimulated with plate-bound anti-CD3, with or without anti-CD28 (1 μg/ml), with proliferation determined by [3H]thymidine incorporation. The results are expressed as the average ± SD of triplicate measurements, and are representative of at least seven independent experiments unless indicated. A, AND TCR transgenic T cells were triggered in the presence of costimulation with anti-CD28 (1 μg/ml). B, AND T cells were cultured in the absence of anti-CD28. C and D, Nontransgenic WT T cells were stimulated with anti-CD3 plus anti-CD28 or anti-CD3 alone, respectively. E, Proliferation of RAG1−/− MRL.AND and RAG1−/− B10.BR.AND T cells was compared in the absence of costimulation (representative of two independent experiments). Magnifications (right panels) of the experiments (left panels) are shown to demonstrate responses at lower anti-CD3 concentrations.
The percentage of dead cells at 0, 24, and 72 h of anti-CD3 incubation in early apoptotic cells at 0, 24, and 72 h of anti-CD3 incubation in the presence or absence of anti-CD28 was similar for all strains, suggesting that the hyperresponsive phenotype is a consequence of increased proliferation of individual cells rather than decreased cell death (data not shown); however, we have noted differences in strain survival in longer term experiments in vivo (27).

We next assessed expression of CD69 and CD40L on MRL AND T cells from MRL mice dis- played a heightened proliferative response upon stimulation by bone marrow-derived DC. Naive CD4+ T cells from AND transgenic and nontransgenic H-2k-matched MRL and control B10.BR and CBA/CaJ mice were cocultured in vitro with DC from the (MRL × B10.BR)F1 and (MRL × CBA/CaJ)F1 backgrounds. We chose DC as APC to provide more physiologic conditions for T cell activation than through I-Ek-bearing transformed cell lines (26) or via anti-CD3. H-2k F1 DC were used in these experiments testing intrinsic differences in T cell hyperresponsiveness because previous studies have shown that APC from the MRL background have (intronically) altered cytokine production, compared with control APC (42, 43), an effect that might aberrantly contribute to MRL T cell activation in syngeneic cocultures.

AND TCR transgenic T cells were cocultured with F1 DC pulsed with agonist PCC 88–104, the weak antagonist peptide linear epitope (Materials and Methods), or without exogenous peptide. In the nominal absence of Ag, immunologic synapses form between T cells and DC eliciting small increases in intracellular calcium and proliferation in T cells (44). Proliferation as determined by [3H]thymidine uptake was significantly increased in the MRL T cells compared with control T cells in all three

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**FIGURE 2.** CD4+ T cells from MRL.AND TCR transgenic mice have enhanced up-regulation CD69 and CD40L following anti-CD3 stimulation, compared with AND T cells from H-2-matched control mice. CD4+ T cells were purified from MRL.AND, B10.AND, and CBA.AND mice, with gating on naive cells (CD44<~ and CD62L<~; >98% naive, data not shown). CD69 expression was determined by flow cytometry with PE-labeled anti-CD69 staining using isotype controls to determine the cut-off intensities. A, Cells were stimulated with plate-bound anti-CD3 (5 μg/ml). B, Cells were stimulated with different concentrations of plate-bound anti-CD3 for 3 h, and CD69 expression determined. C, For CD40L expression, cells were stimulated with plate-bound anti-CD3 (1 μg/ml) with soluble anti-CD28 (1 μg/ml); or with anti-CD3 alone (D) and harvested at different time points. CD40L expression was determined by flow cytometry with PE-labeled anti-CD40L staining using isotype controls to determine the cut-off intensities. Error bars indicate SD of triplicate measurements, *, p < 0.05 for MRL.AND compared with B10.AND and CBA.AND cells. The data are representative of two independent experiments for CD69 expression and four independent experiments for CD40L expression.
conditions. The former had a 1.7-fold increase in proliferative responses when stimulated with the cognate peptide-MHC complex PCC (Fig. 3A), and a 2.5-fold increase when challenged with low affinity (K99Q) peptide-MHC complexes (Fig. 3B). Thus, the differences in proliferation between MRL and control cells were greater with the low affinity stimulus, a notion supported by the observation that the ratio of MRL T cell proliferation vs controls was even 3.5-fold in the absence of exogenous peptide (Fig. 3C).

Non-TCR transgenic MRL CD4+ naïve T cells (CD44low, CD62Lhigh) that were stimulated with DC in the absence of exogenous peptide also displayed increased proliferation compared with controls (Fig. 3D).

To confirm the class II MHC dependence of MRL hyperresponsiveness, we cocultured AND TCR transgenic T cells with bone marrow-derived DC from B6.H-2’s’ mice. In contrast to stimulation with invariant chain-intact DC, coculture with DC from knockout mice defective in class II MHC expression largely abrogated the hyperresponsiveness of MRL. AND T cells observed in the absence of specific peptide (Fig. 3E, compare with 3C). In these experiments, proliferation of T cells from the three different strains in the absence of DC, as well as proliferation of the mitomycin C-treated DC alone served as controls (Fig. 3F).

As we used F1 DC in these experiments, albeit H-2b-matched, the higher uptake of [3H]thymidine in the non-TCR transgenic CD4+ T cells from MRL mice could have resulted from presentation of minor histocompatibility Ags from the B10.BR and/or CBA/CaJ backgrounds. This possibility seemed unlikely, however, because certain experiments were done with TCR transgenic T cells (Fig. 3, A–C), and in those done with non-TCR transgenic mice. MRL T cells proliferated essentially the same following coculture with (MRL × B10.BR)F1 and (MRL × CBA)F1 DC (Fig. 3D).

To further exclude the possibility that abnormalities in MRL DC were contributing to the observed hyperproliferation of MRL T cells, we asked whether AND TCR transgenic T cell proliferation in syngeneic cocultures, in the presence of PCC or K99Q, differed from that in H-2b-matched, but allogeneic cocultures (Fig. 4). In these experiments, proliferation of MRL and control TCR transgenic T cells was essentially independent of the origin of DC,

The DC to T cell ratio was 2:1 at a concentration of 1×10⁶cells/ml. B7-1 and B7-2 and class II MHC were up-regulated by coculture with AND TCR transgenic T cells pulsed with 5μM PCC and K99Q (5μM, respectively). C, [3H]Thymidine incorporation of mitomycin C-treated DC as well as of T cells alone served as controls. The bars represent average ± SD of triplicate measurements. The data are representative of four experiments. n.s., Not significant.
suggesting that the proliferation advantage of MRL T cells over controls is maintained even in the absence of any potential intrinsic difference in DC from the lupus-prone background. Likewise, proliferation of (MRL/H11003 × B10.BR)F1 AND (MRL/H11003 × CBA/CaJ)F1 TCR transgenic T cells in response to PCC or K99Q presented by MRL, B10.BR, and CBA/CaJ DC was similar (data not shown).

MRL T cells produce more IL-2 following TCR engagement

Next naive CD4+ AND T cells from the lupus-prone MRL and control B10.BR and CBA/CaJ strains were stimulated for 2 h with immobilized anti-CD3 (10 μg/ml) in the presence or absence of anti-CD28 (1 μg/ml), followed by treatment with brefeldin A and measurement of intracellular IL-2 by flow cytometry. The population of TCR transgenic MRL T cells producing IL-2 was clearly higher than that of B10.BR and CBA/CaJ T cells. In the presence of anti-CD28, 25% of MRL.AND T cells produced IL-2 compared with 15.3% (B10.BR.AND) and 16.2% (CBA.AND) in the control strains (Fig. 5A). In the absence of anti-CD28, the difference became more apparent with 14.7% of T cells IL-2 positive in the MRL.AND strain compared with 9.6% in the B10.BR.AND and

FIGURE 5. MRL.AND T cells produce more IL-2 following TCR engagement than control cells. A and B, Negatively selected naive CD4+ AND TCR transgenic T cells were stimulated for 2 h with plate-bound anti-CD3 (10 μg/ml) in the presence (A) and absence (B) of anti-CD28 (1 μg/ml), then treated with brefeldin A and stained intracellularly with anti-IL-2. Production of IL-2 was determined by flow cytometry using appropriate isotype controls. Data are representative of five independent experiments. C, MRL.AND and B10.BR AND T cells were stimulated by PCC and K99Q (5 μM each) pulsed (MRL × B10.BR)F1 DC, and IL-2 production was determined by cytometric bead array of supernatant after 72 h of culture. Data are representative of two independent experiments.

FIGURE 6. MRL.AND TCR transgenic T cells display an increased and prolonged rise in intracellular calcium following TCR engagement, as measured at the single cell level. A, Percentage of T cells that display calcium responses induced by cross-linking the TCR with anti-CD3 Ab. AND TCR transgenic T cells were stimulated with 10 μg/ml anti-CD3. A cell with an elevation in [Ca2+]i greater than twice its baseline was classified as “responding”. Note that there is considerable physiologic variability in all three tested strains. The bars denote mean data (n, number of experiments with at least 40 cells included per experiment). B, Averaged calcium responses in T cells stimulated with anti-CD3 Ab. Changes in [Ca2+]i were measured as an increase in the fluorescence intensity F over baseline F0 and corrected for background fluorescence. A single exponential curve was fitted to the data to determine the time constant τ. The fit is shown as the smooth curve superimposed on the data values. The calcium response in MRL.AND cells reached twice the amplitude in comparison to B10.BR.AND and CBA.AND cells and was sustained for a substantially longer period of time (τMRL = 301 s, τB10.BR = 99 s, τCBA = 68 s). τ, Time constant; n, number of averaged cells from five representative experiments. C, Averaged calcium responses in T cells stimulated with 1 μM thapsigargin. The sarcoendoplasmic reticulum calcium ATPase blocker thapsigargin was applied in calcium-free extracellular solution. Increases in [Ca2+]i were measured as described in B. Only responding cells displaying an increase in fluorescence greater than 10% over baseline were included in the analysis. T cells from all three tested strains exhibited similar responses. As compared with anti-CD3 stimulation, the response to thapsigargin was characterized by a slower rate of [Ca2+]i increase and a decaying phase, which returned to baseline levels. n, Number of averaged cells.

C, MRL.AND and B10.BR AND T cells were stimulated by PCC and K99Q (5 μM each) pulsed (MRL × B10.BR)F1 DC, and IL-2 production was determined by cytometric bead array of supernatant after 72 h of culture. Data are representative of two independent experiments.
7.1% in the CBA.AND strain (Fig. 5B). Similar results were obtained for nontransgenic T cells from the three strains (data not shown). Thus, an increased production of IL-2 correlates well with the hyperproliferative phenotype of MRL cells.

Next IL-2 production by MRL.AND and control B10.BR.AND cells was assessed in response to stimulation by bone marrow-matured DC (DC to T cell ratio 2:1), pulsed with the agonist peptide PCC (5 μM) and the APL K99Q (5 μM) (Fig. 5C). A substantial increase in IL-2 production was observed in supernatants from the MRL.AND T cells compared with control B10.BR.AND cells, with the difference between the two enhanced upon stimulation by the low affinity peptide (Fig. 5C).

MRL cells exhibit larger and prolonged calcium transients upon TCR ligation compared with control cells

Naive CD4+ AND TCR transgenic T cells were stimulated by cross-linking the TCR with 10 μg/ml anti-CD3, and increases in the [Ca2+], were measured over a period of 20 min in the presence of 2 mM calcium in the extracellular solution. Cell responses fell into two categories: no measurable change in fluorescence or elevation by >100% over baseline within 25 s. In each experiment at least 40 cells were monitored. Although there was considerable variability in all three strains tested, on average 20, 8, and 21% of B10.BR.AND, CBA.AND, and MRL.AND cells, respectively, displayed fluorescence increases during the monitoring period as previously described (Fig. 6A). The differences in the percent responses were small in relation to the wide range of obtained values. These results suggest a similar level of responsiveness after stimulation of the TCR for all strains. Addition of 50–100 μg/ml anti-CD3 cross-linking goat anti-hamster Ab during recording did not increase the percentage of responding cells for any strain (data not shown), demonstrating that our experimental protocol of anti-CD3 stimulation and subsequent centrifugation of T cells to enhance attachment to the coverslip was sufficient to activate the cells.

We next examined the magnitude and kinetics of the calcium signals. T cells from all three strains displayed a typical biphasic response consisting of a rapid overshoot reaching peak calcium concentration within 25 s and a slow exponential decay to a plateau. Owing to the substantial physiologic variability we noted individual calcium responses differed both in amplitude and time of onset. Single traces were therefore aligned to avoid “filtering out” nonsynchronized signals and averaged before analysis (Fig. 6B). MRL.AND cells displayed an initial elevation of [Ca2+]i twice as large as the B10.BR.AND and CBA.AND control cells. A single exponential curve was fitted to the decaying phase to quantify the time course of the response. Time constants for B10.BR.AND, CBA.AND, and MRL.AND cells equaled 99, 68, and 301 s, respectively. As found for the percentage of responding cells, 50–100 μg/ml goat anti-hamster Ab changed neither the magnitude nor the duration of the signal. These results indicate that T cells from the lupus mouse strain not only generated higher [Ca2+]i levels, but also sustained them for longer periods of time.

To exclude the possibility that the differences in calcium signaling shown for MRL.AND cells (Fig. 6B) were due to larger intracellular stores, T cells were challenged with 1 μM thapsigargin to block the sarcoplasmic reticulum calcium ATPase. Experiments were conducted in calcium-free extracellular solution to ensure the measured increase in fluorescence was solely due to emptying of intracellular stores and there was no contribution from calcium influx via plasma membrane channels. Because the responses were of lower amplitudes compared with anti-CD3 stimulation in normal extracellular solution, cells with an increase in fluorescence >10% over baseline were classified as responding.

Similar responses for B10.BR.AND, CBA.AND, and MRL.AND cells were found (Fig. 6C). When compared with anti-CD3 stimulation (Fig. 6B), the responses to thapsigargin had a slower initial rate of increase, the peak occurred later (100 vs 25 s for anti-CD3), and the signal returned to baseline calcium levels. These results demonstrate that larger intracellular stores cannot account for the enhanced magnitude and sustained calcium transients in MRL.AND cells.

The hyperreactive MRL phenotype appears to be a dominant genetic trait

To determine whether T cell proliferation could be used as a screening assay for studying inheritance of lupus susceptibility, we studied proliferation of (MRL × B10.BR)F1 and (MRL × CBA/CaJ)F1 T cells following anti-CD3 stimulation in the presence or absence of anti-CD28, compared with the parental strains (Fig. 7, A and B, respectively). Proliferation of F1 T cells was almost identical with that of the parental MRL strain. The same was found for AND TCR transgenic T cells from the (MRL × B10.BR)F1 and parental backgrounds; the latter experiment included AKR.AND T cells (Fig. 7C). These results imply that there is a gene or a set of genes in MRL T cells that are responsible for the hyperproliferative phenotype, and that they are dominantly expressed; alternatively, there could be recessive genes in the control backgrounds that down-modulate TCR responsiveness.

![FIGURE 7. (MRL × B10.BR)F1 and (MRL × CBA/CaJ)F1 CD4+ T cells are hyperproliferative following TCR engagement. A and B, Non-TCR transgenic (WT) T cells were stimulated with immobilized anti-CD3 with or without anti-CD28 (1 μg/ml), respectively. Proliferation was assessed as in Fig. 1. The data points for the WT F1 mice represent average ± SD of 12 triplicate measurements from 12 individual mice, all cultured under identical conditions. The data points for the WT parental strains were obtained as in Fig. 1. C, The proliferation assay following anti-CD3 stimulation was performed with F1 AND TCR transgenic T cells and compared with AND parental T cells in the absence of anti-CD28. The data points for (MRL × B10.BR)F1.AND and (MRL × CBA/CaJ)F1.AND represent the average ± SD of 16 triplicate measurements from 16 individual mice. The data points for the parental strains (including AKR) were obtained as in Fig. 1.](http://www.jimmunol.org/)
Discussion
We demonstrate that CD4+ T cells from Fas-intact lupus-prone MRL mice have an altered threshold of activation through the TCR-CD3 complex compared with H-2-matched control strains. T cells from lupus-prone mice were hyperproliferative following anti-CD3 triggering at levels of stimulation that were below the activation threshold in control strains and were more sensitive to low affinity peptide engagement than cognate Ag stimulation. Likewise, the necessity for costimulation in the lupus-prone strain to achieve similar phenotypic responses was less stringent as shown by the proliferation experiments in the absence of anti-CD28. Increased production of IL-2 correlated well with the hyperresponsive MRL phenotype. A defect in early signaling events down-stream of the TCR-CD3 complex, as determined by an enhanced and prolonged rise in intracellular calcium, indicates a mechanism for these observations.

Requirements for T cell activation vary depending on the state of the T cell, the type of APC, and the nature of the TCR ligand (45); thus, it may be difficult to distinguish intrinsic from extrinsic abnormalities. To address these concerns, we isolated comparable populations of naive T cells from lupus-prone and control mice that would enable us to determine whether cells from the former were intrinsically different from those of the latter in terms of T cell activation. Although we did not specifically rest T cells in vitro, we only studied naive T cells, purified by negative selection to avoid activation during the isolation process. We used Fas-intact MRL/Fas-lpr mice as the experimental strain, as these animals develop lupus, albeit at a later time than Fas-deficient MRL/Fas-lpr mice, and their use allowed us to avoid the confounding effect of Fas deficiency on T cell activation (46). We started with T cells from TCR transgenic mice, to more easily compare T cell populations among strains by controlling Ag specificity and affinity, state of activation, and TCR density, although we confirmed observations obtained with TCR transgenic cells with nontransgenic CD4+ T cells. It is conceivable that the MRL and control T cells were not absolutely identical in terms of activation phenotype at baseline, despite the fact that we studied cells that were defined as naive by the presence of surface markers. Nevertheless, it is important to emphasize that the MRL cells develop in an autoimmune environment, and our phenotypic evidence notwithstanding, they could have received different signals from host APC, although expression of activation markers on DC was equivalent across the three strains before and after culture. We also did not analyze CD3 composition of naive cells from MRL and control strains, a potentially relevant point because such differences have been demonstrated upon comparison of heterogeneous cell populations (mixed naive and activated) from patients with SLE and controls (reviewed in Ref. 17).

The finding that CD4+ T cells from MRL lupus-prone mice are hyperproliferative at a lower signal strength than carefully matched cells from control mice suggests that lupus T cells could be activated in vivo after contact with self-peptides having a low affinity for the TCR in the periphery. Such low affinity interactions are critical for physiologic maintenance of the T cell repertoire in normal animals (47, 48). In normal mice tolerance mechanisms that control T cell responses to low affinity self-ligands, even in the setting of costimulation, are intact (40); however, our data suggest these mechanisms can be abrogated in lupus. Identification of a lower threshold for activation in lupus T cells does not preclude other immune abnormalities from contributing to a predisposition to autoimmunity, such as in B cells (49, 50) or APC (42, 43, 51), or in clearance of apoptotic material (52). Rather, we would argue that intrinsic T cell defects lead to tolerance abrogation that, in conjunction with these other abnormalities, promotes T cell-B cell collaboration in secondary lymphoid organs with subsequent pathogenic autoantibody production (28). Such polyclonal T cell activation is a requisite for T cell trafficking to B cell follicles and subsequent oligoclonal B cell help (16). This disease model is consistent with the polygenic susceptibility model for lupus supported by strong experimental evidence (53). The notion that T cell hyperresponsiveness is required for lupus also finds support in the findings that genetic deletions in molecules that enhance TCR signaling in nonautoimmune mice lead to activation of CD4+ T cells at a lower threshold of stimulation with subsequent development of antinuclear Abs and immune complex disease (54, 55).

It is possible that the abnormalities we identified are the result of strain differences in TCR stimulation between the MRL and controls. Nevertheless, the fact that MRL T cells respond differently than three genetically diverse H-2-matched control strains suggests that hyperproliferation in the MRL strain is integral to the lupus phenotype. Ultimately, however, this is a genetic argument, to prove that T cell abnormalities are required for disease. To begin to address this issue, we demonstrated that the proliferation of CD4+ T cells from (MRL × B10.BR)F1 and (MRL × CBA)F1 mice is comparable to that of the MRL parental strain and significantly higher than that of parental B10.BR, CBA/CaJ, and AKR/J T cells. These results imply that there is a gene or a set of genes in MRL T cells that are responsible for the hyperproliferative phenotype, and that they are dominantly expressed; alternatively, there could be recessive genes in the B10.BR or CBA background that down-modulate TCR responsiveness.

We hypothesized that abrogation in early signaling events following TCR engagement and inositol-1,4,5-trisphosphate (InsP3)-mediated calcium release might bias MRL T cells toward a strong proliferative response. We chose to examine the calcium dynamics at the single cell level by confocal microscopy because this approach is sensitive and allows examination of groups of responding and nonresponding cells. Although there were no differences among the three tested strains in the percentage of cells responding to TCR ligation with anti-CD3, the MRL strain displayed a rise in calcium that was greater in magnitude and persisted for a longer period of time. Sustained calcium transients such as plateaus and oscillations are necessary to initiate protein synthesis in T cells, whereas shorter signals do not suffice (56–58). Not surprisingly, altered gene transcription in lupus T cells has been described for calcium-dependent genes such as CD40L, Fas ligand, and c-myc (20, 21, 59, 60).

By challenging with thapsigargin, we found that the cells from the tested strains have similar levels of calcium in their intracellular stores. This indicates that the distinctive calcium response in MRL T cells upon CD3 engagement is not due to fuller stores or increased influx from the extracellular space following store depletion, but rather a result of prolonged activation of the InsP3 receptor. Certain mechanisms have already been suggested to explain this early signaling defect in a variety of cell types, such as mitochondrial hyperpolarization with deficient calcium sequestration from the cytosol (61) or loss of protein kinase A I-mediated phospholipase Cγ1 down-regulation with an increase in InsP3 (62–64). The latter seems less plausible as a mechanism in MRL mice, as a similar percentage of T cells responded to anti-CD3 stimulation, which indicates that InsP3 production is comparable. This is corroborated by findings of equal InsP3 levels in T cells (65) as well as B cells (49) from SLE patients and normal subjects.

A more likely explanation for the defective calcium signaling in MRL cells would be a shift in the receptor calcium-dependent...
inactivation. Phosphorylation of the InsP₃ receptor by the nonreceptor tyrosine kinase Fyn can cause enhanced activity of the receptor even at high cytosolic calcium concentrations (66, 67). Heterogeneous populations of T cells from patients with SLE have altered CD3 structure, associated with abnormal lipid raft composition (68, 69) and heightened tyrosine phosphorylation with increased calcium flux (reviewed in Ref. 17), features of which are common to effector T cells (70). Although our findings were in purified naïve CD4⁺ T cells, a disease related alteration in phosphorylation of the InsP₃ receptor nevertheless would be consistent with the enhanced calcium signals observed in single cells.

We showed that IL-2 levels are increased upon TCR stimulation, a finding in accord with T cell hyperproliferation. In humans with SLE, however, T cells are deficient in IL-2 production, which might be due to increased amounts of phosphorylated CAM-responsive element (CRE) in the absence of specific costimulation (27). Nevertheless, our experiments differed from those using human lupus cells, in which heterogeneous populations in terms of activation status of peripheral blood cells are analyzed. It is not clear whether the latter heterogeneity affects the studies of IL-2 synthesis in cells from patients with SLE. Regardless, these contrasting observations highlight the complexity underlying lupus pathogenesis and the need to correlate human and murine model studies at highly controlled experimental conditions.

Taken together, our data provides evidence for a T cell phenotype in murine lupus that displays an intrinsically lowered threshold of activation. This might ultimately lead to enhanced autoreactive T cell proliferation and enhanced helper functions for B cell autoimmunity.

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Disclosures

The authors have no financial conflict of interest.

References


