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Impaired CD4 T Cell Activation Due to Reliance Upon B Cell-Mediated Costimulation in Nonobese Diabetic (NOD) Mice

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Diabetes in nonobese diabetic (NOD) mice results from the activation of I-A\textsuperscript{\textdelta}-restricted, islet-reactive T cells. This study delineates several characteristics of NOD CD4 T cell activation, which, independent of I-A\textsuperscript{\textdelta}, are likely to promote a dysregulated state of peripheral T cell tolerance. NOD CD4 T cell activation was found to be resistant to antigenic stimulation via the TCR complex, using the progression of cell division as a measure. The extent of NOD CD4 T cell division was highly sensitive to changes in Ag ligand density. Moreover, even upon maximal TCR complex-mediated stimulation, NOD CD4 T cell division prematurely terminated. Maximally stimulated NOD CD4 T cells failed to achieve the threshold number of division cycles required for optimal susceptibility to activation-induced death, a critical mechanism for the regulation of peripheral T cell tolerance. Importantly, these aberrant activation characteristics were not T cell-intrinsic but resulted from reliance on B cell costimulatory function in NOD mice. Costimulation delivered by nonautoimmune strain APCs normalized NOD CD4 T cell division and the extent of activation-induced death. Thus, by disrupting the progression of CD4 T cell division, polarization of APC costimulatory function to the B cell compartment could allow the persistence and activation of diabeticogenic cells in NOD mice. The Journal of Immunology, 2000, 165: 4685–4696.

The nonobese diabetic (NOD) mouse is a model of failed T cell tolerance, culminating in organ-specific autoimmunity (1–3). CD4 T cells selected on the unique I-A\textsuperscript{\textdelta} MHC haplotype are critical for the initiation and progression of disease in NOD mice. However, an I-A\textsuperscript{\textdelta}-restricted T cell repertoire is not sufficient for the initiation of anti-islet autoimmunity (4). Nonautoimmune C57BL/6 (B6) or C57BL/10 (B10) mice congenic for the NOD H-2\textsuperscript{\textdelta} locus (B6.g\textsuperscript{7} and B10.g\textsuperscript{7}, respectively) do not develop spontaneous islet-directed autoimmunity despite the emigration of islet-reactive CD4 T cells to the periphery (5–7). This diabetes-resistant phenotype of B6.g\textsuperscript{7} and B10.g\textsuperscript{7} congenic mice points to the existence of effective peripheral regulatory processes that maintain T cell tolerance to islet \( \beta \) cells. Therefore, in wild-type NOD mice the onset of insulinitis and diabetes is likely to be indicative of a failure in peripheral regulatory mechanisms.

One important mechanism for the regulation of peripheral T cells is activation-induced clonal deletion, which occurs subsequent to a vigorous burst of proliferation in response to antigenic stimulation (8–19). Optimal susceptibility of activated peripheral CD4 T cells to this Ag-driven activation-induced deletion process requires a threshold number of cell divisions (20). A failure to achieve this threshold could lead to the persistence of T cells normally subject to activation-induced regulation. In this work, the division history of activated NOD CD4 T cells was visualized, revealing that initiation of cell division is dependent upon cognate B cell costimulation. This reliance of CD4 T cell activation upon B cell-mediated costimulation in NOD mice is characterized by 1) a premature arrest of CD4 T cell division, which does not allow the threshold number of divisions required for optimal susceptibility to activation-induced deletion to be reached and 2) a resistance of CD4 T cells to TCR-mediated activation. This study demonstrates B cell-dependent aberrances in CD4 T cell activation that could contribute to dysregulated peripheral T cell tolerance in NOD mice.

Materials and Methods

Mice and Abs

NOD/LtJ, NOD.SW (H-2\textsuperscript{\textk}), NOD.Thy1.1 congenic, B6, B6.Thy1.1 congenic, and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 I-A\textsuperscript{\textdelta}\textsuperscript{\textk} mice were purchased from Taconic Farms (Germantown, NY). NOD I-A\textsuperscript{\textbeta}\textsuperscript{\textk} mice were generated by mating NOD I-A\textsuperscript{\textbeta}\textsuperscript{\textk} mice (21) with wild-type NOD mice. All animals used in the described experiments were between 8 and 12 wk old and were housed in specific pathogen-free conditions at the University of Pennsylvania Medical Center. Purified anti-CD3 (2C11), purified anti-C28 (37.51), allophycoerythrin-conjugated anti-CD4 (RM4-5), PE-conjugated anti-\( \text{V}\beta\) (KJ25), PE-conjugated anti-\( \text{V}\alpha\) (RR3-15), and PE-conjugated anti-Thy1.1 (OX-7) were purchased from PharMingen (La Jolla, CA).
Lymphocyte enrichment protocol

Enrichment of T cells or APCs was accomplished by negative selection of splenocytes via magnetic-activated cell sorting (MACS; Miltenyi Biotec, Sunnyvale, CA). APCs were prepared by depleting T cells using anti-CD4, anti-CD8, and anti-Thy-1.2 Abs directly conjugated to MACS beads (Miltenyi Biotec). Highly enriched T cells were prepared by depleting B lymphocytes using anti-B220 directly conjugated to MACS beads (Miltenyi Biotec) and biotinylated anti-I-A<sup>+</sup> (10.3.6) plus streptavidin-MACS beads (Miltenyi Biotec). All depletion protocols yielded >95% efficiency in negative selection of the targeted population as determined by flow cytometry.

5-(and 6-)Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling of lymphocytes

Lymphocytes were labeled with CFSE (Molecular Probes, Eugene, OR) as previously described (22). Briefly, a 5-mM stock solution of CFSE was prepared in DMSO. Lymphocytes isolated from spleens and lymph nodes of mice were resuspended at a concentration of 20–30 × 10<sup>6</sup> cells/ml in serum-free IMDM (Life Technologies, Gaithersburg, MD) at 37°C. An equal volume of a 1:250 dilution of the 5-mM CFSE stock in 37°C IMDM was added to the cell preparation. After a 5-min incubation period at 37°C, the excess CFSE was quenched by adding an equal volume of heat-inactivated FCS (HI-FCS). The CFSE-labeled cells were then washed once and resuspended at the desired concentration in IMDM containing 10% HI-FCS.

In vitro stimulation of CD4<sup>+</sup> T cells

CFSE-labeled cells were plated in 24-well plates at a density of 1 × 10<sup>6</sup> total cells/ml in media containing 10% HI-FCS, varying amounts of anti-CD3 (0–2 μg/ml), and 4 μg/ml anti-CD28 or staphylococcal enterotoxin A (SEA; Sigma, St. Louis, MO) at the designated concentration. For the plate-bound anti-CD3 stimulation assays, 24-well plates were coated with 0.5 ml of purified anti-CD3 in sterile PBS at 5 μg/ml overnight. After this incubation period, the plates were washed twice using sterile PBS to remove the excess unbound Ab. Highly enriched, CFSE-labeled T cells were then plated as described above. For the transwell experiments, 1 × 10<sup>6</sup> cells of the specified type were incubated in the top and bottom compartments separated by a mesh of 5-micron pore size. All cells were incubated for 65–70 h at 37°C in 7% CO<sub>2</sub>. After this incubation period, total output cells of the specified type were incubated in the top and bottom compartments separated by a mesh of 5-micron pore size. All cells were incubated for 65–70 h at 37°C in 7% CO<sub>2</sub>. After this incubation period, total output

Analysis of CD4<sup>+</sup> T cell division history

The fluorescent dye, CFSE, permits the direct visualization of lymphocyte division (22–26). When a CFSE-labeled population of lymphocytes divides, each round of division yields daughter cells that retain only half of the fluorescence intensity characteristic of the preceding parent population. The activated cell population is then analyzed by flow cytometry to determine the number of divisions and absolute number/proportion of daughter cells contained within each division peak. This information allows the direct calculation of the frequency and absolute number of the precursor cells that gave rise to the activated T cells using the total number of cells retrieved (23, 25). Briefly, the number of precursors giving rise to each daughter generation is determined by multiplying the total number of cells under each peak by a multiplication factor, 1/2<sup>i</sup> (where n is the division number). The division profiles determined at the end of the stimulation period can also be used to determine the total number of mitoses (23). This number is closely approximated by using the formula [N × (2<sup>i</sup> − 1)]/2<sup>i</sup>, where N is the total number of cells under each peak, i is the sum of the number of mitoses responsible for generating daughter cells under each division peak allows the determination of the total number of divisions that occurred during the stimulation period. Thus, by using CFSE to track the division history of a stimulated lymphocyte population, it is possible to obtain specific information regarding the frequency and absolute number of dividing cells as well as the number of divisions resulting from any degree of stimulation.

In vivo tracking and activation of CD4<sup>+</sup> T cells

CFSE-labeled splenocytes at a concentration of 2 × 10<sup>6</sup> cells/ml from NOD or C57BL/6 Thy1.1 congenic mice were stimulated with the indicated anti-CD3 and anti-CD28 mAb. CD4<sup>+</sup> T cells from NOD mice were activated with 5 × 10<sup>5</sup> cells/ml of anti-CD3 and 0.03 μg/ml anti-CD28, whereas NOD CD4<sup>+</sup> T cells achieved their maximal division state at 0.03 μg/ml anti-CD3, whereas B6 CD4<sup>+</sup> T cells achieved their maximal division state at 0.03 μg/ml anti-CD3, an ~30-fold higher concentration. A dose-response curve was generated using the calculated total number of mitoses occurring at each concentration of anti-CD3 for both NOD and B6 CD4<sup>+</sup> T cells (Fig. 2B). B6 CD4<sup>+</sup> T cells achieved their maximal division state at 0.03 μg/ml anti-CD3, whereas NOD CD4<sup>+</sup> T cells achieved their maximal division state at 1 μg/ml anti-CD3, an ~30-fold higher concentration. A combination of soluble mitogenic anti-CD3 and anti-CD28 mAb was used to stimulate unfractionated CFSE-labeled splenocytes from NOD and B6 mice. Using conditions providing maximal levels of activation by anti-CD3 and anti-CD28 mAb, CD4<sup>+</sup> T cells from NOD mice exhibited division arrest and failed to generate daughter cells in an advanced division peaks as those achieved by their nonautoimmune B6 counterparts (Fig. 1A). Specifically, comparison of the division profiles of NOD and B6 CD4<sup>+</sup> T cells indicated that the majority (>80%) of activated NOD CD4<sup>+</sup> T cells resided in divisions 1–4, whereas the majority (>70%) of activated B6 CD4<sup>+</sup> T cells were present in divisions 4–7 (Fig. 1B). Assessment of maximal anti-CD3/28-mediated CD4<sup>+</sup> T cell division by nonautoimmune strain splenocyte isolates (B10, B6.g7, BALB/c, and C3H) revealed an identical pattern of division to that of splenic B6 CD4<sup>+</sup> T cells (data not shown).

We also determined the propensity of the precursor pool of CD4<sup>+</sup> T cells for generating daughter cells in any particular division. This analysis provided a precise measure of the division potential of the starting population of stimulated T cells. Calculation of the precursor frequency of CD4<sup>+</sup> T cells that gave rise to activated daughter cells within each division peak revealed that the starting pool of NOD CD4<sup>+</sup> T cells directed the majority of its division effort to peaks 1–4 (Fig. 1C). In contrast, the starting pool of B6 CD4<sup>+</sup> T cells directed the majority of its division effort to peaks 4–6 (Fig. 1C). Importantly, the diminished division potential of NOD CD4<sup>+</sup> T cells was not imposed by the I-A<sup>q</sup> MHC haplotype as evidenced by identical division profiles of NOD.SWR (H-2<sup>q</sup>) and wild-type CD4<sup>+</sup> T cells (data not shown).

NOD CD4<sup>+</sup> T cell division is highly sensitive to modulation of Ag ligand density

The diminished progression of division by maximally stimulated NOD CD4<sup>+</sup> T cells as compared with their nonautoimmune strain counterparts was evident at all concentrations of anti-CD3 tested (Fig. 2A). A dose-response curve was generated using the calculated total number of mitoses occurring at each concentration of anti-CD3 for both NOD and B6 CD4<sup>+</sup> T cells (Fig. 2B). B6 CD4<sup>+</sup> T cells achieved their maximal division state at 0.03 μg/ml anti-CD3, whereas NOD CD4<sup>+</sup> T cells achieved their maximal division state at 1 μg/ml anti-CD3, an ~30-fold higher concentration.

Results

Diminished progression of NOD CD4<sup>+</sup> T cell division upon activation

We have used CFSE to characterize the activation properties of NOD CD4<sup>+</sup> T cells using the extent of cell division as a sensitive measure. A combination of soluble mitogenic anti-CD3 and anti-CD28 mAb was used to stimulate unfractionated CFSE-labeled splenocytes from NOD and B6 mice. Using conditions providing maximal levels of activation by anti-CD3 and anti-CD28 mAb, CD4<sup>+</sup> T cells from NOD mice exhibited division arrest and failed to generate daughter cells in an advanced division peaks as those achieved by their nonautoimmune B6 counterparts (Fig. 1A). Specifically, comparison of the division profiles of NOD and B6 CD4<sup>+</sup> T cells indicated that the majority (>80%) of activated NOD CD4<sup>+</sup> T cells resided in divisions 1–4, whereas the majority (>70%) of activated B6 CD4<sup>+</sup> T cells were present in divisions 4–7 (Fig. 1B). Assessment of maximal anti-CD3/28-mediated CD4<sup>+</sup> T cell division by nonautoimmune strain splenocyte isolates (B10, B6.g7, BALB/c, and C3H) revealed an identical pattern of division to that of splenic B6 CD4<sup>+</sup> T cells (data not shown).
We next determined the sensitivity of NOD vs B6 CD4 T cell activation to MHC ligand density. Splenocytes from I-A<sup>1</sup>/2<sup>−/−</sup>NOD (Fig. 3) and I-A<sup>1</sup>/2<sup>−/−</sup>B6 (data not shown) mice, whose APCs express one-half the cell surface MHC class II density of wild-type I-A<sup>1</sup>/1 counterparts, were used in SEA stimulation assays. As demonstrated in Fig. 4, B6 SEA-mediated CD4<sup>+</sup> T cell activation is unaffected by a one-half reduction in MHC density at a maximal stimulating SEA concentration of 15 μg/ml. At this maximal level of stimulation, the ratio of activated CD4<sup>high</sup> to resting CD4<sup>low</sup> T cells in B6 I-A<sup>−/−</sup> and I-A<sup>−/−</sup> cultures is nearly identical (a ratio of 2.5 in the I-A<sup>−/−</sup> culture and 2.4 in the I-A<sup>−/−</sup> culture; a 4%
impaired activation of NOD CD4 T cells

Incomplete division of activated CD4 T cells leads to their protection from activation-induced death in vitro

Recent work has suggested a dependence of efficient activation-induced apoptosis on a threshold number of cell divisions (20). Therefore, the failure of in vitro anti-CD3-stimulated NOD CD4 T cells to achieve advanced divisions prompted us to assess the efficiency of activation-induced death of these cells. We determined that the majority of cell death, in both NOD and B6 CD4 T cells, occurred after three divisions (Fig. 5A). Compared with maximally stimulated B6 counterparts, NOD CD4 T cells maximally anti-CD3 stimulated failed to undergo cell death as vigorously (35 ± 8% for NOD and 85 ± 4% for B6) (Fig. 5B). However, when the number of cell divisions were equaled for both B6 and NOD CD4 T cells by varying the concentration of anti-CD3, there was an equivalent degree of activation-induced cell death by both B6 and NOD T cells (43 ± 9% for B6 vs 35 ± 8% for NOD) (Fig. 5B). Importantly, quantification of the extent of activation-induced death in the divided CD4+/CFSE gate across the 0- to 2-μg/ml range of anti-CD3 stimulation revealed a linear dependence of cell death upon the extent of cell division in the case of both B6 and NOD CD4 T cells (Fig. 5, C and D, respectively). These data indicate that the reduced susceptibility of NOD CD4 T cells to activation-induced death may be a result of their inability to progress to advanced stages of cell division.

Incomplete division of activated CD4 T cells leads to their protection from in vivo deletion

We next determined whether the diminished capacity of activated NOD CD4 T cells to progress through successive rounds of cell division also prevents deletion in vivo. By using Thy1.1 congenic NOD and B6 T cells, we were able to track CD4 T cells in vivo following adoptive transfer into wild-type (Thy1.2) recipient mice. CD4 T cells from Thy1.1 congenic NOD and B6 mice were CFSE labeled and maximally stimulated in vitro with soluble anti-CD3 and anti-CD28 for 65 h. Cells were then adoptively transferred into wild-type NOD or B6 mice. After a 10-day period, spleens and lymph nodes were isolated from the recipient mice to determine the persistence of the maximally activated Thy1.1+ CD4 T cells (Fig. 6, A and B). Very few of the transferred population of maximally activated B6 CD4 T cells, which were primarily in divisions 4–6 at the time of transfer, were retrieved from the secondary lymphoid organs of the recipient mice 10 days later (Fig. 6B). In contrast, maximally divided NOD CD4 T cells, which were distributed throughout divisions 1–5 at the time of transfer, were persistent 10 days following adoptive transfer into wild-type mice (Fig. 6A). To determine whether the in vivo persistence of activated NOD CD4 T cells is brought about by suboptimal cell division, we also stimulated Thy1.1 B6 CD4 T cells to divide to a half-maximal level using anti-CD3 at 0.005 μg/ml in conjunction with 4 μg/ml of anti-CD28. At this level of stimulation, B6 CD4 T cells divided to the same extent as maximally activated NOD CD4 T cells before in vivo transfer. After a 10-day period, the half
maximally activated B6 CD4 T cells persisted at a level comparable to the maximally activated NOD CD4 T cells (Fig. 6C). The persisting cells were predominantly remaining in divisions 1–3. Overall, these data demonstrate that a correlation exists between the extent of cell division and susceptibility of activated CD4 T cells to elimination from secondary lymphoid organs in vivo.
FIGURE 5. The extent of CD4 T cell activation-induced death is a linear function of the extent of division. A, CD4-gated CFSE-labeled splenocytes were maximally stimulated with 2 μg/ml anti-CD3 and 4 μg/ml anti-CD28, cultured for 65 h, and counterstained with anti-CD4 and 7-AAD to identify dead cells. The dotted line in each panel is placed after division 3 and highlights that the majority of 7-AAD⁺ CD4 T cells are found starting at division 4 in both NOD and B6 cultures. B, Identification of death in the divided gate using the CFSE division profiles of splenic CD4 T cells stimulated with the indicated concentration of anti-CD3 and 4 μg/ml anti-CD28. The dotted line is placed after the undivided peak. The divided CD4⁺ T cell gate is placed to determine the proportion of dead (7-AAD⁺) CD4 T cells at each concentration of anti-CD3. When the CFSE division profile of B6 CD4 T cells is nearly identical with that seen in the case of maximally stimulated NOD CD4 T cells, the extent of cell death is indistinguishable. C and D, Percentage of cell death in the divided CD4⁺ gate is plotted against the fold decrease in CFSE intensity as a measure of the overall extent of cell division. The best-fit lines are drawn through the data set for both B6 (r² = 0.9) (C) and NOD (r² = 0.9) (D) CD4 T cells; the two lines have a nearly identical slope. The vertical dotted lines indicate the maximum limit of NOD CD4 T cell division and correspond to a 12-fold decrease in CFSE intensity. B6 CD4 T cell division surpasses the maximal NOD limit and is associated with a linear increase in CD4 T cell death. Cell death occurred similarly in the case of NOD and B6 CD4 T cells at shared points along the ordinate corresponding to a 1- to 12-fold decrease in CFSE intensity.
We next determined whether the degree of persistence observed in vitro could be recapitulated when NOD CD4 T cells were directly stimulated in vivo. CFSE-labeled splenocytes from Thy1.1 congenic NOD or B6 mice were adoptively transferred into syngeneic wild-type recipients. After an overnight rest period, recipient mice were injected with 25–30 μg of SEA. A maximal burst of proliferation occurred by 48 h following SEA injection in both NOD and B6 mice. By 200 h following SEA injection, a significantly greater proportion of the in vivo SEA-activated CD4 T cells persisted in NOD mice than in B6 mice (Fig. 7A). Fig. 7B is a time course analysis of the in vivo clearance of a SEA-activated Vβ3/Vβ11-bearing CD4 T cell marker population in NOD and B6 mice. Approximately 90% of the activated B6 CD4 T cell population seen at 48 h were deleted in vivo by 200 h following SEA injection. In contrast, only 60% of the activated NOD CD4 T cell population seen at 48 h were subject to in vivo elimination by 200 h following SEA injection. We had previously observed that in vitro SEA-mediated activation of NOD CD4 T cells, similar to anti-CD3-mediated stimulation, resulted in a premature termination of cell division relative to B6 counterparts (Fig. 4). Thus, the long-term in vivo persistence of SEA-activated NOD CD4 T cells is associated with a blunted cell division profile.

The NOD APC compartment imposes the diminished progression of NOD CD4 T cell division

Efficient T cell activation and, in particular, anti-CD3-mediated activation requires the presence of competent APCs as a source of costimulation (27). Given the impaired activation of NOD CD4 T cells, we sought to determine whether these characteristics are T cell intrinsic or are imposed by aberrant NOD APC function. Thus, we assessed the ability of both the B cell and non-B cell APC compartments for delivery of the costimulation necessary to drive effective CD4 T cell activation. In the absence of B cells, NOD CD4 T cells failed to initiate division even upon maximal stimulation with soluble anti-CD3/28 (Fig. 8). In marked contrast, B6 CD4 T cells remained capable of undergoing successive rounds of division in the absence of B cells. The profound unresponsiveness of NOD CD4 T cells in the absence of B lymphocytes was irreversible even upon addition of exogenous rIL-2 (Fig. 8). This latter finding suggests that the proliferative unresponsiveness of NOD CD4 T cells in the absence of B cells does not fit the conventional definition of T cell “anergy” (28). Furthermore, using plate-bound anti-CD3, an APC-independent T cell functional assay, we stimulated highly enriched NOD and B6 T cells in the complete absence of B and non-B cell APCs for 70 h (Fig. 8B). In this plate-bound anti-CD3 assay, in contrast to APC-dependent stimulation with soluble anti-CD3/28, NOD and B6 CD4 T cells exhibited a comparable division profile. These data indicate that NOD CD4 T cells do not exhibit an intrinsic defect in their ability to become activated; rather, the observed NOD CD4 T cell activation defect in response to soluble anti-CD3/28 resides in the inability of the non-B cell NOD APC compartment to provide optimal costimulatory signals. Given this aberrance in NOD APC function, we hypothesized that the suboptimal division profile of activated NOD CD4 T cells in response to soluble anti-CD3/28 is due to a non-B cell APC deficit, which imposes a reliance on B cell-mediated costimulation for CD4 T cell activation. Therefore, purified NOD CD4 T cells were maximally stimulated with soluble anti-CD3/28 in the presence of nonautoimmune B6 APCs. In the presence of these nonautoimmune strain APCs, NOD CD4 T cells
divided to a nearly identical level as their maximally stimulated B6 counterparts (Fig. 9A). This potentiated division of NOD CD4 T cells occurred when these cells were stimulated in the presence of either I-A^d/1 or I-A^d/2 B6 APCs, indicating that the observed enhancement of cell division was not due to an alloresponse directed toward the I-A^d MHC expressed by the B6 APCs. In fact, B6 strain APCs delivered an intrinsically different, MHC-independent, costimulatory signal to CD4 T cells, which mediated an enhanced proliferation of NOD CD4 T cells as compared with that seen upon stimulation in the presence of NOD APCs. This normalization of NOD CD4 T cell division upon receipt of costimulation via B6 APCs also brought about an enhanced degree of activation-induced cell death (Fig. 9B). Furthermore, the linear relationship that existed between cell division and activation-induced death (Fig. 5, C and D) was preserved when NOD CD4 T cells were stimulated in the presence of B6 APCs (Fig. 9B).

Initiation of NOD CD4 T cell division requires cognate interaction with B lymphocytes

In vitro stimulation of T cells using soluble anti-CD3, despite being MHC independent, requires the presence of costimulatory signals from Fc receptor-bearing APCs (29). Data presented above indicate that, in contrast to B6 counterparts, NOD CD4 T cells require B cells for the initiation of anti-CD3-mediated activation.

FIGURE 7. Persistence of CD4 T cells following SEA-mediated activation in vivo. A, CFSE-labeled CD4 T cells from Thy1.1 congenic NOD or B6 mice were i.v. injected into syngeneic wild-type mice followed by an injection of 25–30 μg of SEA. Splenocytes were harvested from recipient mice at 20, 48, 120, and 200 h following SEA injection and analyzed by flow cytometry. A total of 1 × 10^6 CD4^+ events were collected. B, Time course analysis of the in vivo clearance of SEA-activated CD4 T cells in NOD (□) and B6 (▲) mice. By 120 h following SEA injection, ~90% of SEA-activated CD4 T cells were deleted from the B6 recipients, whereas only 60% of the activated cells were deleted from NOD counterparts.

FIGURE 8. A, B lymphocytes are required for efficient anti-CD3-mediated division of NOD CD4 T cells. B cell-depleted, CFSE-labeled splenocytes from B6 and NOD mice were stimulated with 2 μg/ml anti-CD3 and 4 μg/ml anti-CD28 for 65 h. Although B6 CD4 T cells underwent successive rounds of division during the culture period, NOD CD4 T cells were unable to undergo division in the absence of B cells, indicating a functional deficit in the non-B cell NOD APC compartment. This inability of NOD CD4 T cells to divide in the absence of B cells was also observed when cultures were supplemented with 10 ng/ml of rIL-2. B, NOD CD4 T cells are not intrinsically defective in undergoing cell division upon anti-CD3-mediated stimulation. APC-depleted, CFSE-labeled splenocytes from B6 and NOD mice were stimulated using plate-bound anti-CD3 for 70 h. The dotted line is drawn past division 3 to serve as a visual aid indicating a near identical division profile on the part of NOD and B6 CD4 T cells.
We hypothesized that the costimulation provided to NOD CD4 T cells by B lymphocytes is delivered via a cognate interaction between B and T cells. CFSE-labeled, fractionated NOD T cells and B cells were plated in separate compartments of a transwell system while stimulating the T cells with anti-CD3 and anti-CD28 for 65 h. NOD CD4 T cells failed to initiate division despite the presence of B cells in the transwell system, indicating a requirement for cognate T/B cell interactions (Fig. 10A). Furthermore, the soluble products of unfractiobated NOD splenocyte activation with anti-CD3/CD28 did not induce fractionated NOD CD4 T cells to divide in the transwell studies (Fig. 10B).

**Discussion**

This study demonstrates that NOD CD4 T cells require 30- to 35-fold higher TCR/CD3-mediated stimulation to achieve their maximal level of division when compared with their nonautoimmune counterparts. This characteristic also holds true in the case of MHC/SEA-mediated CD4 T cell activation. One implication of the response of NOD CD4 T cells to activation is that there is a range of potentially autoreactive T cells that may not receive sufficient antigenic stimulation to be subject to activation-induced regulation in the periphery. The accumulation of such latent autoreactive T cells provides the potential for the development of autoimmune disease should a milieu favoring their activation arise. Indeed, one such scenario is found in the case of virally induced diabetes (30, 31). Our findings suggest that the aberrant activation requirements of NOD CD4 T cells could lead to inefficient activation of T cells when Ags are presented at a low density. Indeed, this finding may contribute to diabetes resistance of NOD mice heterozygous for I-A^d, which exhibit a reduced expression of I-A^d by their APCs (4).

The resistance of NOD CD4 T cells to the initiation of cell division may render activation on a per cell basis ineffective when a large frequency of T cells compete for the same antigenic ligand(s). It could be argued that competition among CD4 T cells for an antigenic niche would decrease the stimulus received on a per cell basis. Such competition could lead to suboptimal activation of NOD CD4 T cells given their diminished dose responsiveness compared with nonautoimmune counterparts. This latter prediction may be the explanation for an intriguing finding recently described by the Benoist and Mathis laboratory in the BDC2.5 TCR transgene (Tg) model of autoimmune diabetes (6). The T cell compartment of BDC2.5 Tg mice contains a high frequency of potentially diabetogenic CD4 T cells with monoclonal specificity. When the BDC2.5 Tg was fully backcrossed onto the NOD genetic background, diabetogenic T cells were not sufficiently activated to cause diabetes despite impressive homing of Tg T cells to the islets. However, in B6 mice congenic for H-2^q7, the presence of the BDC2.5 TCR Tg readily induces diabetes, pointing to the efficient activation of the diabetogenic Tg T cells on the B6 genetic background. This dichotomy may be explained by our finding that NOD CD4 T cells require a dramatically higher stimulus for activation than their B6 counterparts. Indeed, on the NOD genetic background, it is competition between the supraphysiologic number of Tg-encoded BDC2.5 T cells which may be impeding sufficient activation of individual cells. In contrast, BDC2.5 T cells on the B6 genetic background, due to their significantly lower activation threshold, are not subject to the same competitive force and thereby receive enough stimulation at the single cell level to achieve their full pathogenic potential.

Despite their resistance to activation upon TCR complex ligand, NOD CD4 T cells are capable of dividing to generate activated progeny cells. However, even upon maximal stimulation, NOD CD4 T cell division is prematurely aborted such that the majority of activated progeny cells are distributed in the first three division peaks. This phenotype was also observed in activated CD4 T cells from NZB/NZW F1, and MRL mice, which are prone to spontaneous autoimmunity (data not shown). In contrast, maximally stimulated CD4 T cells from nonautoimmune mice consistently progressed beyond the third cell division. The inability of CD4 T cells to efficiently progress to advanced cell divisions upon stimulation through the TCR complex may be a general characteristic of strains susceptible to spontaneous autoimmunity.

Several recent studies have suggested that a threshold number of cell divisions is required for certain differentiated T cell functions to optimally progress. One such division-dependent process is the commitment of activated CD4 T cells to an IL-4-secreting phenotype that requires a minimum of three cell divisions (24). In light
of this recent report, our finding that NOD CD4 T cells prematurely terminate cell division is a potential explanation for the profound inability of activated peripheral T cells to produce IL-4 (32–34), a deficit that is a shared characteristic of human type I diabetics and NOD mice (35). Indeed, an inefficiency in the differentiation of IL-4-producing “regulatory” Th2 cells has been suggested to allow the development of autoimmune diabetes in NOD mice (3). Another important division-dependent process is the susceptibility of CD4 T cells to activation-induced death. It was recently shown that apoptosis of superantigen-activated T cells occurs most efficiently following three cell divisions (20). Thus, we predicted that the inability of NOD CD4 T cells to efficiently progress to advanced cell divisions may lead to the abnormal persistence of activated T cells. Assessment of the extent of activation-induced death revealed a linear dependence upon the degree of cell division in the case of both activated NOD and B6 CD4 T cells. Our results directly demonstrate that the elimination of CD4 T cells following in vivo activation is a function of their division state. We conclude that long-term persistence of activated NOD CD4 T cells occurs as a consequence of their inability to exceed the division threshold necessary for efficient in vivo activation-induced deletion. In fact, a study by the Holmberg laboratory has indicated that NOD mice mount a significantly more vigorous and prolonged secondary immune response to a foreign immunogen, and that this phenotype correlates with the resistance of NOD T cells to death (36). It is possible that the persistence of suboptimally activated (and, therefore, nondiabetogenic) islet-reactive T cells in the benign insulitis of younger NOD mice (4–12 wk of age) may be the result of a persistent T cell response to islets, much like that seen against foreign immunogens. The factor(s) that allow sufficient activation of the anti-islet T cells in the benign insulitis of younger NOD mice to a diabetogenic phenotype in older NOD mice remain to be elucidated.

Work from the Fathman group indicates that peripheral T cell tolerance to ubiquitous self-Ags can be readily broken in NOD mice. Following immunization with autoantigen, a promiscuous and persistent state of Ag-specific CD4 T cell activation, termed “autoproliferation,” occurred (37). A seemingly contradictory body of work from Delovitch and colleagues documented a marked degree of unresponsiveness by purified NOD CD4 T cells upon TCR complex ligation (34, 38–40). This generalized defect in NOD T cell activation was interpreted to be indicative of a global state of T cell “anergy.” The apparent contradiction between CD4 T cell anergy and promiscuous “autoproliferation” is reconcilable by our results demonstrating that the reduced division capacity of activated NOD CD4 T cells (i.e., anergy) directly leads to the long-term in vivo persistence of these cells, which may account for the “ autoproliferative” phenotype.

In light of the observed aberrances in the activation characteristics of NOD CD4 T cells, we questioned whether these defects are T cell intrinsic or are imposed by the NOD APC compartment. The presented data indicate that NOD CD4 T cells are not intrinsically defective in their ability to become activated. Rather, it is the non-B cell NOD APCs that are unable to provide the costimulation necessary to drive CD4 T cell division, leading to the observed activation defects in APC-dependent T cell functional assays. Indeed, this abnormality in non-B cell NOD APC function is

FIGURE 10. Cognate T/B cell interactions are required for NOD CD4 T cell activation. Fractionated CFSE-labeled NOD B cells (1 × 10⁶) (A) or unFractionated NOD splenocytes (B) were loaded in the top compartment, and 1 × 10⁶ fractionated CFSE-labeled NOD T cells were loaded in the bottom compartment of a transwell system. Cells were cultured with 2 μg/ml anti-CD3 and 4 μg/ml anti-CD28 for a total of 70 h and then analyzed by flow cytometry to determine the extent of CD4 T cell division.
consistent with several studies characterizing a defect in the development and function of these APCs (41–44). Our data indicate that cognate B cell costimulation is required for driving soluble anti-CD3-mediated cell division. Thus, we suggest that B cells may be the most functional APC compartment in the NOD mouse. Indeed, it was recently demonstrated that the progression of diabetes in wild-type NOD mice requires the presence of B lymphocytes, which act as the requisite APCs in the activation of I-Aβ7-restricted diabetogenic T cells (45–50). Moreover, the Sarvetnick laboratory has recently demonstrated that diabetogenesis in NOD mice is dependent upon polarized B cell-mediated Ag presentation to islet-reactive T cells (51). It was shown that when the NOD immune system is modulated to preferentially use macrophages as APCs, the mice are protected from autoimmune diabetes. Furthermore, data from the Flavell group indicate that a dramatic increase in the number of B lymphocytes in the insulitic lesion is correlated with early onset of autoimmune diabetes in receptor interacting protein

References


