

B220⁺ Double-Negative T Cells Suppress Polyclonal T Cell Activation by a Fas-Independent Mechanism That Involves Inhibition of IL-2 Production

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Fas-mediated apoptosis is a key mechanism for elimination of autoreactive T cells, yet loss of function mutations in the Fas signaling pathway does not result in overt T cell-mediated autoimmunity. Furthermore, mice and humans with homozygous Fas^{*lpr*} or Fas ligand^{*gld*} mutations develop significant numbers of B220⁺ CD4⁻ CD8⁻ double-negative (DN) $\alpha\beta$ T cells (hereafter referred to as B220⁺ DN T cells) of poorly understood function. In this study, we show that B220⁺ DN T cells, whether generated in vitro or isolated from mutant mice, can suppress the ability of activated T cells to proliferate or produce IL-2, IL-10, and IFN- γ . B220⁺ DN T cells that were isolated from either *lpr* or *gld* mice were able to suppress proliferation of autologous and syngeneic CD4 T cells, showing that suppression is Fas independent. Furthermore, restoration of Fas/Fas ligand interaction did not enhance suppression. The mechanism of suppression involves inhibition of IL-2 production and its high affinity IL-2R α -chain (CD25). Suppression also requires cell/cell contact and TCR activation of B220⁺ DN T cells, but not soluble cytokines. These findings suggest that B220⁺ DN T cells may be involved in controlling autoreactive T cells in the absence of Fas-mediated peripheral tolerance. *The Journal of Immunology*, 2003, 171: 2421–2426.

Fas and its specific ligand (FasL)² are cell surface proteins whose interaction triggers a cascade of subcellular events that result in apoptosis (1, 2). This mechanism is critical for elimination of autoreactive T cells that escape negative selection in the thymus. However, loss of function mutations in the gene encoding Fas (Fas^{*lpr*}) or FasL (FasL^{*gld*}) does not lead to T cell-mediated disease (3), despite persistence of autoreactive T cells in abnormally high numbers (4). To the contrary, lack of a functional Fas pathway is associated with an Ab-mediated lupus-like disease and impaired delayed-type hypersensitivity (5, 6). More important, Fas^{*lpr*} and FasL^{*gld*} mutations prevent, rather than exacerbate T cell-mediated diseases, including autoimmune diabetes (7–9). Thus, the consequences of loss of function mutations in the Fas signaling pathway are surprisingly inconsistent with the role of the Fas pathway in regulating peripheral tolerance. The underlying mechanism of this paradox is currently not well understood.

A second hallmark of mice (3) and humans (10, 11) with defective Fas signaling pathways is the accumulation of $\alpha\beta$ T cells that lack both CD4 and CD8 coreceptors and express B220 molecules (hereafter referred to as B220⁺ double-negative (DN) T cells). The prevailing view is that B220⁺ DN T cells are aberrant cells of no or limited physiological relevance. However, recent studies show that *lpr* B220⁺ DN T cells, which constitutively express FasL, can kill wild-type (wt) Fas-expressing CD8 T cells and

prolong allograft survival in a Fas-dependent manner (12). Although these studies show the ability of B220⁺ DN T cells to regulate Fas-expressing T cells, the role of B220⁺ DN T cells in regulating autologous T cells with impaired Fas pathway is currently not fully understood. Furthermore, the mechanism by which B220⁺ DN T cells suppress other T cells is unclear.

In this study, we show that B220⁺ DN T cells, whether generated in vitro or isolated from mutant mice, can suppress activation of polyclonally activated autologous and syngeneic T cells in Fas-independent fashion. In addition, Fas-deficient CD4 T cells and Fas-expressing CD4 T cells were suppressed to the same extent by FasL-expressing *lpr* B220⁺ DN T cells showing that suppression of CD4 T cells is not modulated by a Fas-dependent mechanism. Suppression mechanism involves inhibition of IL-2 production and its high affinity IL-2R α -chain (CD25). Suppression also required cell/cell contact and TCR activation of B220⁺ DN T cells, but was cytokine independent. These findings suggest a potential role for B220⁺ DN T cells in immunoregulating autoreactive T cells in mice and humans with defects in the Fas signaling.

Materials and Methods

Mice

AND TCR transgenic mice (13) specific for moth cytochrome *c* (MCC) in the context of I-E^k on B10.BR (H-2^k) background (14) were a generous gift from S. Swain (Trudeau Institute, Saranac, NY). C3H-*lpr/lpr* and C3H-*gld/gld* mice with homozygous loss of function mutations in Fas (Fas^{*lpr*}), FasL (FasL^{*gld*}) molecules and wt (Fas^{wt}) C3H/HeJ (H-2^k) mice were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). All mice were bred and maintained at the Cancer Center of the Johns Hopkins School of Medicine.

Stimulatory beads

Anti-CD3 beads were prepared, as previously described (15). Briefly, 75 mg of anti-CD3 (2C11) mAb in borate buffer was mixed with tosyl-activated 450 Dynabeads (DynaL, Lake Success, NY) and tumbled overnight at 40°C. The beads were then soaked in 5% serum at 40°C to block remaining active sites and stored in PBS until used. Anti-CD3/CD28 beads were

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² Abbreviations used in this paper: FasL, Fas ligand; DN, double negative; MCC, moth cytochrome *c*; wt, wild type.

prepared by mixing anti-CD3 (2C11) and anti-CD28 (37.51) mAbs at a 1:1 ratio and coupled to tosyl-activated 450 Dynabeads, as described above.

Cell purification

We have used negative selection to isolate various subsets of T cells. MCC-specific AND TCR CD4 T cells bearing V α 11V β 3 segments were isolated from spleens of AND/B10.BR mice by negative selection with Dynabeads. Briefly, splenocytes were incubated with a cocktail of biotin-conjugated rat mAbs specific for murine CD8 (53-5.8 mAb), I-E^k (14-4-4S mAb), CD16/CD32 (2.4G2 mAb), and B220 (RA3-6B2 mAb) surface molecules (all were purchased from BD PharMingen, San Diego, CA). Streptavidin-conjugated Dynabeads were used to capture and remove mAb-coated cells, according to the manufacturer's instructions. Purity and specificity of isolated T cells were determined by three-color flow cytometric analysis using mAbs specific for murine V α 11, CD8, and CD4 molecules. Purity of AND TCR CD4 T cells that were used for in vitro generation of B220⁺ DN T cells was always greater than 95%.

We used the same protocol to isolate CD4 T cells from the lymph nodes of C3H-*lpr/lpr* and C3H-*gld/gld* mice. B220⁺ DN T cells were isolated by using a cocktail of biotinylated mAbs specific for the following molecules: CD4 (GK1.5), CD8, I-E^k, and CD16/CD32. Purity of CD4 and B220⁺ DN T cells from *lpr* and *gld* mice that were used in these studies was always greater than 95%, as determined by flow cytometry.

mAbs and flow cytometry

Fluorochrome-conjugated mAbs specific for TCR (clone H57-597), CD4 (clone H129.19), CD8 (clone 53-6.7), CD45R/B220 (clone RA3-6B2), CD25 (clone 3C7), CD44 (clone 30-F11), CD45RB (clone 16-A), and CD62L (clone MEL-14) were purchased from BD PharMingen. Cells stained with fluorescent Abs were analyzed with a FACSCaliber flow cytometer and CellQuest software (BD Biosciences, San Jose, CA).

In vitro generation of B220⁺ DN T cells

Typically, AND T cells (1×10^5) were incubated with anti-CD3/CD28 beads (2×10^6) in wells of a 24-well microtiter plate. Recombinant IL-2 (20 U/ml) and IL-7 (2–10 ng/ml) were added on day 2 and thereafter every 4–5 days to enhance DN T cell survival (16). Media were changed and cells were split, as required. Fresh anti-CD3/CD28 beads were added at frequent intervals of 4–5 days to keep T cells continuously stimulated. B220⁺ DN T cells that were $\geq 85\%$ pure were used in the suppression assay.

Suppression assay

This assay was performed according to the protocol described by Thornton and Shevach (17). Briefly, B220⁺ DN T cells were mixed with CD4 T cells in the presence of anti-CD3 or anti-CD3/CD28 beads in 96-well round-bottom plates in triplicate cultures. After 48 h, 100 ml of supernatant fractions was collected for cytokine analysis and wells were replenished with fresh medium. Proliferation was determined after 72 h with [³H]thymidine that was added during the last 18 h of incubation. Supernatant fractions were assayed for indicated cytokines using capture ELISA (Endogen, Woburn, MA). Each experiment represents at least three independent experiments with similar results.

For neutralization assays, purified Abs specific for IL-2, IFN- γ , and IL-10 cytokines were purchased from BD PharMingen. Pan-specific TGF- β -neutralizing Ab was obtained from R&D Systems (Minneapolis, MN).

Transwell experiment

Transwell experiments were conducted in 24 wells in 0.8 ml of complete tissue culture medium. To measure the effect of soluble cytokines, CD4 T cells (5×10^5) were stimulated in the lower chamber and DN T cells (2.5×10^6) in the upper chamber of Transwell. The semipermeable membrane that separates the upper and lower chamber of Transwell (Corning, Costar, Cambridge, MA) allows diffusion of soluble materials, but not particulate cells. As controls, CD4 T cells were cultured in the lower chamber either alone or mixed with DN T cells. Anti-CD3 beads (5×10^5) were used for stimulation. To study the role of soluble cytokines in the suppression mechanism, supernatants collected from cultures of activated CD4 T cells, B220⁺ DN T cells, or cocultures of CD4 and B220⁺ DN T cells were used to inhibit activation of CD4 T cells.

Real-time PCR

Real-time PCR was performed to measure IL-2 mRNA transcripts in CD4 T cells (5×10^5) that were activated in the presence or absence of B220⁺ DN T cells (2.5×10^6). IL-2 transcripts were also measured in B220⁺ DN

T cells stimulated alone. After 48-h stimulation with anti-CD3 beads, total RNA was isolated from different cultures with the RNeasy Kit (Qiagen, Valencia, CA). Briefly, cells from each sample were disrupted, homogenized, and resuspended in an equal volume of 70% ethanol. Samples were loaded into minicolumns and washed three times. Total RNAs were eluted with water, and concentrations were determined. First strand cDNAs were synthesized from total RNAs using Superscript II (Invitrogen, Carlsbad, CA). Briefly, 1 mg of total RNAs was reverse transcribed with 50 U of Superscript II reverse transcriptase and 150 ng of random hexamers as primers.

Quantitative real-time PCR was performed by adding cDNA to the reaction mixture containing IL-2 primers (Applied Biosystems, Foster City, CA). After 40 cycles, the relative copy numbers of IL-2 mRNA were determined by normalizing the amount of total RNA added to each reaction with 18S rRNA.

Results

Suppression by in vitro generated B220⁺ DN T cells

We have recently described a system to enrich B220⁺ DN T cell population in vitro by chronic anti-CD3/CD28 stimulation of AND TCR transgenic CD4 T cells (15). Using this system, we were able to generate B220⁺ DN T cells that were at least 85% pure (Fig. 1A). We explored the suppressive properties of B220⁺ DN T cells by determining whether they could inhibit proliferation of syngeneic CD4 T cells using an in vitro model system (18–22). Freshly isolated AND TCR CD4 T cells were cultured either alone or with increasing numbers of B220⁺ DN T cells. The different cultures were stimulated with anti-CD3 beads, and proliferation was measured after 72 h by using [³H]thymidine. CD4 T cells proliferated significantly in the absence of B220⁺ DN T cells (Fig. 1B, left panel), but their proliferation was inhibited in the presence of B220⁺ DN T cells in a dose-dependent manner (Fig. 1B, right panel).

Because of the modest proliferative response of naive CD4 T cells stimulated with anti-CD3 in the absence of costimulation (Fig. 1B), it was important to determine whether B220⁺ DN T

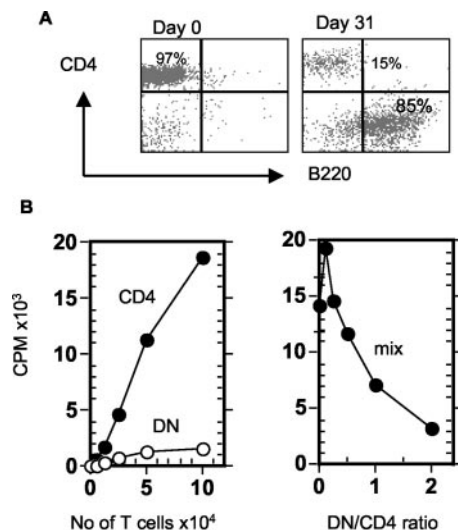


FIGURE 1. In vitro generated B220⁺ DN T cells suppress proliferation of syngeneic AND TCR CD4 T cells. *A*, Continuous anti-CD3 stimulation (*Materials and Methods*) of AND TCR CD4 T cells (*left dot plot*) led to the generation of B220⁺ DN T cells (*right dot plot*). Gates are drawn on TCR⁺ cells. B220⁺ T cells were negative for both CD4 CD8 coreceptors. *B*, CD4, but not B220⁺ DN T cells proliferated in response to stimulation with anti-CD3 beads (*left panel*). B220⁺ DN T cells inhibited proliferation of AND CD4 T cells (*right panel*). AND TCR CD4 T cells (1×10^5) were stimulated with anti-CD3 beads (1×10^5) in the presence of increasing numbers of B220⁺ DN T cells. Proliferation was measured after 72 h using [³H]thymidine. Each point represents the mean of triplicate cultures \pm SEM.

cells could inhibit more robust responses induced by TCR and CD28 stimulation. Stimulation of AND TCR transgenic CD4 T cells with anti-CD3/CD28 beads, as expected, enhanced proliferation by ~5- to 7-fold and led to IL-2 production, but did not lead to B220⁺ DN T cell proliferation (Fig. 2, A and B). Importantly, B220⁺ DN T cells completely suppressed proliferation and IL-2 production of CD28-costimulated CD4 T cells (Fig. 2, C and D). Thus, whereas CD28 costimulation augmented the overall responses of CD4 T cells, it did not abrogate the suppressive ability of the in vitro generated B220⁺ DN T cells.

Suppression by B220⁺ DN T cells from *lpr* mice

To determine the pathophysiologic relevance of the suppressive ability of in vitro generated B220⁺ DN T cells, we determined whether B220⁺ DN T cells that accumulate in Fas-deficient *lpr* mice also have suppressive properties. We isolated B220⁺ DN T cells and CD4 T cells from 16- to 20-wk-old *lpr* mice by negative selection, as described in *Materials and Methods*. Purity of B220⁺ DN and CD4 T cells was greater than 95% (Fig. 3, A and B). All DN T cells expressed B220 and manifested a phenotype of activated T cells, as indicated by high levels of CD44 and low levels of CD62L. CD45RB molecules, which are usually down-regulated on activated T cells, remained highly expressed on B220⁺ DN T cells. The majority of CD4 T cells also exhibited activated phenotype (CD44^{high}; CD62L^{low}; CD45RB^{low}) consistent with the high frequencies of autoreactive T cells in mice with impaired Fas-mediated deletion (Fig. 3B). Functionally, CD4 T cells proliferated in response to anti-CD3 stimulation, whereas B220⁺ DN T cells did not proliferate (Fig. 3C), as described (3).

We next determined whether *lpr* B220⁺ DN T cells could suppress proliferation of autologous CD4 T cells. Our initial experi-

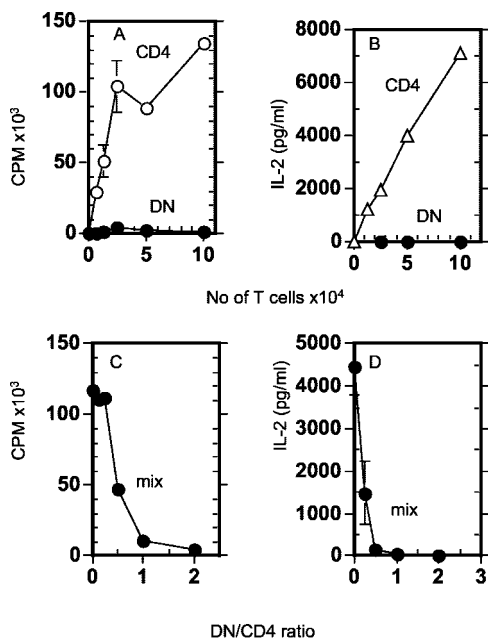


FIGURE 2. B220⁺ DN T cells suppress activation of anti-CD3/CD28-stimulated AND TCR CD4 T cells. *A*, CD4, but not B220⁺ DN T cells proliferated in response to stimulation with anti-CD3 beads. *B*, CD4, but not B220⁺ DN T cells produced IL-2 in response to stimulation with anti-CD3 beads (1×10^5). *C*, B220⁺ DN T cells inhibited proliferation of CD4 T cells. *D*, B220⁺ DN T cells inhibited IL-2 production by AND TCR CD4 T cells. B220⁺ DN T cells were cocultured at different ratios with syngeneic AND TCR CD4 T cells (1×10^5) and stimulated with anti-CD3/28 beads. Proliferation was measured by ³H and IL-2 by ELISA. Each point is the mean of triplicate cultures \pm SEM.

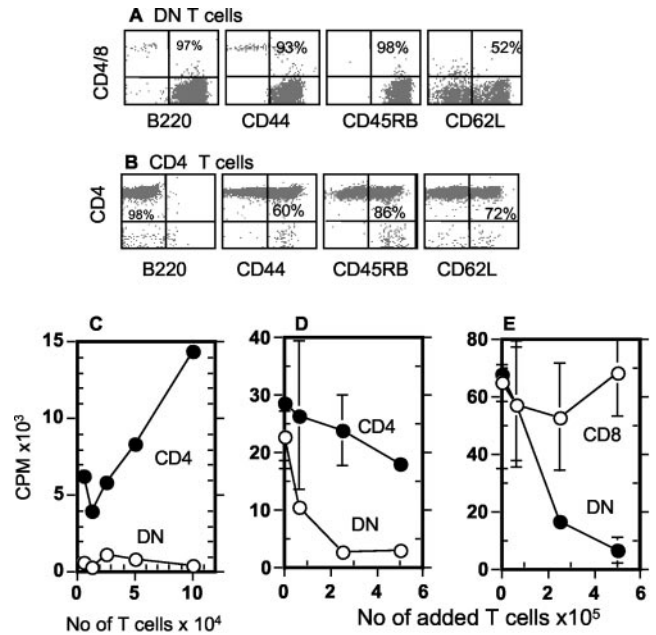


FIGURE 3. B220⁺ DN T cells from *lpr* mice inhibit proliferation of autologous CD4 and CD8 T cells. *A*, Surface phenotypes of *lpr* B220⁺ DN T cells. All gated cells were TCR positive. *B*, Surface phenotype of *lpr* CD4 T cells. All gated cells were TCR positive. Purity of B220⁺ DN and CD4 T cells was >95%. *C*, Proliferation of CD4, but not B220⁺ DN T cells stimulated with increasing numbers of anti-CD3 beads. *D*, Inhibition of proliferation of CD4 T cells (1×10^5) stimulated with anti-CD3 beads (1×10^5) in the presence of increasing numbers of B220⁺ DN T cells. Proliferation of parallel CD4 T cell cultures equivalent in cell numbers to that in corresponding cocultures was used as controls for cell numbers. *E*, Inhibition of proliferation of CD8 T cells (1×10^5) stimulated with anti-CD3 beads (1×10^5) in the presence of increasing numbers of B220⁺ DN T cells or CD8 T cells. Proliferation of parallel CD8 T cell cultures equivalent in cell numbers to that in corresponding cocultures was used as controls for cell numbers. Proliferation was measured by [³H]thymidine after 72 h. Each point is the mean of triplicate cultures \pm SEM.

ments showed that *lpr* B220⁺ DN T cells could suppress CD4 T cells, but they were less potent than the in vitro generated B220⁺ DN T cells (data not shown). Robust suppression, however, was seen at higher DN to CD4 T cell ratio of 5:1 or 2.5:1. Suppression was not significantly affected by the high numbers of cells in the cocultures, as only minimal effect was seen in corresponding cultures that contained equal number of CD4 T cells (Fig. 3D). B220⁺ DN T cells also suppressed proliferation of CD8 T cells in a dose-dependent manner, and no suppression was seen in control cultures that contain equivalent numbers of CD8 T cells (Fig. 3E).

Although stimulation of CD4 T cells with anti-CD3/28 beads resulted in vigorous proliferation and cytokine production, it did not alter the suppressive ability of B220⁺ DN T cells. Proliferation, IL-2, IL-10, and IFN- γ production of anti-CD3/28-stimulated CD4 T cells were inhibited by B220⁺ DN T cells in a dose-dependent manner (Fig. 4). These results show that *lpr* B220⁺ DN T cells possess a significant ability to suppress activation of autologous CD4 and CD8 T cells.

Role of the Fas pathway

Significant accumulation of B220⁺ DN T cells also occurs in *gld* mice with homozygous loss of function mutations in the FasL gene. We determined whether B220⁺ DN T cells from *gld* mice share the suppressive properties of B220⁺ DN T cells that accumulate in *lpr* mice. We purified B220⁺ DN T cells from 16- to

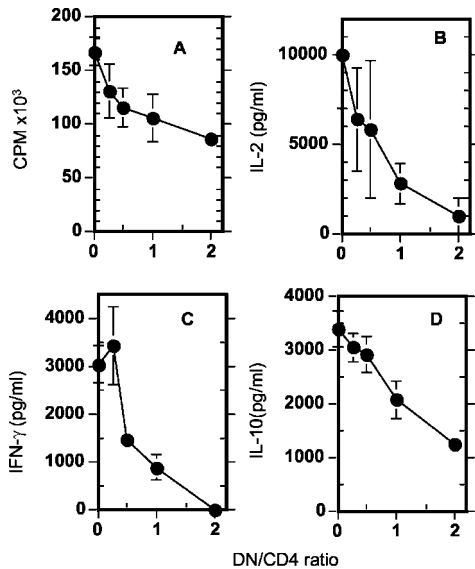


FIGURE 4. CD28 engagement does not abrogate B220⁺ DN T cell-mediated suppression. *A*, B220⁺ DN T cells inhibited proliferation of anti-CD3/CD28-stimulated CD4 T cells. *B–D*, Inhibition of IL-2, IFN- γ , and IL-10, respectively. CD4 T cells were stimulated with anti-CD3/CD28 beads in the presence of increasing numbers of B220⁺ DN T cells. Proliferation and cytokine production were measured, as described above. Each point was mean of triplicate cultures \pm SEM.

20-wk-old C3H-*gld/gld* mice and tested their ability to suppress activation of autologous (FasL^{*gld*}) or syngeneic (FasL^{*lpr*}) CD4 T cells. All cell preparations were >95% pure (data not shown). B220⁺ DN T cells from *gld* mice potently suppressed proliferation of CD4 T cells from *lpr* and *gld* mice in a dose-dependent fashion (Fig. 5*B*). They also inhibited IL-10 and IFN- γ production (data not shown). Therefore, it appears that suppression is a general

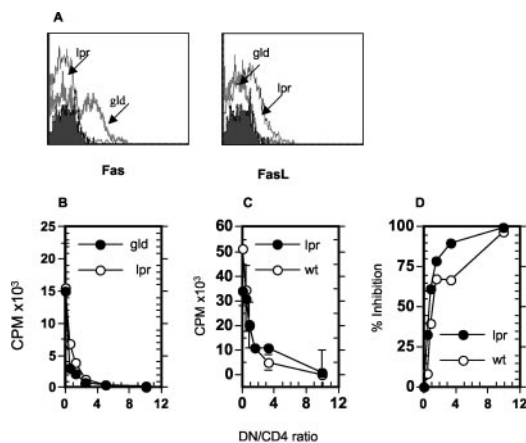


FIGURE 5. Fas/FasL interactions do not augment B220⁺ DN T cell-mediated suppression. *A*, Fas (left) and FasL (right) expression among B220⁺ DN T cells from *lpr* (thin line, open histogram) and *gld* (thick line, filled histogram) mice. Filled histograms represent isotype-matched negative controls. *B*, B220⁺ DN T cells from *gld* mice suppressed proliferation of autologous and *lpr* CD4 T cells. CD4 T cells (1×10^5) were mixed with B220⁺ DN T cells at various ratios in the presence of anti-CD3 beads (1×10^5). *C*, B220⁺ DN T cells suppressed proliferation of syngeneic (*lpr*) and wt CD4 T cells to similar extents. B220⁺ DN T cells from *lpr* mice were incubated with autologous or wt syngeneic CD4 T cells in the presence of anti-CD3 beads. Each point is the mean of triplicate cultures \pm SEM. *D*, Percentage of inhibition after normalization of suppression to the proliferation of control *lpr* and wt CD4 T cells, respectively.

function of murine B220⁺ DN T cells associated with impaired Fas-mediated apoptosis.

The ability of B220⁺ DN T cells from *lpr* and *gld* mice to suppress activation of autologous CD4 T cells shows that suppression is Fas independent. However, B220⁺ DN T cells from *lpr* mice constitutively express FasL (Fig. 5*A*, left dot plot) and are reported to induce apoptosis of wt (Fas^{wt}) CD8 T cells in a Fas-dependent fashion (12). We therefore determined whether expression of Fas on CD4 T cells augments their suppression. *lpr* B220⁺ DN T cells inhibited proliferation of Fas-expressing wt and Fas-deficient *lpr* CD4 T cells to similar extent (Fig. 5, *C* and *D*), showing that offered Fas/FasL interaction does not modulate B220⁺ DN T cell-mediated suppression of CD4 T cells. We further confirmed these results by CFSE analysis. Proliferation of CFSE-labeled CD4 T cells from *lpr* and wt mice was inhibited by *lpr* B220⁺ DN T cells to similar extent, as determined by the dilution of CFSE (data not shown). In addition, the level of apoptosis, as measured by Annexin V, among *lpr* and wt CD4 T cells cocultured with *lpr* B220⁺ DN T cells was similar (data not shown). Thus, B220⁺ DN T cell-mediated suppression appears to be Fas independent and is not modulated by Fas/FasL interactions.

B220⁺ DN T cell-mediated suppression involves inhibition of IL-2 transcription and up-regulation of its high affinity receptor (CD25)

T cell-mediated suppression can be mediated via direct contact, soluble cytokines, or both (18, 23). To determine whether suppression is contact dependent or mediated by cytokines, we performed a Transwell experiment. Separation of simultaneously activated B220⁺ DN and CD4 T cells by a semipermeable membrane of Transwell abrogated suppression, whereas significant suppression occurred in mixed cultures (Fig. 6*A*). Furthermore, supernatant fractions from mixed or separate cultures of activated CD4 T cells or B220⁺ DN T cells did not suppress proliferation of CD4 T cells, confirming that suppression is cytokine independent (data not shown). Neutralization of potentially suppressive cytokine IL-10 and TGF- β and other cytokines also did not abrogate suppression (data not shown). Suppression required TCR activation because *lpr* B220⁺ DN T cells were unable to suppress Ag-specific proliferation of AND TCR transgenic CD4 T cells stimulated with MCC/I-E^k beads (Fig. 6*B*, left panel). In contrast, in vitro generated AND Tg B220⁺ DN T cells readily suppressed proliferation of syngeneic AND Tg CD4 T cells (Fig. 6, right panel). Thus, it appears that suppression of CD4 T cells requires direct contact and TCR activation, but is cytokine independent.

Because very small amounts of IL-2 were detected in the coculture of CD4 T cells and B220⁺ DN T cells (Fig. 4*B*), lack of IL-2 could be the limiting factor that impaired CD4 T cell proliferation. Addition of exogenous IL-2 to the cocultures of *lpr* B220⁺ DN and CD4 T cells significantly abrogated suppression at low DN to CD4 T cell ratio (2.5:1) and partly reversed suppression at a higher ratio of 5:1. These results show IL-2 can overcome suppression and that lack of IL-2 plays a role in the suppression mechanism (Fig. 6*C*). Abrogation of suppression was not due to proliferation of B220⁺ DN T cells, which did not proliferate in response to anti-CD3 stimulation even in the presence of IL-2 (data not shown).

To further understand the mechanism of suppression, it was important to determine whether IL-2 was produced by CD4 T cells, but consumed by *lpr* B220⁺ DN T cells (IL-2 sink) or, alternatively, *lpr* B220⁺ DN T cells impaired the ability of CD4 T cells to produce IL-2. Differentiation between these mechanisms was achieved by the analysis of IL-2 mRNA levels in different cultures

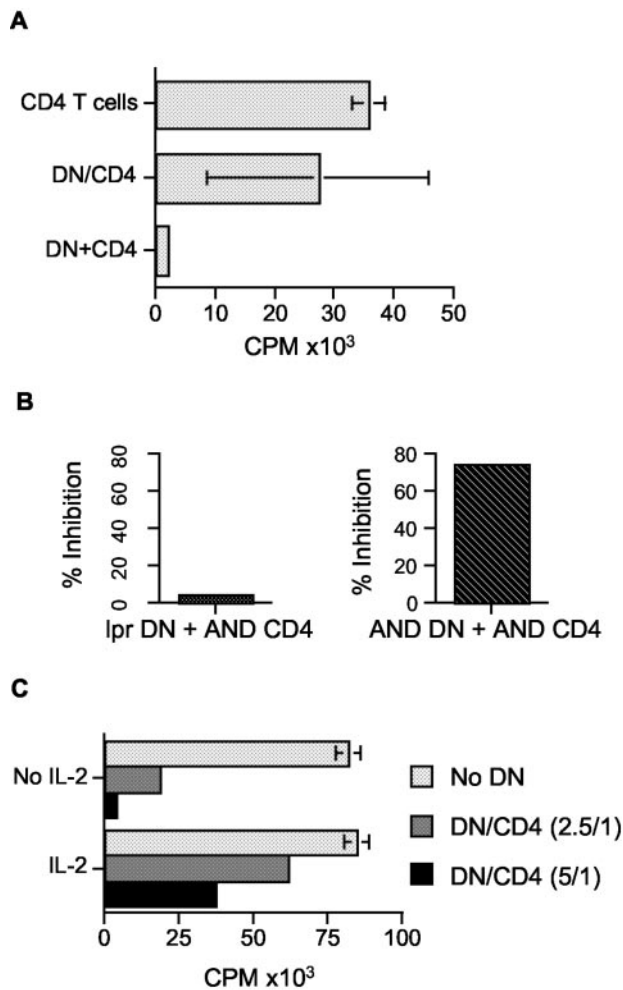


FIGURE 6. B220⁺ DN T cell-mediated suppression is contact dependent and requires TCR activation. *A*, Transwell experiments showing that *lpr* B220⁺ DN T cells (2.5×10^6) suppressed proliferation of CD4 T cells (5×10^5) when the two cell types were mixed together (DN + CD4), but not when stimulated in separate (DN/CD4) chambers of the Transwell. Anti-CD3 beads (5×10^5) were used for stimulation. Each point is a mean of triplicate cultures \pm SEM. *B*, Suppression requires TCR activation. *Left panel*, *lpr* B220⁺ DN T cells failed to suppress proliferation of MCC-stimulated AND Tg CD4 T cells. MCC-specific AND TCR transgenic CD4 T cells (1×10^5) cultured alone or in the presence of B220⁺ DN T cells (2.5×10^5) were stimulated with I-E^k/MCC beads (1×10^5). There was no detectable alloreactive proliferation between AND TCR transgenic CD4 T cells and B220⁺ DN T cells, which share the same MHC haplotype (H-2^k). *Right panel*, AND B220⁺ DN T cells inhibited proliferation of syngeneic AND Tg CD4 T cells. MCC-specific AND TCR transgenic CD4 T cells (1×10^5) cultured alone or in the presence of B220⁺ DN T cells (1×10^5) were stimulated with I-E^k/MCC beads (1×10^5). Proliferation was measured after 72 h using [³H]thymidine. Results were represented as percentage of inhibition of suppression. *C*, IL-2 abrogates suppression. CD4 T cells were cultured alone or in the presence of *lpr* B220⁺ DN T cells at 5:1 or 2.5:1 ratio and stimulated with anti-CD3 beads. IL-2 (100 U) was added to the indicated cultures at the time of stimulation. Proliferation was measured after 72 h, as described above. Each point was the mean of triplicate cultures \pm SEM.

using quantitative real-time PCR. There was almost complete inhibition of IL-2 transcription by CD4 T cells stimulated in the presence of *lpr* B220⁺ DN T cells, whereas high numbers of IL-2 mRNA transcripts were detected in CD4 T cells stimulated in the absence of B220⁺ DN T cells (Fig. 7A). No IL-2 mRNAs were detected in B220⁺ DN T cell stimulated alone consistent with their

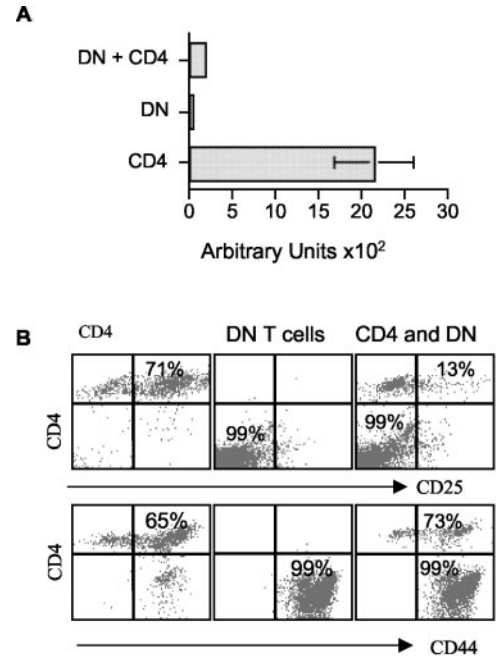


FIGURE 7. Suppression mechanism involves inhibition of IL-2 transcription and its high affinity receptor (CD25). *A*, *lpr* B220⁺ DN T cells inhibit IL-2 transcription. CD4 T cells (5×10^5) were stimulated with anti-CD3 in the presence or absence of *lpr* B220⁺ DN T cells (2.5×10^6). The relative expression of IL-2 as determined by quantitative real-time PCR using IL-2 primers and probes on cDNA prepared from various cultures is shown. Normalization of cDNA content was done on 18S; numbers represent a ratio of relative IL-2 mRNA to 18S for each culture. Each point is the mean of two cultures \pm SEM. *B*, B220⁺ DN T cells inhibit CD25 up-regulation. CD4 and B220⁺ DN T cells were cultured either alone or in mixed cultures and stimulated with anti-CD3 beads. CD25 and CD44 expression levels on each cell type were measured after 48 h. Electronic gates were drawn on TCR⁺ cells. CD4 T cells and B220⁺ DN T cells in the mixed cultures were distinguished by their differential expression of CD4 coreceptor. B220⁺ DN T cells were also negative for CD8 coreceptor.

failure to produce IL-2. Because the ratio of DN to CD4 cells was 5:1 and DN cells do not express IL-2 mRNA, the decreased transcript levels in the cocultured cells might have resulted from a cell dilutional effect. However, the inhibition of IL-2 mRNA in DN/CD4 cocultures was so potent and the differences in the levels of IL-2 message between CD4 cultures and CD4 and DN cocultures were in the range of 15- to 20-fold and thus could not be accounted for by the dilutional effect alone. In addition, inhibition of IL-2 mRNA transcription provides an explanation for the suppression of IL-2 production in the DN/CD4 T cell cocultures (Figs. 2D and 4B). The alternative possibility that IL-2 was produced by CD4 T cells, but consumed by B220⁺ DN T cells is unlikely, as B220⁺ DN T cells (Fig. 7B) did not up-regulate CD25 (IL-2R α -chain), and thus their ability to act as IL-2 sink is significantly impaired. Thus, the failure of suppressed CD4 T cells to produce IL-2 implicates inhibition of IL-2 transcription as mechanism of suppression and rules out consumption of IL-2 by *lpr* B220⁺ DN T cells as a cause of suppression.

We also determined whether B220⁺ DN T cells could suppress CD25 up-regulation. Expression of CD25 is necessary for efficient IL-2 uptake and proliferation of activated T cells. B220⁺ DN T cells potently inhibited CD25 up-regulation. Very few CD4 T cells expressed CD25 when stimulated in the presence of B220⁺ DN T cells (Fig. 7B, right top panel), whereas the CD44 expression was not inhibited (Fig. 7B, right bottom panel). In positive control cultures of CD4 T cells that were stimulated in the absence of *lpr*

B220⁺ DN T cells, high levels of both CD25 (Fig. 7B, left top panel) and CD44 molecules (Fig. 7B, left bottom panel) were detected. B220⁺ DN T cells did not express CD25 molecules when stimulated alone or in the presence of CD4 T cells (Fig. 7B, middle and right top panels). These results show that B220⁺ DN T cell-mediated suppression involves inhibition of transcription of IL-2 and its high affinity receptor.

Discussion

These studies show that B220⁺ DN T cells, whether in vitro generated or isolated from *lpr* or *gld* mice, can potently suppress proliferation of activated CD4 T cell and prevent cytokine production. Suppression is Fas independent and is not influenced by Fas/FasL interaction. Suppression mechanism involves inhibition of IL-2 production and requires cell/cell contact and TCR activation, but not soluble cytokines. These findings suggest a potential immunoregulatory role for B220⁺ DN T cells in animals and humans with impaired Fas-mediated tolerance.

Fas-induced apoptosis is critical for elimination of effector T cells and maintenance of immune homeostasis. To allow productive immune response, activated cells remain relatively resistant to Fas-mediated apoptosis during the early phase of activation, proliferation, and differentiation into effector cells (2). Production of IL-2 as well as undergoing cell cycle are among the factors that sensitize activated T cells for Fas-mediated apoptosis (2). IL-2 facilitates Fas-mediated signal by inhibition of the transcription and expression of Fas-associated death domain-like IL-1 β -converting enzyme-inhibitory protein (24). Failure of suppressed cells to produce IL-2 could thus reduce the impact of Fas engagement on the intensity of suppression. Consistent with this view, we did not detect differences in the level of suppression of Fas-deficient and Fas-expressing CD4 T cells by FasL-*lpr* B220⁺ DN T cells (Fig. 5, C and D). In contrast, *lpr* B220⁺ DN T cells were reported to kill target cells in the presence of IL-2 and when preactivated CD8 T cells were used (12). Thus, it appears that the activation status and presence or absence of IL-2 are important factors that influence whether target cells are suppressed or killed by *lpr* B220⁺ DN T cells. The potential ability of *lpr* B220⁺ DN T cells to induce apoptosis of Fas-sensitive cells puts some restraints on the use of *lpr* B220⁺ DN T cells for adoptive immunotherapy, as it may lead to nonspecific cytotoxicity. Consistent with this view (3), syngeneic transfer of *lpr/lpr* (25), but not *gld/gld* (26) bone marrow into irradiated wt mice has been reported to result in a wasting disease. Efforts should therefore focus on using the equally potent, but potentially safe, FasL-deficient, *gld* B220⁺ DN T cells for adoptive immunotherapy studies.

In summary, our data show that B220⁺ DN T cells derived from various sources are capable of suppressing proliferation of autologous and syngeneic T cells by a Fas-independent mechanism that involves inhibition of IL-2 and its high affinity receptor. Suppression also requires cell/cell contact and TCR activation of B220⁺ DN T cells, but not soluble cytokines. These results offer novel insights into the relationship between the different components of T cell tolerance network. In addition, these studies may help better understanding of the pathogenesis of autoimmune lymphoproliferative syndrome in humans and have broad implications for other autoimmune disorders.

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