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TCR/Self-Antigen Interactions Drive Double-Negative T Cell Peripheral Expansion and Differentiation into Suppressor Cells[1]

John J. Priatel, Oliver Utting, and Hung-Sia Teh[2]

Mature CD4$^-$CD8$^-$ $\alpha\beta^+$ T cells (DNTC) in the periphery of TCR transgenic mice are resistant to clonal deletion in cognate Ag-expressing (Ag$^+$) mice. Previously, we have characterized DNTC populations bearing the alloreactive 2C TCR in Ag-free (Ag$^-$) and Ag$^+$ mice. Despite appearing functionally anergic when challenged with cognate Ag in vitro, Ag-experienced DNTC exhibit markers of activation/memory, a lowered threshold of activation, ex vivo cytolytic activity, and the ability to rapidly secrete IFN-γ. Remarkably, these memory-like DNTC also possess potent immunoregulatory properties, competing effectively for bystander-produced IL-2 and suppressing autoreactive CD8$^+$ T cell proliferation via a Fas/FasL-dependent cytolytic mechanism. The fact that DNTC recovered from Ag$^+$ mice possess markers and attributes characteristic of naive CD8$^+$ T cells that have undergone homeostasis-induced proliferation suggested that they may be derived from a similar peripheral expansion process. Naive DNTC adoptively transferred into Ag-bearing hosts rapidly acquire markers and functional attributes of DNTC that have continually developed in the presence of Ag. Thus, the peripheral selection and maintenance of such autoreactive cells may serve to negatively regulate potential autoimmune T cell responses. *The Journal of Immunology,* **2001, 167: 6188–6194.**

Peripheral T cell tolerance is essential to avoid self-inflicted damages caused by autoimmunity. To ensure its maintenance, a number of mechanisms exist, including the intrathymic deletion of autoreactive T cells (1), the induction of peripheral T cell anergy by self Ags (2), and clonal ignorance (3). T cell anergy is defined as a cellular state in which a cell is viable but exhibits decreased proliferation and cytokine production upon engagement of the Ag receptor. In contrast to intrathymic clonal deletion, the silencing of autoreactive T cells through T cell anergy may be precarious, as a failure to maintain this nonresponsive state would result in dire consequences. However, autoreactive cells may play a regulatory role. Recent studies emphasize a role for regulatory T cells in down-regulating responses toward self and allogeneic Ags (4). Investigations on lymphopenic animals, regardless of their derivation, have shown that they have a high incidence of developing autoimmune diseases (4). As these types of autoimmunity can be circumvented by the administration of T cell populations of a specific cell surface phenotype, it suggests that T cells may play an active role in their own suppression (4).

In addition to regulating their responses to cognate Ag, T cells also function in maintaining the size of the peripheral T cell pool (5). For example, the adoptive transfer of naive CD4$^+$ or CD8$^+$ T cells into a lymphopenic environment results in them abandoning their quiescent state and undergoing extensive proliferation. Such homeostasis-driven expansion does not result from stimulation with cognate Ag, rather it requires low-affinity interactions with self peptides/MHC complexes (5). However, neither costimulation through CD28 nor accessibility to IL-2 appears critical for this process (6). Although the slow proliferation driven by homeostatic forces does not result in the up-regulation of acute activation markers like CD69 and CD25 or effector cell formation, these dividing cells do acquire markers and characteristics of memory T cells (6–8). In contrast to naive T cells, CD8$^+$ T cells derived from homeostatic expansion mount accelerated responses to cognate Ag, possess significant CTL activity, and produce IFN-γ without a requirement for prior stimulation. Conversion of naive cells into memory ones through lymphopenia-induced homeostatic proliferation may account for the increased frequency of memory/activated T cells in lymphopenic individuals such as patients suffering with AIDS or immunosuppressive regimens (9).

The peripheral lymphoid organs of $\alpha\beta$ TCR transgenic mice contain significant numbers of CD4$^-$CD8$^-$ T cells that express the transgenic $\alpha\beta$ TCR (DNTC[3]; Ref. 10). Unlike conventional cells, DNTC from TCR transgenic mice do not express endogenous TCRα genes (11), they develop independently of class I MHC molecules (12, 13), and they are resistant to clonal deletion in cognate Ag-expressing mice (10, 12). We have previously characterized mature DNTC bearing the alloreactive 2C TCR in cognate Ag-free (H-2^b) and Ag-expressing (H-2^d) mice (14). The 2C TCR recognizes the naturally occurring peptides p2Ca and QL9 (both derived from the same ubiquitous protein) in the context of L^d (15, 16), and it is positively selected by K^b MHC class I molecules (17). To examine the effects of chronic exposure to physiological levels of cognate antigenic ligand, we performed functional analyses on DNTC from Ag-free (Ag$^-$) and Ag$^+$ mice. Initial comparisons of these two populations revealed that DNTC from Ag$^+$ mice appeared functionally anergic as they hypoproliferate and produce little or no IL-2 in response to Ag stimulation

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[3] Abbreviations used in this paper: DNTC, double-negative (CD4$^-$CD8$^-$$\alpha\beta^+$) T cell; LN, lymph node; ccDNA, competitor cDNA.

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(14). However, they differ from conventional in vivo anergized T cells (18, 19) because this type of anergy is reversible by the addition of exogenous IL-2. Interestingly, DNTC from Ag^+ mice also possess a lowered activation threshold as they respond to a low-affinity ligand by up-regulating CD25 and CD69 (20). By contrast, DNTC from Ag^- mice are refractory to stimulation by the low-affinity ligand. In this study, we demonstrate that this autoreactive population has a number of features in common with naive $CD8^+$ T cells that have undergone homeostatic proliferation and conversion into memory-like T cells. Furthermore, DNTC from Ag^+ mice can sequester bystander-produced cytokine to drive their proliferation and cytolytic effector function. Importantly, these DNTC could suppress autoreactive $CD8^+$ T cell proliferation by using a Fas/FasL-dependent cytolytic mechanism. Collectively, our data suggest a novel mechanism for the preservation of self-tolerance. This mechanism is dependent on the differentiation of DNTC into regulatory effector cells following a self Ag-driven peripheral expansion process that resembles lymphopenia-induced $CD8^+$ T cell homeostatic proliferation.

Materials and Methods

Mice

Breeders for the H-2^b 2C TCR transgenic mice were kindly provided by Dr. D. Y. Loh (Washington University, St. Louis, MO). The 2C TCR transgenic mice were bred onto the C57BL/6 (H-2^b) background. H-$2^{b/d}$ 2C were F_1 mice generated by mating H-2^b 2C mice with DBA/2 (H-2^d). C57BL/6-*lpr/lpr* mice were obtained from The Jackson Laboratory (Bar Harbor, ME). H-2^b 2C mice were also crossed onto a Fas-deficient (*lpr*) background. Mice were housed under pathogen-free conditions in the Department of Microbiology and Immunology's animal facility at the University of British Columbia (Vancouver, Canada). All studies followed the guidelines set by the university's Animal Care Committee and the Canadian Council on Animal Care.

CFSE labeling

DNTC (1×10^7/ml) were labeled with 1 μM CFSE (Molecular Probes, Eugene, OR) in PBS for 10 min at room temperature. After stopping the reaction with the addition of an equal volume of FCS, cells were washed four times with complete media.

APCs and peptides

The peptide transporter-deficient cell lines T2-L^d and T2-K^b were created by transfection of the human (T × B) hybridoma T2 with either L^d or K^b mouse MHC class I molecules (21). The p2Ca (LSPFPFDL; Ref. 22) and pOVA (SIINFEKL; Refs. 16, 23, and 24) peptides were synthesized at the University of British Columbia's Nucleic Acid-Protein Service Unit.

Proliferation assays

DNTC were purified as previously described (14). Ten thousand DNTC were stimulated with 5×10^4 mitomycin C-treated T2-L^d cells loaded with 1 μM p2Ca peptide. DNTC proliferation was compared with p2Ca/APCs alone or with 10 ng/ml rIL-2 or rIL-15 (R&D Systems, Minneapolis, MN). Cells were cultured in a 200-μl volume in a U-bottom 96-well plate. After 72 h, cultures were pulsed with 1 μCi of [^{3}H]thymidine for 8 h to assess proliferation.

RT-PCR

RNA was purified from Ag-stimulated DNTC. Briefly, 1×10^5 DNTC were incubated with 5×10^4 L^d-expressing APCs (T2-L^d) loaded with 1 μM p2Ca peptide in a U-bottom 96-well plate for 8 h. Cells from 48 wells were pooled per sample, and RNA was prepared using the RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Following reverse transcription, cDNA samples were normalized for hypoxanthine phosphoribosyltransferase content using competitive-quantitative PCR before analyzing IL-2, TNF-α, and TGF-β expression. The primers used to detect expression of these cytokines and the competitor, pPQRS plasmid, have been described previously (25). To detect IL-15 expression, primers specific to two different exons were used to amplify a 290-bp fragment by RT-PCR. The sequences of the primers read as follows: 5′-ACCACTTTATACACTGACAGTGAC-3′ and 5′-CTCGCATGCAGTCAGGACGTGTTG-3′. Digitized images of ethidium-stained agarose gels were inverted in Adobe Photoshop (Adobe Systems, Mountain View, CA).

Flow cytometry

Single cell suspensions from lymph nodes (LNs) were subjected to RBC lysis by ammonium chloride. All incubations were done in FACS buffer (2% FCS in PBS) on ice for 15 min. All Ab reagents were purchased from BD PharMingen (San Diego, CA) except for anti-mouse CD8-Tricolor, which was purchased from Caltag Laboratories (Burlingame, CA), and 1B11 Ab (26), which was purchased from H. J. Ziltener (University of British Columbia). Data was acquired using a FACScan and it was analyzed by CellQuest software (BD Biosciences). For intracellular IFN-γ detection, LN cells (5×10^5) were stimulated with T2-L^d cells loaded with 1 μM p2Ca for 8 h. To block cytokine secretion, Golgi Plug (BD PharMingen) was added 3 h into the incubation. At the finish of the stimulation, the cells were labeled with cell surface markers before fixing with 2% paraformaldehyde in PBS. After permeabilization with 0.3% saponin in PBS, cells were stained intracellularly, washed, and analyzed by flow cytometry.

Coculture to monitor bystander proliferation

Fifty thousand labeled DNTC were preactivated for 24 h with Ag (T2-L^d plus 1 μM p2Ca) before the addition of either media alone or 5×10^4 2C $CD8^+$ T cells. After culture for an additional 2 days, DNTC proliferation was tracked by flow cytometry.

Coculture to monitor DNTC suppressor activity

Fifty thousand unlabeled DNTC were preactivated with cognate Ag for 24 h before the addition of 5×10^4 CFSE-labeled 2C $CD8^+$ T cells. Labeled $CD8^+$ T cells were either added to wells containing activated DNTC or Ag alone. Proliferation of $CD8^+$ T cells was measured by flow cytometry 3 days following their stimulation.

Direct ex vivo CTL assays

DNTC were purified from peripheral LN. TAP-deficient cells (T2-K^b or T2-L^d) expressing either K^b or L^d were ^{51}Cr labeled. Washed targets (1×10^4) were mixed with various ratios of effectors and were incubated in 200 μl of media in 96-well V-bottom plates. Assays were done in the presence and absence of 1 μM p2Ca peptide. After 5 h of incubation, supernatants were collected and counted. The spontaneous release varied between 10 and 15% of the maximum release counts. All assays were performed in triplicate. Percent maximum release was calculated as 100% × [cpm (experimental well) − cpm (spontaneous release)]/[cpm (maximum release) − cpm (spontaneous release)].

CTL assays with ex vivo activated effectors

DNTC or 2C $CD8^+$ T cell effectors were generated by a 3-day stimulation with Ag and IL-2. To produce targets, $CD8^+$ T cells were positively selected by magnetic separation using the MiniMACS system (Miltenyi Biotec, Auburn, CA) according to manufacturer's specifications. After activating the purified $CD8^+$ T cells with ConA and IL-2 for 40 h, blasts were labeled with ^{51}Cr for 1 h at 37°C. Washed targets (1×10^4) were incubated with 1×10^5 effectors for 5 h before collection of supernatants for counting. The pOVA and p2Ca peptides were used at a final concentration of 1 μM. Fas/FasL interactions were blocked by the addition of 10 μg/ml recombinant Fas-Fc fusion protein (Immunex, Seattle, WA).

Adoptive transfers

Naive DNTC and $CD8^+$ T cells were recovered from the LNs of H-2^b 2C TCR transgenic mice. Ag-experienced DNTC were obtained from H-$2^{b/d}$ 2C TCR transgenic mice. Purified T cell populations were labeled with CFSE, and 1×10^6 cells were transferred per mouse via tail vein injections. C57BL/6 (B6) and BDF_1 (B6 × DBA/2 F_1) recipient animals were subjected to 600 rad of gamma irradiation 5 h before cell transfer. Proliferation assays were set up as described above except that 2×10^3 $1B2^+$ cells were added per well.

Results

DNTC from Ag^+ mice possess markers and attributes of $CD8^+$ T cells that have undergone homeostatic expansion

A comparison of the proportion of DNTC in Ag^- and Ag^+ mice revealed a 2-fold increase in DNTC in the peripheral lymphoid organs of Ag^+ mice (Fig. 1*A*). Associated with this increased incidence, there is a ~50% increase in the DNTC yield from Ag^+ mice. Despite the persistence of autoreactive DNTC, Ag^+ mice

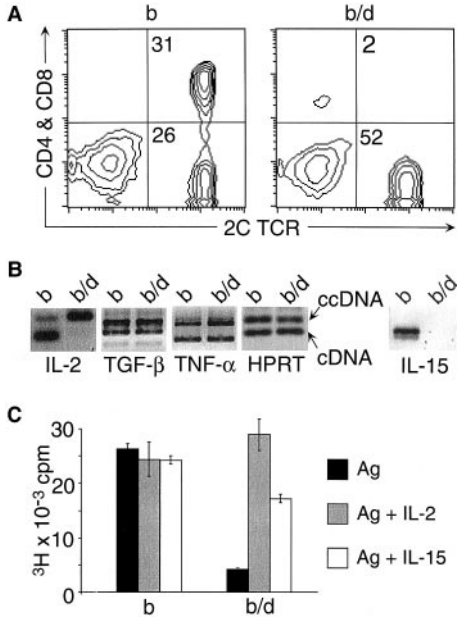

FIGURE 1. DNTC from Ag^+ mice fail to transcribe cytokines necessary for Ag-driven proliferation. *A*, DNTC in TCR transgenic mice are resistant to clonal deletion in Ag^+ (b/d) mice. LN T cells were incubated with 1B2, an anti-clonotypic 2C TCR Ab, anti-CD4-PE, and anti-CD8-PE and were analyzed by FACS. *B*, H-$2^{b/d}$ DNTC express diminished levels of IL-2 and IL-15 after Ag stimulation. Arrows signify the positions of the wild-type cDNA and the competitor cDNA fragments. *C*, Ag-induced proliferation of H-$2^{b/d}$ (b/d) DNTC is augmented by either exogenous IL-2 or IL-15. The TAP-deficient cell line T2-L^d (21) was used as an APC and was loaded with 1 μM p2Ca peptide. Assays were done in triplicate and the error bars represent the SD.

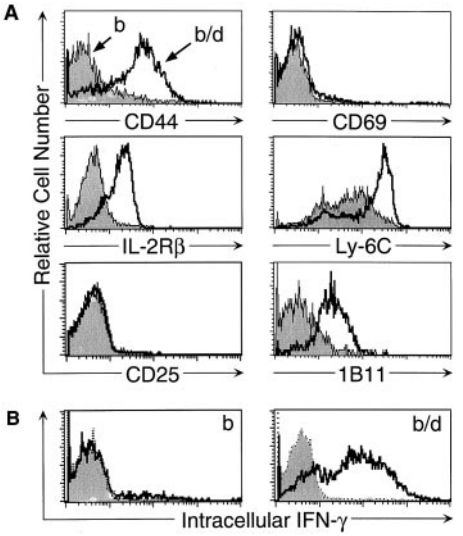

FIGURE 2. DNTC from Ag^+ mice possess qualities of memory $CD8^+$ T cells. *A*, Ag-experienced DNTC (bold line) lack expression of acute activation markers CD69 and CD25 but express memory/activation markers CD44, IL-2Rβ, Ly-6C, and 1B11. Shaded histograms denote expression of the indicated marker by DNTC from Ag^- mice. *B*, DNTC from Ag^+ mice rapidly express IFN-γ after TCR activation. Shaded histograms represent isotype control staining.

fail to display overt signs of autoimmunity. We have previously noted that tolerance induction of these self-reactive cells may involve T cell anergy (14). Consistent with T cell anergy, DNTC from Ag^+ mice transcribe severely reduced amounts of IL-2 following challenge with antigenic ligand (Fig. 1*B*). These cells are also defective in IL-15 synthesis, but they express equivalent levels of TNF-α and TGF-β. However, unlike other models of T cell anergy (27), DNTC from Ag^+ mice do not express elevated levels of IL-10 (data not shown). The defective response by DNTC from Ag^+ mice to Ag could be overcome by the addition of either exogenous IL-2 or IL-15 (Fig. 1*C*). Reconstitution of the proliferative response by IL-15 is likely due to the high expression of IL-2Rβ, a receptor for IL-15 (28), by these cells.

DNTC from Ag^+ mice express surface markers present on both memory and naive $CD8^+$ T cells that have undergone homeostatic expansion in lymphopenic hosts (6–8). Thus, DNTC from Ag^+ mice express high levels of the CD44, Ly6C, and IL-2Rβ memory markers (Fig. 2*A*). They also parallel memory T cells in that they do not express the acute activation markers CD25 and CD69 (Fig. 2*A*). In addition, H-$2^{b/d}$ DNTC also expressed the 1B11 epitope, a recently described marker for effector $CD8^+$ T cells (29, 30). Further corroborating a memory phenotype, DNTC from Ag^+ mice are capable of synthesizing high levels of IFN-γ rapidly after stimulation (Fig. 2*B*). By contrast, neither DNTC from Ag^- nor naive $CD8^+$ T cells produced detectable levels of the cytokine during the same test period (Fig. 2*B* and data not shown).

DNTC from Ag^+ mice use cytokines produced by activated bystander T cells and possess suppressor activity

DNTC from Ag^+ mice up-regulate CD25 when stimulated either by the low- (p2Ca/K^b) or the high- (p2Ca/L^d) affinity ligands (Ref. 20; Fig. 3*A*). By contrast, DNTC from Ag^- mice up-regulate CD25 only in response to the high-affinity ligand. To test whether this heightened capacity of Ag-experienced DNTC to up-regulate CD25 makes them effective competitors for growth factors produced during the course of a normal immune response, we designed a cell coculture experiment. Ag-stimulated DNTC, labeled with CFSE (31) to track cellular proliferation, were incubated for 3 days in the presence or absence of 2C $CD8^+$ T cells and their proliferation was compared. As the DNTC and added $CD8^+$ T cells express the same TCR, they can both respond to the same Ag. DNTC from Ag^- (H-2^b) mice proliferated well to Ag alone (Fig. 3*B*). By contrast, the majority of DNTC from Ag^+ (H-$2^{b/d}$) mice have died after 3 days of stimulation, as evidenced by the loss of CFSE-labeled cells. The few cells that survived responded very poorly to Ag stimulation (Fig. 3*B*). Strikingly, the proliferation of H-$2^{b/d}$ DNTC was greatly enhanced by the presence of activated 2C $CD8^+$ T cells or exogenous IL-2, whereas the proliferation of the H-2^b DNTC was not further augmented by these additions. This result indicates that H-$2^{b/d}$ DNTC can compete effectively for growth factors produced by neighboring activated $CD8^+$ T cells.

After observing the bystander effect, we investigated whether H-$2^{b/d}$ DNTC could regulate the proliferation of $CD8^+$ T lymphocytes. A similar coculture experiment was performed to test this possibility. This time CFSE-labeled 2C $CD8^+$ T cells were either cultured with Ag alone or with preactivated unlabeled DNTC from either Ag^- (H-2^b) or Ag^+ (H-$2^{b/d}$) 2C mice. The results indicate that the H-$2^{b/d}$ DNTC were very effective suppressors of Ag-induced $CD8^+$ T cell proliferation. By contrast, the H-2^b DNTC were ineffective suppressors (Fig. 3*C*). Most striking was the reduction in CFSE-labeled $CD8^+$ T cell number in wells containing H-$2^{b/d}$ DNTC. As exogenous IL-2 did not reverse this suppressive effect, mechanisms other than limiting amount of this cytokine were sought to explain this suppressive effect.

Mechanism of DNTC suppression

As $CD8^+$ T cells undergoing lymphopenia-induced homeostatic expansion displaycytolytic activity (6–8) and H-$2^{b/d}$ DNTC also

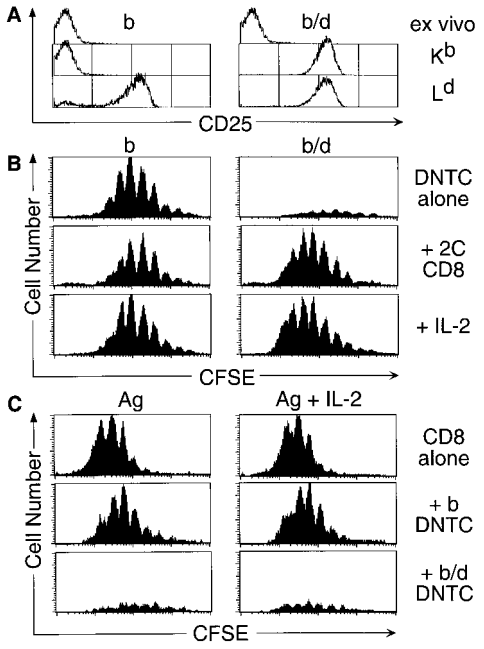

FIGURE 3. Immunoregulatory properties of DNTC from Ag^+ mice. *A*, DNTC from Ag^+ (b/d) mice rapidly up-regulate CD25 upon stimulation with either low- (K^b) or high- (L^d) affinity ligands. Cells were stimulated for 8 h with 1 μM p2Ca in the presence of either K^b- or L^d-expressing APCs. *B*, Bystander-activated T cells promote proliferation of DNTC from Ag^+ (b/d) mice. DNTC, ± culture with 2C $CD8^+$ T cells, were stimulated with APCs loaded with 1 μM p2Ca. DNTC proliferation after a 3-day incubation period was tracked by CFSE labeling (31). Only viable cells, determined by forward and side scatter, were included in the analyses. The bottom row of histograms represents proliferation of DNTC when stimulated with Ag and exogenous IL-2. *C*, DNTC from Ag^+ mice suppress $CD8^+$ T cell proliferation. Purified 2C $CD8^+$ T cells, with or without coculture with DNTC from either Ag^- (b) or Ag^+ (b/d) mice, were stimulated with 1 μM p2Ca-loaded APCs. 2C $CD8^+$ T cell proliferation was tracked by CFSE labeling after 3 days of stimulation. H-$2^{b/d}$ DNTC suppression of $CD8^+$ T cell proliferation was not reversed by exogenous IL-2.

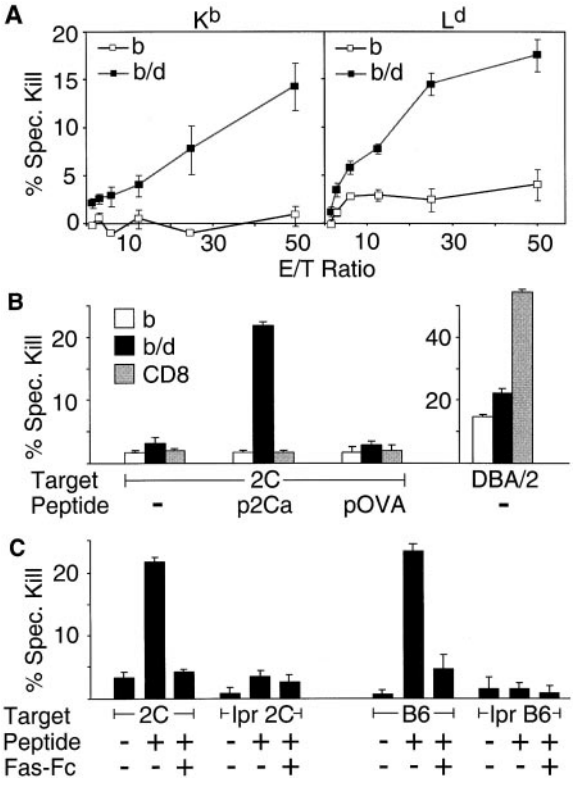

FIGURE 4. DNTC from Ag^+ mice possess ex vivo cytolytic activity and a heightened Fas/FasL-dependent killing capacity after activation. *A*, Ex vivo H-$2^{b/d}$ DNTC exhibit cytolytic activity toward targets expressing either high- (p2Ca/L^d) or low- (p2Ca/K^b) affinity ligands. DNTC killing was less than 3% in the absence of peptide. *B*, Activated H-$2^{b/d}$ DNTC can kill $CD8^+$ Con A blasts expressing low-affinity ligand. DNTC from Ag^- (b) and Ag^+ (b/d) mice and 2C $CD8^+$ T cell effectors were generated by 3 days of stimulation with Ag and IL-2. ^{51}Cr-labeled $CD8^+$ T cell targets, either 2C (H-$2^{b/b}$) or nontransgenic DBA/2 (H-$2^{d/d}$), were prepared from 2-day-activated Con A blasts. A fixed E:T ratio of 10:1 was used. All assays were done in triplicate and the error bars represent the SD. *C*, DNTC kill targets in a Fas/FasL-dependent manner and irrespective of TCR specificity. DNTC from Ag^+ mice were activated and their cytolytic activity against $CD8^+$ Con A blasts was determined as described in *B*. DNTC from Ag^+ mice kill wild-type (2C) but not Fas-deficient (*lpr* 2C) 2C $CD8^+$ T cell blasts. DNTC kill nontransgenic (B6) but not Fas-deficient (*lpr*) $CD8^+$ T cells. Disruption of Fas/FasL interaction by addition of Fas-Fc fusion protein blocks killing by activated DNTC.

express the cytotoxic effector marker, 1B11, we sought to determine whether DNTC from Ag^+ mice also possess cytolytic activity. To investigate this possibility, DNTC from either H-$2^{b/d}$ or H-2^b 2C mice were purified and incubated with ^{51}Cr-labeled T2-K^b and T2-L^d APCs loaded with 1 μM p2Ca peptide. Remarkably, H-$2^{b/d}$ DNTC exhibited spontaneous cytolytic activity against APCs expressing either the high-affinity (p2Ca/L^d, $K_A = 2 \times 10^6$ M^{-1}) or low-affinity (p2Ca/K^b, $K_A = 3 \times 10^3$ M^{-1}) ligands (Ref. 23; Fig. 4*A*). By contrast, H-2^b DNTC displayed no killer activity toward p2Ca/K^b-bearing targets and a very weak, if any, cytolytic activity toward p2Ca/L^d-expressing ones.

We next determined whether H-$2^{b/d}$ DNTC could kill $CD8^+$ target cells expressing physiological levels of the low-affinity ligand. The effectiveness of Ag-activated H-$2^{b/d}$ DNTC to kill CD8 Con A blasts from H-2^b 2C mice was tested in a standard ^{51}Cr-release assay. It was found that Ag-activated H-$2^{b/d}$ DNTC killed 2C $CD8^+$ blasts efficiently only in the presence of 1 μM p2Ca peptide (Fig. 4*B*). This killing was specific for the p2Ca peptide, as the addition of the K^b binding peptide pOVA (16, 24) did not render them susceptible to lysis. By contrast, neither Ag-activated H-2^b DNTC nor H-2^b 2C $CD8^+$ T cells could kill these targets even when they were supplemented with the p2Ca peptide. This finding indicates that H-$2^{b/d}$ DNTC possess a broader range of target specificity than either H-2^b DNTC or 2C $CD8^+$ T cells. Both Ag-activated DNTC from H-2^b or H-$2^{b/d}$ 2C mice were effective killers of DBA/2 (H-2^d) Con A blasts, which express the cognate Ag, in the absence of exogenously added p2Ca peptide (Fig. 4*B*). As expected, Ag-activated $CD8^+$ T cells from H-2^b 2C mice were the most efficient killers of DBA/2 targets.

Two major pathways for T cell mediated cytotoxicity exist, one Fas based and the other perforin based (32). To determine the relative contribution of Fas/FasL signaling to DNTC killing of $CD8^+$ T cell targets, we used both Fas-deficient targets and recombinant Fas-Fc fusion protein (33) to abrogate Fas/FasL interaction. Interestingly, H-$2^{b/d}$ DNTC exhibited greatly reduced killing activity toward Fas-deficient 2C $CD8^+$ targets as compared with wild-type (Fas^+) targets (Fig. 4*C*). Corroborating a major role of Fas signaling in DNTC cytotoxicity, the killing of wild-type 2C $CD8^+$ blasts was blocked by the addition of Fas-Fc fusion protein. Furthermore, the killing of $CD8^+$ T cells was not dependent on them expressing the 2C TCR (Fig. 4*C*). Nontransgenic $CD8^+$ T cells could be killed as effectively as 2C TCR-expressing cells.

Therefore, the H-$2^{b/d}$ DNTC killing is dependent upon the expression of Ag (in this case the low-affinity ligand, p2Ca/K^b) and Fas on the target cell surface.

DNTC require interaction with cognate Ag to drive their peripheral expansion

As DNTC from Ag^+ mice share characteristics with $CD8^+$ T cells stimulated by homeostatic proliferation, we sought to test whether these cells may be derived from a similar process. First, we examined whether cognate Ag stimulation (H-$2^{b/d}$ splenocytes) of DNTC from Ag^- mice induced the expression of the acute activation markers CD25 and CD69. In agreement with our hypothesis, DNTC did not up-regulate these acute activation markers at either 16 h (Fig. 5*A*) or 40 h (data not shown) post-stimulation, whereas $CD8^+$ T cells rapidly expressed these markers. To test the ability of naive DNTC to undergo expansion in vivo, we injected CFSE-labeled cells into sublethally irradiated Ag^- (H-$2^{b/b}$) or Ag^+ (H-$2^{b/d}$) mice and analyzed their proliferation 1 wk after transfer. As a control for homeostatic expansion, we also transferred CFSE-labeled $CD8^+$ T cells bearing the same 2C TCR into sublethally irradiated Ag^- or Ag^+ hosts. In agreement with others (6), we found that 2C $CD8^+$ T cells undergo homeostatic expansion in Ag^- mice (Fig. 5*B*). In stark contrast to $CD8^+$ T cells, DNTC from Ag^- mice fail to expand in irradiated Ag^- hosts (Fig. 5*B*). This observation is similar to one recently describing the fact that 2C DNTC do not proliferate when placed into lymphopenic RAG1-deficient (H-$2^{b/b}$) animals (34). Therefore, low-affinity interactions of self peptides/MHC of the H-$2^{b/b}$ background are insufficient to drive DNTC proliferation. Dissimilar to homeostatic proliferation, $CD8^+$ T cells injected into Ag^+ recipients undergo rapid proliferation, completely losing their CFSE fluorescence 1 wk after transfusion (Fig. 5*B*). By contrast, exposure of DNTC to cognate Ag in vivo results in a rate of proliferation that is higher than $CD8^+$ T cell homeostatic expansion but lower than Ag-driven $CD8^+$ T cell growth (Fig. 5*B*). In accord with their natural memory phenotype, H-$2^{b/d}$ DNTC undergo a slow peripheral expansion after transfer into syngeneic irradiated recipient animals. Consequently, although H-$2^{b/d}$ DNTC do not respond well to L^d in vitro, these cells are well equipped for peripheral expansion in vivo. Collectively, these data imply that DNTC require high avidity interactions to induce a slow homeostatic-like growth.

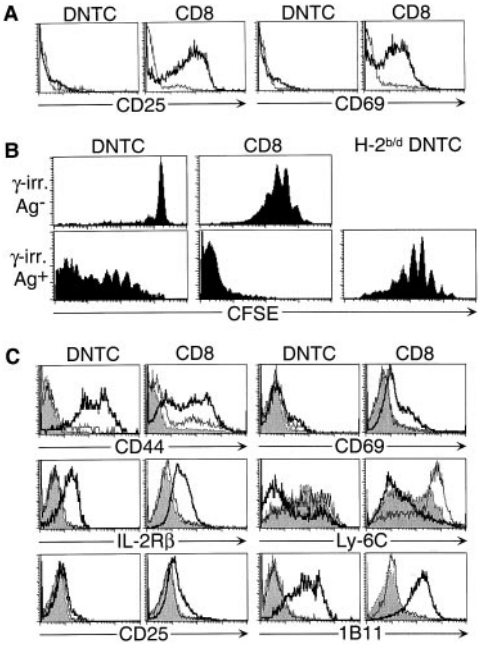

FIGURE 5. Peripheral DNTC expansion requires interactions with cognate Ag. *A*, 2C $CD8^+$ T cells, but not DNTC, up-regulate the acute activation markers CD25 and CD69 upon cognate Ag exposure. DNTC or $CD8^+$ T cells from Ag^- 2C mice were activated with H-$2^{b/d}$ splenocytes for 16 h and were assessed for the expression of CD25 and CD69 by flow cytometry. *B*, Expansion of DNTC in vivo requires interaction with cognate Ag. Purified CFSE-labeled naive T cells (either $CD8^+$ T cells or DNTC from H-2^b 2C mice) were injected into irradiated (γ-irr.) Ag^- (B6; H-$2^{b/b}$) or Ag^+ (BDF_1; H-$2^{b/d}$) mice, and their in vivo proliferation was monitored 1 wk later. In addition, CFSE-labeled H-$2^{b/d}$ DNTC were also adoptively transferred into irradiated Ag^+ recipients to establish their expansion potential in vivo. In contrast to 2C $CD8^+$ T cells, self peptides presented by the H-2^b background are insufficient to drive DNTC homeostatic proliferation. *C*, DNTC proliferation driven by cognate Ag is accompanied by expression of markers associated with homeostatic expansion. One week following cell transfer, spleen and LNs were recovered from recipient animals and were analyzed by flow cytometry. The histograms illustrate expression of various markers on T cells injected into either Ag^- (thin line) or Ag^+ (bold line) mice. Shaded histograms with dotted lines represent naive DNTC and $CD8^+$ T cells from nonmanipulated H-2^b 2C mice.

Next, we examined the markers expressed by $CD8^+$ T cells and DNTC residing in Ag^-- and Ag^+-irradiated hosts for 1 wk. Coinciding with the lack of proliferation in the lymphopenic Ag^- conditions, H-2^b DNTC retain their naive cell surface phenotype (Fig. 5*C*). In contrast, $CD8^+$ T cells undergoing homeostasis-induced proliferation have an increased incidence of memory-like T cells, expressing higher levels of CD44, IL-2Rβ, and Ly-6C. Contrary to cells parked in an Ag^- host, H-2^b DNTC exposed to cognate Ag in vivo acquire an activated/memory state bearing high levels of CD44, IL-2Rβ, and 1B11 (Fig. 5*C*). However, they do not up-regulate the acute activation markers CD69 and CD25. By contrast, exposure of $CD8^+$ T cell to cognate Ag in vivo is associated with the up-regulation of both acute activation markers (Fig. 5*C*). This less vigorous response by DNTC to cognate Ag may be responsible for their resistance to clonal deletion in Ag^+ mice. These results suggest that TCR/cognate Ag interaction is required for the peripheral expansion of DNTC.

We have found DNTC from Ag^+ mice were able to respond to the low-affinity (p2Ca/K^b) ligand. To determine whether DNTC from Ag^- mice could undergo this functional change following homeostatic expansion in Ag^+ mice, we injected purified H-2^b DNTC into Ag^+ mice. Seven days later, the injected DNTC were purified from Ag^+ hosts and were stimulated with either the low- or the high-affinity ligand of the 2C TCR ± exogenous IL-2. This response was compared with proliferative responses of DNTC, which were parked in Ag^- mice, or by naive DNTC from Ag^- mice (Fig. 6). As expected, naive DNTC from H-2^b 2C TCR transgenic mice responded well to the high-affinity (p2Ca/L^d) ligand regardless of whether exogenous IL-2 is added. However, these cells lacked the capacity to respond to the low-affinity (p2Ca/K^b) ligand even in the presence of exogenous IL-2. By contrast, DNTC that had undergone homeostatic expansion in Ag^+ hosts gave a low proliferative response to both the high- or low-affinity ligands in the absence of an exogenous source of IL-2. Strikingly, this poor response toward either ligand was abrogated by the addition of exogenous IL-2 (Fig. 6). Interestingly, DNTC isolated from Ag^- hosts also responded weakly to p2Ca/K^b plus IL-2 even though they had proliferated minimally after transfer.

In agreement with a previous report (6), $CD8^+$ T cells that have undergone homeostatic expansion in Ag^- host also exhibit increased sensitivity to Ag stimulation (Fig. 6). We found that these $CD8^+$ T cells can now respond weakly to the low affinity-ligand plus IL-2. The $CD8^+$ T cells recovered from Ag^- mice also mounted a stronger response toward p2Ca/L^d as compared with

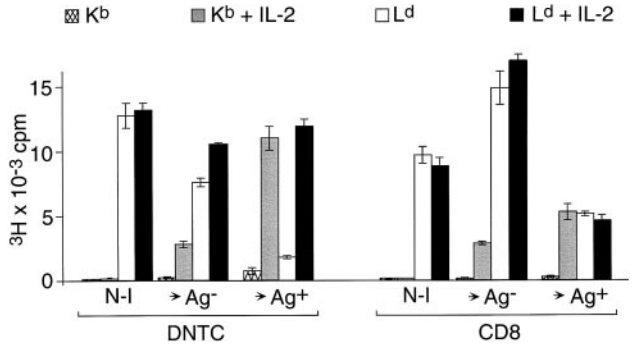

FIGURE 6. DNTC recovered from Ag^+ hosts have a lower threshold for activation, but require exogenous IL-2 to promote Ag-induced proliferation. Adoptively transferred DNTC residing in Ag^+ hosts for 1 wk possess a lowered threshold of activation, enabling them to respond to the low-affinity ligand, p2Ca/K^b. DNTC and $CD8^+$ T cells from H-2^b 2C mice were recovered from spleen and LNs of irradiated B6 ($\rightarrow Ag^-$) and BDF_1 ($\rightarrow Ag^+$) host animals 1 wk after transfer. Recovered cells were stimulated with either T2-K^b or T2-L^d loaded with 1 μM p2Ca in the presence and absence of exogenous IL-2. Proliferation of adoptively transferred cells was compared with purified cell populations derived from noninjected (N-I) H-2^b 2C mice. Equivalent numbers of $2C^+$ cells, determined with the clonotypic 1B2 Ab, were added per well.

naive 2C $CD8^+$ T cells. By contrast, $CD8^+$ T cells recovered from Ag^+ mice proliferated poorly toward p2Ca/L^d, and the addition of IL-2 had no effect on this response. This observation is consistent with the observation that $CD8^+$ T cells recovered from Ag^+ mice are functionally anergic and that this state is irreversible by exogenous IL-2 (19).

Discussion

Our analysis of DNTC suggests that they represent a novel suppressor cell population whose function is to negatively regulate immune responses. As DNTC acquire regulatory properties only after exposure to self Ags in vivo, it suggests that a self Ag-driven peripheral expansion plays a pivotal role in the acquisition of immunoregulatory function. Rather than stimulating rapid growth, effector cell formation, and acute activation markers, exposure to cognate Ag initiates DNTC to undergo slow expansion and memory conversion. For instance, DNTC having undergone self Ag-driven peripheral expansion possess markers of T cell memory, a lowered threshold of activation, an ability to rapidly express IFN-γ after TCR stimulation, and ex vivo cytolytic activity. However, DNTC from Ag^+ mice differ from conventional T cells or those from Ag^- mice in that they respond poorly when challenged with cognate Ag in vitro and fail to synthesize IL-2. As they possess potent cytolytic activity and express cognate Ag, their poor proliferation in vitro may be in part due to fratricide. Despite the potential for self-self killing, the fact that exogenous IL-2 can overcome this proliferation defect strongly suggests that diminished cytokine production plays the prominent role in their defective response. Consequently, these regulatory DNTC are dependent on stimulation by both self Ag and bystander-produced cytokine for their proliferation and heightened cytolytic activity. Furthermore, the inability to synthesize cytokines required for their proliferation limits their autoimmune potential. The reliance on the Fas-based pathway of cytotoxicity may focus DNTC killing on activated T cells or APCs that have up-regulated Fas expression. The aforementioned properties make DNTC particularly adept at sensing their environment, directing their killer/suppressor function toward cells mediating immunity or autoimmunity.

Because this slow expansion/differentiation process appears similar to one recently described for homeostasis-induced proliferation of naive $CD8^+$ T cells (5), we sought to determine whether they may share a similar derivation. To verify this hypothesis, naive DNTC were adoptively transferred to an Ag^+ host for 1 wk. After this test period, these previously naive T cells had undergone a slow expansion and memory conversion. Interestingly, these DNTC had not only acquired most of the markers present on DNTC from Ag^+ mice, but they had also adopted similar functional attributes. Although it is still not clear whether DNTC that have developed in the presence of Ag (i.e., H-$2^{b/d}$ DNTC) are completely equivalent to naive DNTC that have recently been exposed to Ag for a short period of time (i.e., H-2^b DNTC placed into a H-$2^{b/d}$ animal for a period of 1 wk), we do find the similarities, in particular the functional attributes, between these two cell types to be impressive. The fact that either cell type can proliferate robustly to either the low- or high-affinity ligand when supplied with exogenous IL-2 is striking. In addition, Ag-experienced DNTC, regardless of origin, share the expression of a wide array of markers. Collectively, our results suggest that a process resembling a mechanism for maintaining peripheral $CD8^+$ T cell numbers may be used to ensure self tolerance. These observations provide a rationale and basis for the persistence of autoreactive T cells among the repertoire of healthy individuals.

Recently, it has been demonstrated that DNTC clones from TCR transgenic mice possess suppressor-like activity and mediate Ag-specific suppression (35). Although suppression by DNTC clones also involved Fas/FasL interaction, their proposed mechanism of killing differs. According to their model, DNTC clones obtain Ag from APCs through TCR-mediated endocytosis, and they re-express these newly acquired molecules on their cell surface. Subsequently, cognate Ag-specific $CD8^+$ T cells become susceptible to cytotoxicity by their recognition of cognate Ag on the cell surface of DNTC clone, initiating Fas/FasL signaling and programmed cell death. However, our data does not fit with such a model, as H-$2^{b/d}$ DNTC can kill either 2C TCR-expressing or nontransgenic $CD8^+$ T cells, which express a wide array of TCR, with equivalent efficiency. Therefore, the killing of $CD8^+$ T cells by DNTC is not dependent on recognition of a cognate Ag on DNTC by the $CD8^+$ T cell. In our studies, killing is dependent on recognition of the Ag on the target cell by the TCR of the DNTC. Exposure of DNTC to self Ag in vivo results in a lowering of the triggering threshold (20). This enables DNTC to respond to a greater variety of self Ags and hence, to regulate the response of a larger repertoire of T cells. Therefore, DNTC may play an important role in maintaining peripheral T cell tolerance and down-regulating immune responses by killing activated T cells, particularly those reactive against self Ags. The dominant role of the Fas/FasL in DNTC mediated suppression suggests that the inability of such regulatory T cells to function may contribute to the autoimmunity observed in animals that are deficient in either Fas (*lpr/lpr*) or FasL (*gld/gld*) expression (36, 37).

DNTC are relatively rare in healthy mice, comprising 3–5% of the peripheral T cell pool. The heightened incidence of DNTC in lymphoproliferative, graft-vs-host, and autoimmune disease suggests either a pathogenic or immunoregulatory role for this subset of T cells (38). The origin of DNTC in TCR transgenic mice has been a subject of considerable interest. Although DNTC in TCR transgenic mice have been suspected to arise from the forced expression of the $\alpha\beta$ transgene in $\gamma\delta$ precursors (11), it is possible that they instead represent a distinct lineage of $\alpha\beta$ cells. Our analyses of spleens from normal mice revealed that although both $\alpha\beta$ and $\gamma\delta$ DNTC possess similar cell surface markers as regulatory DNTC expressing the 2C TCR, only activated $\alpha\beta$ DNTC from

normal mice are effective killers of syngeneic $CD8^+$ T cell blasts (data not shown). Interestingly, NK T cell ($NK1.1^+$ DNTC) killing was also biased toward the Fas/FasL-based pathway. In contrast to mainstream T cells, $CD4^+$ and $CD4^-CD8^-$ NK T cell subsets exhibit a restricted TCR diversity and are largely restricted by the invariant CD1 molecule (39). Recently, the mouse CD1-restricted repertoire has been described to be dominated by a few autoreactive TCR families (40). The fact that these cells possess semivariant, autoreactive TCRs and a natural memory phenotype suggests that they may be derived from a self Ag-driven selection and peripheral expansion (39, 40). Additionally, CD1 has been implied to play a critical role in the deletion of activated $CD8^+$ T cells (41). Based on the similarities between regulatory DNTC from 2C mice and $CD4^-CD8^-NK1.1^+\alpha\beta TCR^+$ cells in normal mice, it is conceivable that the forced expression of the $\alpha\beta$ transgenic TCR in immature T cells may have induced their differentiation along the NKT cell lineage. In summary, we have shown that self Ags mediate the peripheral expansion and conversion of $\alpha\beta$ DNTC from TCR transgenic mice into potent immunoregulatory cells. These self-reactive regulatory cells are uniquely poised to suppress immune responses and prevent autoimmunity. The existence of DNTC in normal mice with a similar cell surface phenotype and cytolytic function suggests that a similar process may operate in normal mice.

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