Homeostatic T cell proliferation in a T cell-dendritic cell coculture system

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Contributed by Herman N. Eisen, December 31, 2001

Naïve T cells do not proliferate in normal individuals in the absence of antigen stimulation, but they proliferate spontaneously when T cells are severely depleted. We show here that coculture of syngeneic dendritic cells (DC) with naïve T cells expressing a single T cell receptor also results in T cell proliferation in the absence of foreign antigen. As in lymphopenic mice, where T cell proliferation depends upon DC, this response in the coculture system requires interaction of the T cells' T cell receptor with self-peptide–MHCs on DC. This *in vitro* proliferation also requires soluble factors, including IL-15 secreted by DC, and can be inhibited potently by cell–cell contact with $CD4^+CD25^+$ regulatory T cells. The coculture system described may illuminate mechanisms that maintain stable numbers of T cells in normal individuals.

After severe T cell depletion, induced by ionizing radiation, chemotherapy, or virus infection, the residual T cells undergo proliferation (1). Similarly, when small numbers of naïve T cells are adoptively transferred into syngeneic lymphopenic hosts, such as mice deficient in recombination activating gene-1 ($RAG^{-/-}$), or into sublethally irradiated mice, the transferred T cells also proliferate (2–6). The proliferating CD8 T cells acquire the cell surface markers and functional properties of antigen-stimulated memory T cells (7–9). The spontaneous T cell proliferation and differentiation into memory-like T cells under lymphopenic conditions is referred to as homeostasis-driven or lymphopenia-induced response (10–13).

Although a deliberate antigen challenge is not required, the homeostasis-driven T cell response depends upon engagement of the T cell receptor (TCR) with self-peptide–MHCs (pepMHC). Thus, CD8 T cells do not proliferate when small numbers are transferred into lymphopenic hosts lacking MHC class I, such as mice deficient in β_2-microglobulin (β_2m; refs. 2–4). The coreceptor CD8 also promotes homeostatic proliferation as $CD8^+$, but not $CD8^-$ (and $CD4^-$), 2C T cells proliferate in syngeneic $RAG^{-/-}$ recipients (14). In addition, homeostatic T cell proliferation depends upon the presence of lymphopenia (or "space"), because it occurs only in T cell-deficient hosts but not in hosts with normal numbers of T cells. Recently, cytokine IL-7 and IL-12 were shown to promote homeostatic CD8 T cell proliferation in lymphopenic mice (15–17), indicating that soluble stimulating factors may provide a concrete basis for the notion of space.

Despite the progress made in understanding many aspects of homeostatic T cell proliferation and differentiation, much remains to be elucidated. For example, the cell type that provides the required self-pepMHC complexes has not been defined. Whether other soluble factors, besides IL-7 and IL-12, also promote homeostatic T cell proliferation is unknown. Similarly, it is unknown whether the absence of naïve T cell proliferation in normal hosts reflects active inhibition among T cells. Part of the difficulty in clarifying the cellular and molecular bases for homeostatic T cell proliferation arises from the need to analyze this process in intact animals, which are not readily manipulated. To overcome this difficulty, we have developed an *in vitro* T cell-dendritic cell coculture system, in which T cells undergo proliferation in a TCR- and self-pepMHC-dependent manner. By using this system, we show here that: (*i*) dendritic cells (DC), but not other antigen-presenting cells, provide the required self-pepMHC complexes; (*ii*) IL-15 secreted by DC promotes the T cell response; and (*iii*) $CD4^+CD25^+$ regulatory T cells appear to actively inhibit homeostatic T cell proliferation.

Materials and Methods

Mice. 2C TCR transgenic mice, on the recombination activating gene-1 deficient ($RAG^{-/-}$) background (2C/RAG), had been backcrossed with C57BL/6 (B6, H-2^b) mice for 10 generations. $RAG^{-/-}$ mice were backcrossed with B6 mice for 13 generations. B6 mice and mice deficient in β_2m or TCRβ and TCRδ genes on the B6 background were from The Jackson Laboratory. F5 TCR transgenic mice on the H-2^b background were bred onto the $RAG^{-/-}$ background. All mice were kept in a specific pathogen-free facility and used between 6 and 12 weeks of age.

Cell Preparation. Lymph node cells from 2C/RAG mice, consisting of >95% $1B2^+$ 2C T cells, were labeled with carboxyfluorescein diacetate-succinimidyl ester (CFSE) and then either added to cultures or transferred into $RAG^{-/-}$ recipients. $CD4^+CD25^+$ T cells were isolated by FACS sorting of spleen and lymph node cells of B6 mice (>95%). To prepare splenic dendritic cells, 2×10^6 B16 melanoma cells secreting granulocyte/macrophage colony-stimulating factor (GM-CSF; ref. 18) were injected s.c. into B6 or $\beta_2m^{-/-}$ mice. Ten days later, spleens were removed and spleen cells were incubated with anti-CD11c beads followed by magnetic sorting (MACS). The isolated DC were routinely >95% $CD11c^+$. The freshly purified DC were immature but spontaneously acquired the mature phenotype after overnight culture in medium (Fig. 3 and data not shown). Lymph node cells from mice deficient in TCRβ and TCRδ genes were used as sources of B cells (>95% $B220^+$). Macrophages were prepared from peritoneal lavage of B6 mice or B6 mice that were injected i.p. with thioglycollate 4 days earlier. Macrophages in the lavage were allowed to adhere to the plates 3 h at 37°. The purity of macrophages ($CD11b^+$) was 96%.

Antibodies, Cytokines, and Flow Cytometry. Fluorochrome-conjugated antibodies to CD8, CD44, CD69, CD25, Thy-1.2, CD11c, and CD11b were purchased from PharMingen. The clonotypic antibody, 1B2, specific for the 2C TCR, was conjugated to biotin. Cells first were blocked with unconjugated anti-FcR antibody, stained in PBS containing 0.5% BSA and 0.1% NaN_3, and then analyzed on a FACScalibur, collecting 10,000–1,000,000 live cells (PI^-) per sample. Recombinant murine IL-5 and IL-12 were from R & D Systems, and IL-5, IL-6,

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Abbreviations: TCR, T cell receptor; pepMHC, peptide–MHC protein; β_2m, β_2-microglolin; RAG, recombination activating gene-1; DC, dendritic cells; CFSE, carboxyfluorescein diacetate-succinimidyl ester; ROC, reaggregated organ culture; GM-CSF, granulocyte/macrophage colony-stimulating factor; RPA, RNase protection assay; LPS, lipopolysaccharide.

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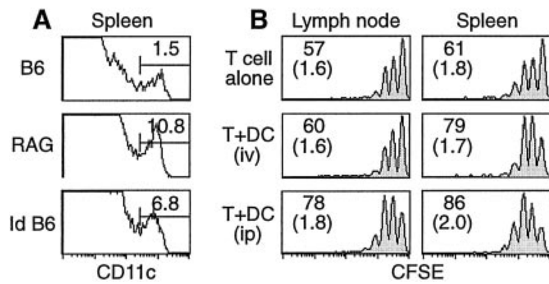

Fig. 1. Dendritic cells promote T cell proliferation. (*A*) Splenocytes from normal C57BL/6 (B6) mice, $RAG^{-/-}$ mice, and sublethally irradiated (Id) B6 mice were assayed for CD11c. Numbers indicate percentages of $CD11c^+$ DC. Data shown are from one representative mouse of three mice per group. (*B*) CFSE-labeled 2C T cells (2×10^6) from lymph nodes of 2C/RAG mice were injected i.v. into $RAG^{-/-}$ recipients alone or together with dendritic cells (2×10^7) i.v. or i.p. Four days later, cells from lymph nodes and spleens of the recipients were assayed for 1B2, CD8, and CFSE. CFSE profiles of $1B2^+CD8^+$ 2C cells are shown. Numbers indicate the percentages of cells that proliferated. Numbers in parentheses indicate the average divisions of cells that proliferated.

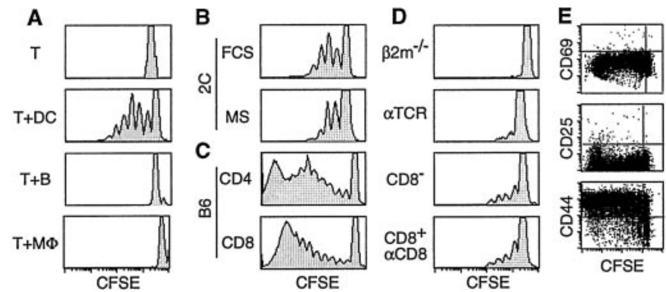

Fig. 2. T cell proliferation in T-DC cocultures. (*A*) CFSE-labeled 2C T cells (4×10^5) were cultured in 24-well plates alone (T) or with purified DC (2×10^6), B cells (B, 2×10^6), or macrophages (MΦ, 2×10^6). Proliferation of 2C T cells were assayed 5 days later, gating on $1B2^+CD8^+$ 2C cells. Representative data from one of five independent experiments are shown. (*B*) The same as in *A*, except that either 10% FCS or 2.5% heat-inactivated mouse serum (MS) was used. (*C*) CFSE-labeled T cells from B6 mice were cultured with syngeneic DC for 5 days. CFSE profiles of TCR^+CD4^+ and TCR^+CD8^+ cells are shown. Representative data from one of two experiments are shown. (*D*) CFSE-labeled 2C cells were cultured with DC from $\beta_2m^{-/-}$ mice, and CFSE profiles of $1B2^+CD8^+$ 2C cells at day 5 are shown ($\beta_2m^{-/-}$). In the other three cultures, CFSE-labeled 2C cells were cultured with DC from B6 mice; in one culture, CFSE profiles of $1B2^+CD8^-$ 2C cells at day 5 are shown ($CD8^-$); in the other two cultures, 2C cells were preincubated with the anti-2C TCR antibody, 1B2 Fab (αTCR, 10 μg/ml), or anti-CD8α antibody (αCD8, 10 μg/ml) at 4°C for 30 min and then cultured with DC. CFSE profiles of $1B2^+CD8^+$ 2C cells at day 5 are shown. Representative data from one of two experiments are shown. (*E*) The same as in *A*, except that the expression of CD44, CD25, and CD69 by $1B2^+CD8^+$ 2C T cells in T-DC cocultures is shown as a function of CFSE intensity.

IL-7, IL-9, IL-13, IL-15, and RANTES were from (PeproTech, Rocky Hill, NJ). Human recombinant IL-2 was a kind gift from Roche (Gipf-Oberfrick, Switzerland). Neutralizing antibodies specific for IL-5, IL-13, and RANTES were from R & D Systems. Anti-IL-9 antibody was from PharMingen. Antibodies to IL-7, IL-7R, and IL-2Rβ were isolated from supernatants of hybridomas, which were kindly provided by Philippa Marrack (National Jewish Center).

T Cell–DC Coculture. For suspension cultures, CFSE-labeled 2C T cells (usually 400,000/well) were mixed with various numbers of purified DC in 24-well plates in RPMI 1640 medium containing 10% heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For reaggregated organ cultures (ROCs), T cells and DC were mixed together in suspensions and then pelleted by centrifugation. After removal of the supernatant, cell pellets were pipetted as a standing drop onto a nucleopore filter placed on a Gelfoam sponge in RPMI 1640 medium with supplements. Cells were cultured at 37° in a humidified 5% CO_2 incubator for 5 days before analysis.

RNase Protection Assay (RPA). RNA was isolated from DC cultures or DC plus T cell cultures before and after culture at the indicated lengths of time. RPA was performed by using the RiboQuant MultiProbe RPA System (PharMingen). Four kits containing cDNAs encoding various mouse cytokines and chemokines were used as templates for the T7 polymerase-directed synthesis of $[^{32}P]$UTP-labeled antisense RNA probes. Ten micrograms of each target RNA was hybridized. Quantification of band intensities was performed by PhosphorImager. The signals from specific transcripts were normalized to signals from housekeeping gene L32 in each lane to adjust for loading differences.

Results

Cotransfer of DC Enhances Proliferation of Naïve 2C T Cells in $RAG^{-/-}$ Recipients. DC constitutively express high levels of MHC classes I and II (19, 20) and were hypothesized to provide self-pepMHC for homeostatic T cell proliferation (21). T cell proliferation in lymphoid organs of lymphopenic mice could result from an increased abundance of DC relative to T cells in these organs. To examine this possibility, we determined the abundance of DC in the spleen of B6, $RAG^{-/-}$, and irradiated B6 mice. DC were more abundant in splenocytes from $RAG^{-/-}$ mice (≈11%) and irradiated B6 mice (≈7%) than from normal B6 mice (≈1.5%, $P < 0.001$, Fig. 1*A*). Upon transfer of small numbers (1×10^6) of naïve $CD8^+$ T cells expressing the 2C TCR into $RAG^{-/-}$ recipients, <0.5% of splenocytes were 2C T cells 2 days after transfer (data not shown). Thus, there are >20-fold more DC than T cells in the spleens of $RAG^{-/-}$ recipients. In contrast, in an intact B6 mouse or a 2C TCR transgenic mouse on the $RAG^{-/-}$ background (2C/RAG), >30% of spleen cells are T cells; i.e., there are many more T cells than DC.

To determine whether an increased abundance of DC in lymphoid organs of lymphopenic mice promotes homeostatic T cell proliferation, we cotransferred CFSE-labeled naïve 2C T cells and purified DC into nonirradiated syngeneic $RAG^{-/-}$ hosts. 2C T cells were adoptively transferred i.v., and, at the same time, DC were either injected i.v. or i.p. Higher percentages of 2C cells proliferated multiple times in recipients that received 2C cells and DC than in those that received 2C cells alone: the difference was especially pronounced in the spleen after i.p. injection of DC (Fig. 1*B*), perhaps because many i.v. injected DC were trapped in lungs and failed to reach lymph nodes and spleen. It is clear, nevertheless, that injected DC enhanced homeostatic T cell proliferation in lymphopenic hosts.

DC Promote Proliferation of Naïve T Cells *in Vitro*. To examine further the role of DC in homeostatic T cell proliferation, we tested whether syngeneic DC can stimulate T cell proliferation in culture in the absence of antigen. When cultured alone for 5 days, naïve 2C T cells did not proliferate. In contrast, when cultured together with DC for the same length of time, a large fraction of 2C cells proliferated (Fig. 2*A*). Similarly, 2C cells proliferated when cultured with DC in a ROC on a filter support (see below) or when cultured with DC generated by GM-CSF stimulation of bone marrow cells *in vitro* (data not shown). 2C cells also proliferated in culture when FCS was replaced with heat-inactivated mouse serum (Fig. 2*B*), indicating that T cell response was not caused by components in FCS. As with monoclonal 2C T cells, significant fractions of $CD4^+$ and $CD8^+$ T cells from normal B6 mice also proliferated when cocultured with syngeneic DC for 5 days (Fig. 2*C*). In contrast, 2C cells did

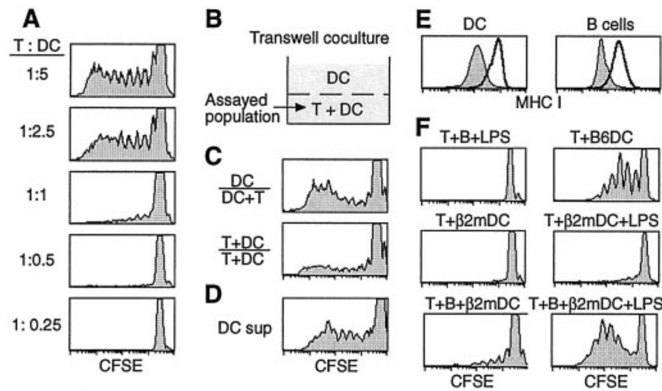

Fig. 3. Soluble factors secreted by DC promote T cell proliferation *in vitro*. (*A*) CFSE-labeled 2C cells (4×10^5) were cultured with different numbers of DC for 5 days. Representative CFSE profiles of $1B2^+CD8^+$ cells are shown. Numbers indicate the ratio of T cells to DC. (*B*) A schematic diagram of transwell coculture. (*C*) CFSE-labeled 2C cells (4×10^5) were cultured with equal numbers of DC in the lower chamber, and DC (1×10^6) alone or together with unlabeled 2C cells (4×10^5) were added into the upper chamber. Five days later, 2C cells in the lower chamber were assayed for proliferation (CFSE profiles), gating on $1B2^+CD8^+$ 2C cells. Representative data from one of three experiments are shown. (*D*) CFSE-labeled 2C cells (4×10^5) were cultured with equal numbers of DC in the presence of supernatants from DC cultures. Representative CFSE profiles of $1B2^+CD8^+$ cells at day 5 are shown. (*E*) Comparison of MHC class I (K^b) on DC before (shaded area) and after culture (bold line) for 24 h and on resting (shaded area) and LPS-activated (bold line) B cells. (*F*) Representative CFSE profiles of $1B2^+CD8^+$ cells at day 5 in various cultures. T+B+LPS, 2C cells plus B cells in the presence of LPS (100 ng/ml); T+B6DC, 2C cells plus DC from B6 mice; T+β_2mDC, 2C cells plus DC from $\beta_2m^{-/-}$ mice; T+β_2mDC+LPS, 2C cells plus DC from $\beta_2m^{-/-}$ mice in the presence of LPS; T+B+β_2mDC, 2C cells plus B cells and $\beta_2m^{-/-}$ DC; T+B+β_2mDC+LPS, 2C cells plus B cells and $\beta_2m^{-/-}$ DC in the presence of LPS. Cells were cultured in ROCs with 5×10^5 T cells, 1×10^6 B cells, or 5×10^5 DC.

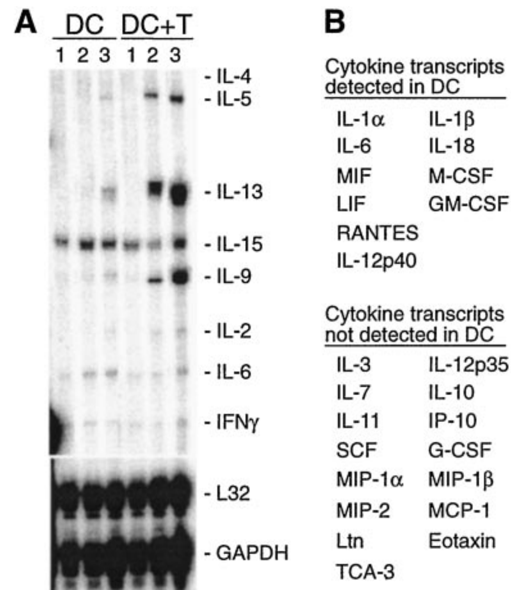

Fig. 4. Quantification of cytokine transcripts in total RNA of DC and DC plus T cells before and after culture. (*A*) DC were cultured alone or together with 2C cells at a 5:1 ratio. RNA was harvested from cultured cells at day 1, 2, or 3. Cytokine transcripts were assayed in total RNA by RPA. Cytokine transcripts are labeled. L32 and glyceraldehyde-3-phosphate dehydrogenase serve as controls for RNA amounts in different lanes. (*B*) Summary of cytokine expression by DC after culture.

not proliferate when cocultured with syngeneic B cells or macrophages (Fig. 2*A*), indicating that DC are uniquely able to stimulate T cell proliferation *in vitro*.

The spontaneous T cell proliferation in T-DC cocultures resembles homeostatic T cell proliferation in lymphopenic mice. First, T cell proliferation in the coculture system required the engagement of TCR with self-pepMHC complexes expressed by DC. Thus, 2C T cells did not proliferate when cultured with DC from $\beta_2m^{-/-}$ mice or with DC from normal B6 mice in the presence of the Fab fragment of the 1B2 antibody specific for the 2C TCR (Fig. 2*D*). Second, coreceptor CD8 is important; thus, $CD8^-$ 2C cells proliferated very little in T-DC cocultures and proliferation of $CD8^+$ 2C cells was severely blocked by anti-CD8 antibody treatment. Finally, proliferation of 2C cells was associated with the up-regulation of CD44 whereas the CD69 and CD25 were not induced (Fig. 2*E*), indicating the acquisition of memory cell surface markers without overt activation.

Soluble Factors Secreted by DC Promote T Cell Proliferation *in Vitro*. The T-DC cocultures in which T cells undergo homeostatic proliferation provide a system to investigate the requirement of other factors that affect this process. For this purpose, we first determined the level of DC required to support T cell proliferation in suspension culture. As shown in Fig. 3*A*, 2C cells proliferated when DC were in 2.5- or 5-fold excess, but proliferation was minimal when the DC/T ratio was 1 or less.

To investigate whether DC secrete soluble factors that promote T cell proliferation, transwell cocultures were used. Because T cells appeared to be at the threshold of proliferation when equal numbers of DC and T cells were cultured, 400,000 each of 2C T cells and DC were placed in the bottom chamber of the transwell and various numbers of DC were placed in the upper chamber. The chambers were separated by a 0.4-μm membrane that is permeable to proteins and small molecules but not to cells (Fig. 3*B*). When 1×10^6 or more DC were placed in the upper chamber, a significant fraction of 2C cells in the bottom chamber proliferated (Fig. 3*C*). When 2C cells were added into the upper chamber as competitors, 2C cells in the bottom chamber proliferated less. Furthermore, when supernatants collected from DC cultures (in the absence of T cells) were added to T-DC cocultures (1:1), T cells also proliferated (Fig. 3*D*).

In addition, soluble factors secreted by DC also promoted T cell proliferation in cocultures in which self-pepMHC complexes were provided by B cells. One possible reason for the failure of B cells alone to support T cell proliferation in cocultures (Fig. 2*A*) is that B cells express lower levels of MHC class I than DC (Fig. 3*E*). Consistently, T cells did not proliferate when cocultured with B cells and DC from $\beta_2m^{-/-}$ mice (Fig. 3*F*). Stimulation of B cells with lipopolysaccharide (LPS) significantly increased the level of MHC as well as CD40 and B7.2 expression (Fig. 3*E* and data not shown), but LPS-activated B cells alone still were not sufficient to promote T cell proliferation (Fig. 3*F*). However, the addition of DC from $\beta_2m^{-/-}$ mice to cocultures of T cells and LPS-activated B cells resulted in T cell proliferation.

Together, these results demonstrate that DC secrete soluble factors that stimulate homeostatic T cell proliferation in T-DC cocultures.

DC Secrete Multiple Cytokines After Culture *in Vitro*. To identify DC-derived soluble factors that promote T cell proliferation, supernatants from DC cultures were subjected to ultrafiltration through a 3-kDa cutoff membrane. The retentate but not the filtrate supported T cell proliferation when T cells were cultured with equal numbers of DC (data not shown), indicating that the soluble factors are >3 kDa in molecular mass. Accordingly, we analyzed cytokine gene expression by DC by using an RPA.

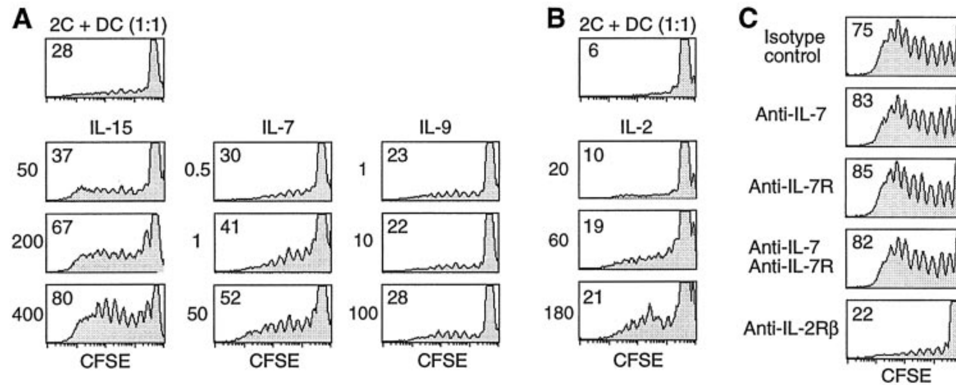

Fig. 5. IL-15 promotes T cell proliferation in T-DC cocultures. (*A*) CFSE-labeled 2C cells (4×10^5) were cultured with equal numbers of DC in medium or in the presence of different concentrations (numbers outside the rectangles) of IL-15, IL-7, or IL-9 (ng/ml). CFSE profiles of 1B2$^+$CD8$^+$ cells at day 5 are shown. Numbers inside rectangles indicate percentages of T cells that proliferated. Representative data from one of four experiments are shown. (*B*) The same as in *A*, except that IL-2 was added (units/ml). (*C*) CFSE-labeled 2C cells (4×10^5) were preincubated with anti-IL-7, anti-IL-7Rα, both anti-IL-7 and anti-IL-7R, anti-IL-2Rβ, or isotype control antibodies for 30 min at 4°C and then cultured with DC (1×10^6). Representative CFSE profiles of 1B2$^+$CD8$^+$ 2C cells at day 5 are shown. Numbers indicate the percentages of T cells that proliferated.

Freshly isolated splenic DC expressed only low levels of IL-15, IL-18, and MIF (Fig. 4 and data not shown), but, after culture for just 1 day, DC expressed many cytokines, including IL-1, IL-6, IL-12p40, RANTES, GM-CSF, M-CSF, and lymphocyte inhibitory factor. The expression of most of these cytokine genes persisted over the 5-day culture period, and the level of IL-15 transcript was increased after culture (data not shown). IL-2, IL-4, IL-7, IL-10, and IL-12p35 transcripts were not detected in the total RNA of DC either before or after culture.

The cytokine transcripts in total RNA of DC plus T cells also were assayed at various times. Similar transcript levels for IL-15, IL-18, MIF, IL-1, IL-6, RANTES, GM-CSF, M-CSF, and lymphocyte inhibitory factor were detected in total RNA from DC plus T cell cocultures and from DC cultures alone (data not shown), indicating that T cells did not significantly alter the profile of transcription of these cytokine genes by DC. However, several new cytokine transcripts, including IL-5, IL-13, IL-9, and perhaps IL-2, were detected in total RNA from T cell–DC cocultures (Fig. 4*A*). [Both IL-2 and IL-15, but not IL-4 and IL-7, were detected in T-DC culture supernatants by ELISA (data not shown).] Because DC alone did not express these transcripts, their expression likely was induced in T cells after coculture with DC.

IL-15 Secreted by DC Promotes T Cell Proliferation *in Vitro*. To test which cytokine promotes T cell proliferation, 2C cells and DC were cultured in suspension at a 1:1 ratio in the presence of various amounts of exogenous cytokines. In the absence of any added cytokines, very few 2C cells proliferated (Fig. 5*A*, top image). With increasing amounts of IL-15, increasing proportions of 2C cells proliferated (Fig. 5*A Left*). Exogenously added IL-2 also promoted T cell proliferation although to a lesser extent (Fig. 5*B*). In contrast, the addition of IL-5, IL-6, IL-9, IL-12, IL-13, or RANTES had no discernible effect (Fig. 5*A* and data not shown), although the same amounts of cytokines have been shown to exhibit other biological activities (e.g., refs. 22 and 23). Consistently, addition of antibodies specific for IL-5, IL-9, IL-13, and RANTES did not inhibit T cell proliferation (data not shown). Thus, IL-6, IL-12, and RANTES secreted by DC and IL-5, IL-9, and IL-13 secreted by T cells probably do not promote T cell proliferation alone in cocultures with DC.

IL-7 has been reported to promote naïve T cell survival and homeostatic proliferation in lymphopenic mice (15, 16, 24). Although IL-7 transcripts were not detected by RPA and IL-7 was not detected by ELISA in DC culture supernatants (detection limit: 20 pg/ml), it could be that these assays were not sufficiently sensitive. To investigate the role of IL-7 in the cocultures, exogenous IL-7 was added to T-DC (1:1) cocultures and was shown to promote T cell proliferation in a dose-dependent manner (Fig. 5*A Center*). In a complementary approach, blocking antibodies specific for IL-7, IL-7R, or both were added to T-DC cocultures. In the absence of these antibodies, $\approx$75% of T cells proliferated after 5 days in culture, and this response was not affected by adding antibodies individually or together (Fig. 5*C*). In contrast, a blocking antibody specific for the common β-chain of the IL-2 and IL-15 receptors almost completely inhibited T cell proliferation. These results show that IL-15 and probably IL-2, but not IL-7, promote T cell proliferation in the T-DC coculture system.

Inhibition of T Cell Proliferation by CD4$^+$CD25$^+$ T Cells. In addition to competition for self-pepMHC on DC and for stimulating soluble factors, it is possible that active inhibition among T cells contributes to the lack of naïve T cell proliferation in normal hosts. In lymphopenic hosts, in contrast, this inhibition would be relieved by the reduced T cell density. To examine this possibility, a ROC was used. In a ROC, an equal number of 2C T cells and DC were centrifuged into a cell pellet and then cultured on a filter placed on a Gelfoam sponge. Probably because of increased cell—cell interaction in this arrangement, T cells proliferated at a 1:1 ratio of T to DC in ROC, and more T cells ($\approx$80%) proliferated in these cultures than in suspension culture (Fig. 6*A*).

The effect of increasing numbers of T cells on the proliferation of T cells expressing the same TCR was investigated first. CFSE-labeled 2C cells and DC were cultured at a 1:1 ratio in ROC in the presence of increasing numbers of unlabeled 2C cells. In the absence of unlabeled 2C cells, 78% of CFSE-labeled 2C cells proliferated by 5 days (Fig. 6*A*). In the presence of 1-, 3-, and 7-fold more unlabeled 2C cells, the proportions of CFSE-labeled 2C cells that proliferated decreased to 52%, 34%, and 12%, respectively (Fig. 6*B*). Thus, as in mice, increasing numbers of T cells in cocultures result in diminished T cell proliferation, probably because of (*i*) competition between labeled and unlabeled 2C cells for the same self-pepMHC and/or stimulating soluble factors and (*ii*) increased inhibition among T cells resulting from an increased T cell density.

To distinguish between these possibilities, we carried out ROC as above except that unlabeled CD8 T cells expressing a different TCR (F5) were used. In the presence of a 1-, 3-, 7-, and 15-fold

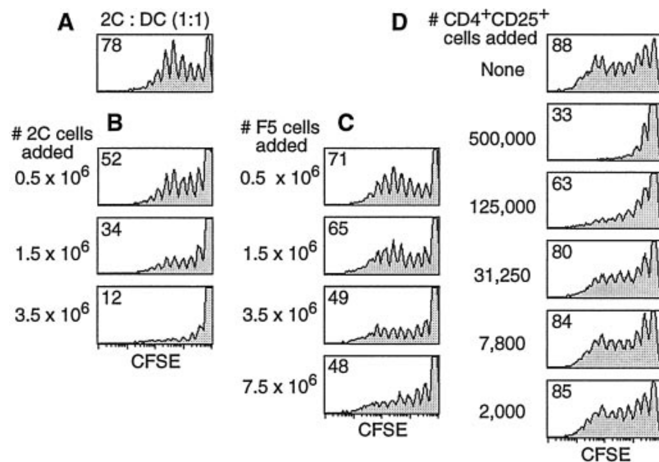

Fig. 6. Inhibition of T cell proliferation by $CD4^+CD25^+$ regulatory T cells in T-DC cocultures. (*A*) CFSE-labeled 2C cells (5×10^5) were cultured with equal numbers of DC in ROCs. Representative CFSE profiles of $1B2^+CD8^+$ 2C cells at day 5 are shown. Numbers inside rectangles indicate percentages of T cells that proliferated. (*B* and *C*) The same as in *A*, except that increasing numbers of unlabeled 2C or F5 T cells were added (numbers outside the rectangles). Representative data from one of three experiments are shown. (*D*) The same as in *B* and *C*, except that various numbers of unlabeled $CD4^+CD25^+$ T cells were added.

excess of unlabeled F5 cells, 71%, 65%, 49%, and 48% of 2C cells proliferated (Fig. 6*C*), indicating that F5 cells are significantly less potent than 2C cells in inhibiting 2C cell proliferation. F5 TCR recognizes an influenza virus peptide in association with class I D^b molecule and, therefore, probably does not compete for the same self-pepMHC complexes with the 2C TCR, which recognizes other peptides in association with K^b.

In contrast, the addition of $CD4^+CD25^+$ regulatory T cells (25, 26) in T-DC ROC more severely inhibited the proliferation of CFSE-labeled 2C cells than did equal numbers of 2C or F5 cells (Fig. 6*D*). The $CD4^+CD25^+$ T cells were approximately three times more potent than unlabeled 2C cells in decreasing the percentages of 2C cell proliferation to about 33% (Fig. 6 *A*, *B*, and *D*). Because $CD4^+CD25^+$ regulatory T cells recognize peptides in complexes with MHC class II molecules, their effect on 2C cell proliferation probably is not through competition for the same self-pepMHC complexes but, rather, by direct inhibition or by limiting stimulatory soluble factors. Consistently, when $CD4^+CD25^+$ regulatory T cells were separated from 2C cells in transwell cocultures, no inhibition was observed (data not shown). The addition of IL-10 or type β transforming growth factor in T-DC cocultures did not inhibit T cell proliferation (data not shown). Thus, $CD4^+CD25^+$ regulatory T cells likely exert their inhibitory effect directly through interaction with 2C T cells or indirectly through interaction with DC.

Discussion

We show here that naïve T cells proliferate when cultured with syngeneic DC in the absence of exogenous antigen and that this *in vitro* response closely resembles lymphopenia-induced proliferation in mice (10–13). In both the *in vitro* and *in vivo* responses: (*i*) T cell proliferation requires engagement of the T cells' TCR with self-pepMHC complexes on DC; (*ii*) coreceptor CD8 is required for an optimal response; (*iii*) DC abundance is essential; and (*iv*) the proliferating T cells are not activated to express CD69 and CD25. Based on these findings, T cell proliferation in the T-DC coculture system appears to be fundamentally the same as homeostasis-driven T cell proliferation in lymphopenic hosts. Compared with recently reported spleen or lymph node organ cultures for analysis of homeostatic T cell proliferation (16), the T-DC coculture system described here appears to be better defined and more easily manipulated.

DC have been assumed but not demonstrated to provide pepMHC complexes for homeostatic T cell proliferation in lymphopenic hosts (21). By using the *in vitro* coculture system, we now have shown that, among antigen-presenting cells, DC, but not B cells and macrophages, promote T cell proliferation. Although B cells normally express much higher levels of MHC class I proteins than nonprofessional antigen-presenting cells, the level on B cells is still too low to support T cell proliferation in cocultures (Fig. 3). In addition, B cells do not secrete stimulating soluble factors. Thus, both resting and LPS-activated B cells did not elicit T cell proliferation, whereas LPS-activated B cells, which express elevated levels of MHC class I, were able to support T cell proliferation in cultures in the presence of $\beta_2m^{-/-}$ DC, which probably provide stimulating soluble factors. As with resting B cells, the class I MHC levels expressed by other cell types is likely too low to support homeostatic T cell proliferation in lymphopenic individuals.

The ability of DC to stimulate T cell proliferation in cultures is consistent with the known properties of DC (19, 20) and the ability of these cells to stimulate T cell proliferation in autologous mixed lymphocyte reaction (27). Mature DC in spleen and lymph nodes express high levels of MHC and costimulatory (CD80 and CD86) and adhesion [intercellular adhesion molecule-1 (ICAM-1) and ICAM-3] molecules. *In vitro*, DC stimulate intense T cell membrane ruffling and crawling of T cells over the DC surface (28). Recently, immunological synapses were observed between DC and naïve T cells in the absence of exogenic antigen (28). A DC-induced, antigen-independent intracellular Ca^{2+} increase in T cells also has been shown with a variety of T cells expressing different TCR (28). In contrast, similar T cell responses were not observed in cultures when DC were replaced by B cells (29), monocytes (30), or fibroblasts (31) that expressed MHC class II, ICAM, and B7. Together, these observations and our present findings strongly suggest that DC are the essential providers of the self-pepMHC complexes needed for homeostatic T cell proliferation in lymphopenic hosts.

Although soluble factors secreted by DC are required to promote T cell proliferation in the coculture system and in lymphopenic mice, there are clear differences in their cytokine requirements. For example, IL-7 was shown to be critical for homeostatic T cell proliferation in mice (15, 16), whereas we did not detect IL-7, as transcripts or protein, in cultured DC or DC plus T cells. T cell proliferation in cocultures was enhanced by exogenous IL-7 but not blocked by anti-IL-7 and anti-IL-7R antibodies (Fig. 5), indicating that IL-7 is not essential for T cell proliferation in this system.

In contrast, IL-15 appears to be critical. The expression of IL-15 is detected in freshly isolated DC and induced upon culture (Fig. 4). T cell proliferation in T-DC cocultures was enhanced by the addition of IL-15 and severely blocked by antibody to the β-chain of the IL-15 (and IL-2) receptors (Fig. 5). In mice, however, IL-15 seems to promote proliferation of memory, but not naïve, CD8 T cells (refs. 32 and 33; Ananda Goldrath, personal communication). The effect of IL-15 *in vitro* probably was not caused by selective proliferation of memory 2C cells, because (*i*) the requirement for TCR–pepMHC interaction and (*ii*) $<5\%$ $CD44^{hi}$ 2C T cells in 2C/RAG mice and high percentages of 2C cell proliferation, especially in ROCs ($\approx 80\%$). IL-15 may act together with other cytokines to promote homeostatic T cell proliferation. Consistent with this possibility, many cytokines were induced in cultured DC (Fig. 4*B*) and others (IL-2, IL-5, IL-9, and IL-13) were induced in T cells cocultured with DC. Although IL-5, IL-6, IL-9, IL-13, and RANTES, added individually, did not stimulate T cell proliferation and antibodies specific for IL-5, IL-9, and IL-13 did not block this T cell

response, the addition of IL-2 enhanced T cell proliferation in the coculture system.

Two broad mechanisms can be envisioned to account for the lack of homeostatic T cell proliferation in normal hosts (21). One is too much competition among T cells for self-pepMHC complexes and stimulatory soluble factors. The other is active inhibition of T cell proliferation by T cells themselves. Because of difficulties in distinguishing between these mechanisms in intact mice, we have started to examine this issue in the T-DC coculture system. The evidence so far suggests that both mechanisms are operational under the *in vitro* conditions. Thus, proliferation of CFSE-labeled 2C cells was inhibited more severely by unlabeled 2C cells than by unlabeled F5 cells (Fig. 6), indicating competition for particular self-pepMHC complexes, analogous to T cell competition for antigen-bearing antigen-presenting cells (34).

$CD4^+CD25^+$ regulatory T cells were much more potent than unlabeled 2C cells in inhibiting 2C cell proliferation (Fig. 6). The effect of $CD4^+CD25^+$ T cells *in vitro* is consistent with the inhibition of T cell proliferation in lymphopenic mice by $CD4^+CD25^+$ T cells (35) or by large numbers of $CD4^+$ T cells that contain the $CD25^+$ fraction (21). The greater effect of $CD4^+CD25^+$ T cells than unlabeled 2C cells cannot be attributed to the competition for the same self-pepMHC complexes, because 2C cells recognize class I MHC–peptide complexes whereas $CD4^+CD25^+$ regulatory T cells recognize class II MHC–peptide complexes. The $CD4^+CD25^+$ T cells did not inhibit 2C T cell proliferation when the cells were separated by a permeable membrane, indicating that inhibition is not mediated by stable soluble factors. In contrast, unlabeled 2C cells inhibited proliferation of CFSE-labeled 2C cells even when the two were separated by a permeable membrane (Fig. 3), indicating competition for soluble stimulating factors. Notably, the addition of IL-10 or type β transforming growth factor, two inhibitory cytokines secreted by $CD4^+CD25^+$ regulatory T cells (25, 26), did not reduce T cell proliferation in cocultures. Thus, the effect of $CD4^+CD25^+$ regulatory T cells on homeostatic T cell proliferation does not depend on competition for soluble factors or self-pepMHC complexes; instead, these cells evidently exert their inhibitory effect directly through contact with responding T cells or indirectly through interaction with DC. The T-DC coculture system described here likely will facilitate the analysis of these and other mechanisms underlying homeostatic T cell proliferation in lymphopenic hosts.

We thank Tara Schmidt and Carroll McKinley for technical assistance, Glenn Paradis for cell sorting, Dr. David Kranz for 2C/RAG mice, Dr. D. Kioussis for F5 mice, Dr. N. Mach for GM-CSF-producing tumor cell line, Dr. Ananda Goldrath for sharing unpublished data, and members of the Chen and Eisen laboratories for helpful discussions. This work was supported in part by National Institutes of Health Grants AI44478 and AI50631 (to J.C.) and AI44477 and CA60686 (to H.N.E.) and a Cancer Center Core Grant (to Richard Hynes). Q.G. is supported partly by an Anna Fuller postdoctoral fellowship. D.P. is supported as a StressGen fellow.

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