Severely Impaired Clonal Deletion of CD4+ T Cells in Low-Dose Irradiated Mice: Role of T Cell Antigen Receptor and IL-7 Receptor Signals

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Severely Impaired Clonal Deletion of CD4+ T Cells in Low-Dose Irradiated Mice: Role of T Cell Antigen Receptor and IL-7 Receptor Signals

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Systemic administration of high doses of soluble Ag induces peripheral CD4+ T cell tolerance in unmanipulated hosts. To test whether tolerance is modified under conditions of transient lymphopenia, we tracked the response of 5C.C7 TCR-transgenic CD4+ T cells to i.v. moth cytochrome c peptide in mice that received low-dose gamma irradiation 10 days previously. This model was chosen because it does not support spontaneous lymphopenia-induced proliferation of 5C.C7 cells, allowing the study of Ag-specific responses without interference from simultaneous spontaneous proliferation. Clonal expansion in response to i.v. peptide was increased in irradiated mice, while clonal deletion was severely impaired in comparison with untreated animals. Amplified TCR triggering was observed in irradiated hosts, consistent with dendritic cell activation leading to enhanced Ag presentation. Failure of deletion was accompanied by persistent T cell activation and accumulation of Th1 effector cells. Up-regulated expression of IL-7R and the prosurvival protein Bcl-xL was associated with clonal persistence. Cells with memory and naive phenotypes were both represented within persistent clones, but no Th1 function could be demonstrated within the long-term memory population. Failure of clonal deletion in irradiated hosts represents a novel mechanism limiting TCR diversity in a lymphopenic environment and may contribute to subsequent autoimmunity. The Journal of Immunology, 2006, 177: 8320–8330.

Lymphopenia is a well-recognized risk factor in a subset of autoimmune diseases (1–4). The discovery that lymphopenia drives spontaneous T cell proliferation (also termed homeostatic or lymphopenia-induced proliferation (LIP)3 (5–10)) has focused attention on the mechanistic relationship between LIP and autoimmune disease (11). LIP is driven by low-affinity TCR engagement and involves T cells with avidity for endogenous peptide:MHC complexes, including self-peptide MHC complexes (12–14). Increased access to IL-7 in lymphopenic hosts is believed to play a significant role in LIP (15). The proliferative process results in conversion of naive T cells to an activated/memory phenotype and acquisition of cytokine-producing capacity (10), cytotoxic function (16), and the ability to enter nonlymphoid target tissues (12). Although these are all prerequisites for the initiation of an autoimmune attack in the case of self-reactive T cells, LIP is not sufficient on its own to cause autoimmune disease in animals that lack a genetic predisposition (17, 18). LIP is also known to occur in humans, as shown by the expansion of T cells of memory phenotype and the restricted T cell repertoire that results from peripheral T cell reconstitution after chemo/radiotherapy (19) or treatment of HIV after the onset of lymphopenia (20). Although autoimmune disease is more frequent under these circumstances (21, 22), it is by no means universal.

In contrast to LIP, acute exposure of naive self-reactive T cells to self-Ag in lymphosufficient animals leads to tolerance via clonal deletion rather than clonal persistence. Why this antiserf response differs from antiserf LIP is not clear. To determine whether the lymphopenic environment itself is responsible for clonal persistence of cells that would otherwise undergo deletion, we administered soluble peptide by the i.v. route, a method that is widely regarded as a mimic of peripheral deletion in response to high-affinity self-Ag. Our model made use of 5C.C7 TCR-transgenic CD4+ T cells (12, 23–25), which do not undergo LIP after adoptive transfer into the transiently lymphopenic environment created by low-dose total body irradiation (our unpublished observation and Ref. 26). We could thus separate the effects of LIP and specific Ag exposure. In contrast, previous studies of 5C.C7 cells in more severely lymphopenic hosts indicated that they underwent extensive LIP (27). In lightly irradiated animals, the immune response to i.v. peptide was more vigorous and persistent than the response seen in T cell-sufficient hosts and cells developed significant Th1 effector function for several weeks after the Ag response. Importantly, long-term clonal persistence was associated with high-level expression of IL-7R, reminiscent of spontaneous LIP. These data indicate that cells responding to a tolerogenic stimulus in a lymphopenic environment develop the capacity to mount significantly larger effector responses. Self-directed effector function in lymphopoenic animals may serve as a crucial initiating factor for autoimmune disease in individuals with additional genetic susceptibility factors. In addition, persistent clonal expansion in response to foreign Ags present during lymphopenic episodes has the capacity to contribute to skewing of the TCR repertoire, over and above the oligoclonal expansion resulting from LIP.
Materials and Methods

Mice

All lines of transgenic mice were bred and housed under specific pathogen-free conditions in the Centenary Institute (CI) Animal Facility. Approval for all animal experimentation was obtained from the Institutional Ethics Committee at the University of Sydney. TCR-transgenic mice expressing the 5C.C7 TCR (Vα11* Vβ3*) specific for the COOH-terminal epitope of moth cytochrome c (MCC) in the context of IE (28, 29) were bred on a B10.BR Rag2−/− background (H-2b, CD45.2). C57BL/6 (CD45.1) mice were obtained from the Animal Resources Centre (ARC, Perth, Australia). B10.BRL mice (B6.SJLPtprca mice backcrossed to B10.BR Rag2−/−H-2k, CD45.1 homozygous line) were bred in the CI Animal Facility.

Adaptive transfer of T cells and immunization

For the assessment of LIP, cells from pooled peripheral and mesenteric lymph nodes (LN) of adult B6.SJLPtprca (CD45.1) mice at 10–12 wk of age were labeled with 5 μM CFSE ( Molecular Probes) as described (30, 31). Aliquots of 5 × 106 cells were injected i.v. into the lateral tail vein of sex- and age-matched C57BL/6 (CD45.2) mice exposed to 450 rad gamma irradiation (32) 10 days before the cell transfer. Three weeks after adoptive cell transfer, host LN and spleen cells were stained with appropriate mAbs and analyzed by flow cytometry. Naive 5C.C7 TCR-transgenic CD4+ T cells were obtained as pooled LN cells of 5C.C7 TCR-transgenic B10.BR Rag2−/− (CD45.2) mice. After labeling with CFSE as above, 1–3 × 106 cells were injected i.v. into male B10.BRL (IE–CD45.1) hosts, which received 450 R whole body irradiation 10 days before T cell transfer. Two days later, mice were challenged i.v. with 10 μg of MCC87–103, KaneradiylLKQATK (MCC peptide; MCCip); Queensland Institute of Medical Research, Brisbane, Australia) in PBS. The fate of TCR-transgenic T cells was analyzed 3, 10, 31, and 80 days after MCCp challenge.

Dendritic cell (DC) surface phenotype

Spleens and LN of control and irradiated mice were collected, minced, and digested with collagenase/DNase as described (32). Single-cell suspensions were stained for DC markers without a density gradient enrichment step to avoid cell loss. Non-specific staining due to FcR binding was blocked with anti-CD16/32 (clone 2.4G2; CI). For intracellular staining with Bcl-xL, cells were stained for cell surface markers, fixed with 2% paraformaldehyde. Intracellular staining was conducted on FACStarPlus, DiVa, or LSRII flow cytometers. Data acquisition and analysis following the gating strategy outlined previously (12). The fold change in 5C.C7 TCR-transgenic CD4+ T cells was divided by 2, the second by 4, and so on. The percentage of cells recruited into cell division (R) was calculated using equation 1:

\[
R = 100 \left(1 - \left(1 - \frac{n}{n_i}\right) / 2\right)\%
\]

where \(n_i\) is the number of cells in the ith division peak.

The proliferative capacity (P) (average number of daughter cells generated per recruited cell) was calculated using equation 2:

\[
P = \frac{\sum n_i}{\sum n_i / 2}
\]

Results

Naive 5C.C7 TCR-transgenic CD4+ T cells fail to undergo LIP in irradiated hosts

Polyclonal populations of CD4+ and CD8+ T cells undergo LIP when adoptively transferred into irradiated or T cell-deficient hosts, resulting in the generation of a large number of CD4+ and CD8+ cells with an activated/memory phenotype (10, 16). Such proliferation is dependent on TCR specificity and does not involve the entire repertoire. We chose a known LIP-resistant TCR specificity, namely that of 5C.C7 TCR-transgenic T cells on a raq2−/− background (W.-P. Koh and B. Fazekas de St. Groth, unpublished observation) to study the response to systemic Ag in irradiated hosts without interference from concurrent LIP. Compared with the commonly used protocol for the induction of LIP, we selected a lower dose of irradiation (450 R instead of 600–750 R) and delayed T cell transfer until day 10, to minimize the effects of acute radiation damage. Use of a lower dose of irradiation lessened initial lymphopenia (80% immediate reduction in T cell numbers after 450 R, as compared with over 95% after 600 R), while the delay in T cell transfer allowed partial reconstitution of host T cells to 40 and 30% of normal CD4+ and CD8+ T cell counts, respectively, by the time of T cell transfer.

Naive raq2−/− 5C.C7 TCR-transgenic T cells adoptively transferred 10 days postirradiation into CD45.1 congenic hosts failed to proliferate in the partially reconstituted hosts (Fig. 1A). The number of donor 5C.C7 TCR T cells recovered from the spleen and LN of irradiated hosts was similar to that recovered from untreated control animals and remained stable over 80 days (Fig. 1B). We confirmed that these mildly lymphopenic conditions did indeed support LIP of polyclonal T cell populations by measuring proliferation of adoptively transferred polyclonal CD4+ and CD8+ T cells (Fig. 1C). These data indicate that 5C.C7 TCR T cells do not receive sufficiently strong TCR signals to undergo proliferation in irradiated hosts, at least in the experimental set-up used here, and that this model is therefore ideal for studying the effect of lymphopenia on Ag-specific responses, in the absence of LIP.
Impaired CD4+ T cell deletion in irradiated mice after exposure to i.v. peptide

To test whether irradiation of the host would influence Ag-dependent deletion, we adoptively transferred CFSE-labeled naive 5C.C7 TCR-transgenic CD4+ T cells into intact hosts or hosts irradiated with 450 R 10 days earlier. When the recipients were challenged with soluble MCCp i.v., 5C.C7 T cells mounted a vigorous response to the MCCp in both irradiated and control mice with a peak on day 3 (Fig. 2).

Although the absolute number of 5C.C7 TCR T cells recovered from LNs and spleens differed between individual experiments (Table I), the number of 5C.C7 TCR T cells in secondary lymphoid organs was always significantly higher in the irradiated group (mean ± SEM, 7.4 x 10^7 ± 0.7) as compared with the control group (4.9 x 10^7 ± 0.7). Indeed, by day 10, more than 90% of transgenic T cells in the control group had been deleted (Fig. 2), and the number of residual cells continued to decline until day 80. In the irradiated group, there was an initial 5.7-fold decrease in transgenic cell number at day 10 in the spleen, but no further reduction occurred even on day 80 when the lymphoid compartment was fully reconstituted (Fig. 2B and Table I). In the LNs, cell numbers continued to decline slowly until day 31 but then remained stable.

Visualizing T cell fate by tracking cell division

To better understand the difference in the response to i.v. peptide in irradiated and control animals, we compared CFSE dilution profiles of transferred 5C.C7 TCR T cells after MCCp exposure (Fig. 3 and Table II). First, we found significant differences in the initial response. Thus, analysis of irradiated hosts revealed the presence of more cells in divisions 5–7 (Fig. 3, day 3), more pronounced TCR down-regulation (measured by Vβ11 mean fluorescence intensity, Table II), and generation of a higher mean number of progeny per recruited cell (measured by proliferative capacity (Cp), Table II), consistent with the higher number of 5C.C7 TCR T cells recovered from irradiated compared with intact hosts (Fig. 1B and Table I). Second, CFSE-intermediate cells (divisions 3–5) accumulated in the irradiated hosts (Fig. 3, days 10 and 31), suggesting that these cells were at least partially protected from deletion. Indeed, 70% of all 5C.C7 TCR T cells in these hosts remained CFSE intermediate (divisions 2–7) at day 10, and over 50% remained CFSE intermediate for at least 80 days. CFSE-negative cells (division 7) in irradiated hosts were also protected from deletion, as indicated by stable numbers between days 10 and 80 (Fig. 3). In contrast, the profound drop in 5C.C7 TCR T cell numbers in the control group indicated that initial deletion occurred in CFSE-intermediate and CFSE-negative subsets, although the relative decrease in the CFSE-intermediate division peaks between days 3 and 31 (Fig. 3) also showed that CFSE-intermediate cells were more efficiently deleted than CFSE-negative cells. By day 80, only a small number of CFSE-negative cells survived in nonirradiated hosts.

Activation of APCs in irradiated mice

The combination of TCR down-regulation and higher proliferative capacity observed in irradiated animals suggested increased TCR signaling (33). Because the strength of TCR engagement is determined in part by the context of Ag presentation, we compared the
10 days earlier. On the next day, mice received 10⁶/iH9262/g of MCC87–103 (MCCp) via the i.v. route, and the T cell response was measured on days 3, 10, 31, and 80 after Ag challenge. A, Representative dot plots of LN cells at indicated times after MCCp challenge, gated for donor 5C.C7 TCR T cells (Vα11⁺ CD45.1⁺). The percentage of donor 5C.C7 cells is indicated above the gate. B, Absolute number of donor 5C.C7 TCR-transgenic CD4⁺ T cells per recipient (spleen or pooled LNs, as indicated). Bars represent mean ± SEM of three independent experiments (□, control; ■, irradiated hosts).

The frequency of Ag-specific IFN-γ- and IL-2-producing cells (our unpublished observation). To test whether Ag-experienced T cells surviving in irradiated hosts differentiated into cytokine-producing effector cells, we studied cytokine production by 5C.C7 TCR T cells after in vitro restimulation with MCCp (Fig. 5). The frequency of Ag-specific IFN-γ- and IL-2-producing cells was significantly higher in the spleen and LNs of irradiated hosts than in control hosts (Fig. 5B). The percentage of donor 5C.C7 cells is indicated above the gate. B, Absolute number of donor 5C.C7 TCR-transgenic CD4⁺ T cells per recipient (spleen or pooled LNs, as indicated). Bars represent mean ± SEM of three independent experiments (□, control; ■, irradiated hosts).

Table I. Deletion of naive 5C.C7 TCR T cells in control and irradiated (IRR) hosts after i.v. challenge with MCCp

<table>
<thead>
<tr>
<th>Experiment (E)</th>
<th>Vari11⁺ × 10⁶ ± SEM/Mouse (pooled lymph nodes)</th>
<th>Vari11⁺ × 10⁶ ± SEM/Mouse (spleen)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 10</td>
</tr>
<tr>
<td>E1 Control</td>
<td>0.10 ± 0.04</td>
<td>1.17 ± 0.45</td>
</tr>
<tr>
<td>E1 IRR</td>
<td>0.08 ± 0.01</td>
<td>2.47 ± 0.30</td>
</tr>
<tr>
<td>E2 Control</td>
<td>0.13 ± 0.01</td>
<td>1.95 ± 0.24</td>
</tr>
<tr>
<td>E2 IRR</td>
<td>0.13 ± 0.02</td>
<td>2.06 ± 0.31</td>
</tr>
<tr>
<td>E3 Control</td>
<td>0.17 ± 0.04</td>
<td>4.06 ± 0.53</td>
</tr>
<tr>
<td>E3 IRR</td>
<td>0.13 ± 0.01</td>
<td>5.54 ± 0.15</td>
</tr>
<tr>
<td>E4 Control</td>
<td>0.14 ± 0.03</td>
<td>2.33 ± 0.36</td>
</tr>
<tr>
<td>E4 IRR</td>
<td>0.18 ± 0.06</td>
<td>4.16 ± 0.47</td>
</tr>
<tr>
<td>E(1–4) Control</td>
<td>0.13 ± 0.01</td>
<td>2.44 ± 0.34</td>
</tr>
<tr>
<td>E(1–4) IRR</td>
<td>0.16 ± 0.03</td>
<td>3.51 ± 0.46</td>
</tr>
</tbody>
</table>

*Naive 5C.C7 TCR T cells (Vα11⁺) from CD45.2⁺/− hosts were adoptively transferred into B10.BRL (CD45.1⁺) hosts, either untreated or irradiated (450 R) 10 days earlier. Mice were challenged i.v. with 10 μg of MCCp on the next day and were sacrificed 3, 10, or 31 days after challenge. LN and spleen cell suspensions were analyzed for the presence of donor Vari11⁺ cells; the absolute number of donor (CD45.2⁺ Vα11⁺) Vari11⁺ cells per recipient was calculated. Data are expressed as mean ± SEM, three to six animals per group; na, not analyzed.
hosts than in those of controls (Fig. 5A). Furthermore, the number of IFN-γ- and IL-2-secreting cells increased between days 10 and 31 after Ag challenge (Fig. 5B). Up to 75% of total cytokine-producing cells were found in the spleens of irradiated animals, whereas no effector cells could be detected in those from control mice (Fig. 5B and data not shown). Importantly, there was a distinct Th1 pattern of cytokine production because we could detect no IL-4- or IL-10-positive cells in the irradiated animals at any time (data not shown). In contrast, some IL-4- and IL-10-positive cells could be detected in the control mice, albeit at a very low frequency (<1%). Accumulation of Th1 effectors in irradiated animals after i.v. challenge with MCCp was transient and no effector memory cell differentiation was observed, as we found no cytokine-producing cells at day 80 after MCCp challenge in vitro (Fig. 5B) or in vivo (data not shown). Our results demonstrate that CD4+ T cells surviving in irradiated hosts for 30 days after i.v. peptide administration underwent differentiation into Th1 effector cells. However, at late time points, the surviving cells appeared to be anergic.

Expression of prosurvival cytokine receptors and antiapoptotic proteins by 5C.C7 TCR CD4+ T cells in irradiated hosts

Expression of CD127 (IL-7Rα) and CD122 (IL-2/15Rβ) by CD8+ T cells has been previously associated with differentiation into memory cells (15, 34) consistent with the known ability of IL-7 and IL-15 to act as prosurvival cytokines. We studied the expression of CD127, CD122, CD25 (IL-2Rα), CD132 (common receptor β-chain (c)), and the prosurvival protein Bcl-xL by 5C.C7 TCR-transgenic CD4+ T cells after i.v. exposure to MCCp in control and irradiated mice (Fig. 6). All cytokine receptor chains were down-regulated early in the response (Fig. 6, A and B). Subsequently, cells reacquired expression of CD127 and CD132 whereas expression of CD25 and CD122 remained low. Remarkably, a significantly higher proportion of transgenic cells in the irradiated hosts were CD127 positive on day 10 (61% as compared with 44% in control, Fig. 6B, left panel). The absolute number of CD127-positive cells in irradiated hosts remained constant up to 80 days after MCCp challenge, whereas total number of CD127-negative
cells progressively declined with time (Fig. 6B, right panel), indicating a survival advantage of CD127-expressing cells in irradiated hosts; this was not the case in control mice where CD127-positive and CD127-negative cells underwent deletion at a similar rate. Furthermore, we observed differential up-regulation of CD127 on the CFSE-intermediate subset, which increased with every consecutive division, so that up to 80% of CFSE-negative T cells expressed CD127 by day 10 (Fig. 6D, upper panel). This pattern of CD127 expression correlated with the enhanced survival in irradiated hosts of both CFSE-intermediate and CFSE-negative cells (Fig. 3). The concomitant increase in CD127 and CD132 expression by both CFSE-intermediate and CFSE-negative T cells in irradiated hosts (Fig. 6, A and B) is consistent with the expression of a fully functional IL-7R mediating survival signals via expression of prosurvival proteins such as bcl-2 and Bcl-xL. Indeed, there was a small but significant increase in the level of Bcl-xL expression by 5C.C7 TCR T cells from the irradiated animals as compared with nonirradiated controls (Fig. 6C). Furthermore, analysis of Bcl-xL expression as a function of cell division (Fig. 6D, lower panel) revealed that <15% of CFSE-intermediate and CFSE-negative T cells were Bcl-xL positive in nonirradiated controls; in contrast, Bcl-xL expression in irradiated animals increased with every consecutive division, so that >60% of CFSE-negative T cells were Bcl-xL positive. Indeed, up-regulation of CD127 and Bcl-xL as a function of cell division number was remarkably similar in irradiated hosts (Fig. 6D, right panel), suggesting that improved survival of Ag-experienced CD4+ T cells in irradiated hosts is mediated by IL-7.

**Activation phenotype of surviving 5C.C7 TCR CD4+ T cells after Ag exposure in irradiated hosts**

Up-regulation of IL-7R on a subset of effector CD4+ T cells has been associated with transition to activated/memory cells (35-37). However, the cytokine data described above suggested that no long-term memory function developed after i.v. Ag challenge. We therefore examined the expression of cell surface markers associated with the memory phenotype (Fig. 7). On day 10, CFSE-negative cells displayed a more differentiated phenotype in irradiated than in control hosts, with increased expression of CD54 and decreased CD62L, CD69, and CD27. In contrast, the CFSE-intermediate cells in irradiated hosts expressed lower levels of CD54 and CD44 than the CFSE-negative cells and a significant proportion of the population was still CD69 positive and CD62L negative. By day 31, the phenotype of CFSE-negative cells was comparable between the two
FIGURE 6. Expression of cytokine receptors and Bcl-xL during the response of naive TCR-transgenic CD4⁺ T cells to i.v. peptide in control and irradiated hosts. In the experiment described in the legend to Fig. 2, 5C.C7 TCR T cells from LN were analyzed for the expression of (A) IL-2Rα (CD25), IL-2/IL-15Rβ (CD122) and IL-2Rγc (CD132), (B and D) IL7Rα (CD127), and (C and D) Bcl-xL on the indicated days after Ag challenge. A, Representative dot plots of CFSE vs CD25, CD122 and CD132, gated on Vα11⁺CD45.1⁺ cells. The percentage of positive cells is indicated above the gate. B, Representative dot plots, gated on Vα11⁺CD45.1⁻ cells (left panel). The percentage of cells in each quadrant is indicated; na, not analyzed. Absolute numbers of CD127⁻ and CD127⁺ cells per mouse were calculated and plotted as shown in the right panel for LNs (each circle represents a mean value for one independent experiment). ○, CD127-negative cells; ●, CD127-positive cells. C, Bcl-xL expression on days 4, 10, and 31 after Ag challenge. Bold line, control+MCCp; shaded histogram, irradiated+MCCp; dashed, Ab control (rabbit anti-GFP). D, CFSE profiles of 5C.C7 T cells on day 10 after Ag challenge of irradiated and control mice were analyzed for CD127 and Bcl-xL expression. Left panels, The gating strategy: numbers within dot plots indicate division number. Right panels, The percentage of CD127-positive or Bcl-xL-positive cells for each cell division number. Data are from one representative experiment; ○, control+MCCp; ●, irradiated+MCCp.
groups and typical of memory cells. Interestingly, however, the residual CFSE-intermediate cells in the irradiated mice showed an unusual phenotype, with expression of CD62L, CD69, CD54, CD27, and CD44 at levels comparable to those of naive cells. This phenotype persisted to day 80 (data not shown). To test whether this was the result of differential deletion of memory phenotype cells within the CFSE-intermediate population, we calculated the absolute number of CD44-positive and CD44-negative cells in the CFSE-negative and CFSE-intermediate compartments in irradiated hosts (Fig. 7B). The total number of CFSE-intermediate cells declined from $0.38 \times 10^6$ to $0.28 \times 10^6$ between days 10 and 31 after Ag challenge, whereas the number of CFSE-negative cells increased from $0.13 \times 10^6$ to $0.27 \times 10^6$, consistent with a slow loss of CFSE fluorescence over that period. In contrast, the number of CD44low cells increased from $0.27 \times 10^6$ to $0.43 \times 10^6$, and the number of CD44high cells decreased from $0.24 \times 10^6$ to $0.12 \times 10^6$, while the absolute numbers of 5C.C7 cells on days 10 and 31 remained stable ($0.51 \times 10^6$ and $0.55 \times 10^6$, respectively), consistent with the data in Fig. 1. Thus, the shift from CD44high to CD44low appeared to result from reversion rather than differential loss of CD44high cells.

**Discussion**

T cells responding to i.v. peptide in an intact host normally undergo deletion after an initial burst of proliferation (23). The results presented here show that the response to i.v. peptide is qualitatively and quantitatively different under lymphopenic conditions. Importantly, we have excluded the effect of simultaneous LIP by using a model in which MCC-specific CD4+ T cells do not undergo LIP. Deletion of MCC-specific cells in response to i.v. peptide occurred within a week in control animals but was markedly impaired in irradiated hosts (Fig. 2). Lack of deletion affected both Ag-experienced T cells that were fully divided (CFSE negative) and cells that had undergone less than eight cell divisions (CFSE intermediate, Fig. 3). Our observation is consistent with that of Williams et al. (38) who observed the absence of clonal deletion accompanied by breaking self-tolerance in response to the superantigen staphylococcal enterotoxin B in nude mice reconstituted with syngeneic T cells. However in that case, a proportion of the cells responding to staphylococcal enterotoxin B would have simultaneously been undergoing LIP, and therefore the possibility that LIP itself prevents tolerance could not be excluded. We also observed accumulation of Th1 effector cells within the

**Figure 7.** Expression of cell surface activation markers during the response of naive TCR-transgenic CD4+ T cells to i.v. peptide. In the experiment described in the legend to Fig. 2, 5C.C7 TCR T cells from LN were analyzed for the expression of activation markers on the indicated days after Ag challenge (A). Representative dot plots are shown, gated on Vα11+CD45.1+ cells. Results are representative of three independent experiments. The percentage of cells in each of the quadrants is indicated. B, Dot plots of CD44 and CFSE expression in irradiated mice (left panel) were further analyzed to calculate the absolute number of LN 5C.C7 TCR T cells in each quadrant (right panel). The row and column totals are provided for each analysis, together with the absolute cell number (boxed) at lower right. Similar results were obtained in two additional independent experiments.
CFSE-negative population over the first month of the response (Fig. 5). Importantly, although lymphopenia was sufficient to induce Th1 effector cell differentiation and persistence of a CFSE-negative population with the surface phenotype of memory cells (Fig. 7), the response did not convert to a fully immunogenic pattern, as indicated by the lack of long-term persistence of Th1 memory, defined by cytokine production upon in vitro (Fig. 5) and in vivo recall (data not shown). Thus, the CFSE-negative cells surviving to day 80 appeared to be anergic. CFSE-intermediate cells that had undergone less than eight rounds of division during the initial response did not make cytokines and expressed lower levels of activation markers such as CD44 than did the CFSE-negative cells. Indeed, the overall percentage of CD44low cells increased from 53 to 78% between days 10 and 31, without a change in cell numbers, suggesting reversion to a CD44high phenotype (Fig. 7). We have previously shown that the very few residual CFSE-intermediate cells that survive peptide-mediated deletion in normal hosts are also CD44low (23). In LIP, CFSE-intermediate cells are also known to express lower levels of memory markers than the fully divided CFSE-negative population (39). Thus, CFSE-intermediate cells surviving in lymphopenic hosts may represent partially or abortively differentiated cells that revert to a naïve phenotype.

Initial expansion of Ag-specific T cells in response to soluble Ag was accelerated in irradiated hosts (Fig. 2); when considered in association with more profound TCR down-regulation and higher proliferative activity per recruited precursor (Table II), these findings are indicative of increased TCR triggering (40). Moreover, our data indicate that neither acute radiation effects nor concomitant TCR signals responsible for LIP are implicated in this effect. Because the number of Ag-specific T cells and dose of Ag were fixed, stronger TCR triggering in irradiated mice can thus be attributed to the host environment. It has been suggested that macrophage activation and release of proinflammatory cytokines following irradiation may contribute to homeostatic expansion after irradiation (41, 42). However, in our experiments, adoptive T cell transfer was delayed for 10 days after irradiation to allow acute radiation damage to resolve. To test whether amplified TCR triggering in lymphopenic hosts was contingent upon an increase in the stimulatory capacity of APCs, we studied DC phenotype at the time of Ag challenge and found that expression of both MHC class II and costimulatory molecules was increased on DCs as well as B cells. Although B cells do not activate naïve T cells in a steady state (43, 44), it could be argued that they might contribute to Ag presentation if microenvironmental damage due to irradiation had disturbed the follicular location of B cells. However, the total number of B cells in secondary lymphoid organs was severely reduced at the time of Ag challenge, whereas the absolute number of DCs had recovered completely by 2 wk after irradiation due to rapid myeloid reconstitution (data not shown). Thus, it is likely that enhanced stimulatory capacity of DCs in irradiated, lymphopenic hosts was indeed responsible for the increase in T cell responsiveness. In support of this scenario, we previously found that DCs from intact T cell deficient (rag-1 knockout) mice express higher levels of costimulatory molecules which enhance early Ag-specific activation and proliferation in a similar way to irradiation (W.-P. Koh, C. Power, and B. Fazekas de St. Groth, unpublished observations). Importantly, rapid phase LIP of CD4 T cells is also known to depend on costimulation (33, 45). Taken together, these results indicate an important contribution of costimulation to the effect of lymphopenia on the immune response. Because LIP is TCR mediated, it can be argued that increased costimulation in lymphopenic hosts might provide stronger TCR triggering for otherwise average-affinity TCRs. We suggest that an increase in TCR triggering and resultant accumulation of MCC-specific T cells in response to i.v. peptide challenge of irradiated hosts is due not only to the increased number of MCC:pMHC complexes per DC but to the increase in the costimulatory context of Ag presentation.

Because peptide-induced deletion in vivo occurs via apoptosis of Ag-experienced T cells (46, 47), the increased expression of the antiapoptotic protein Bcl-xL observed here (Fig. 6D) could explain T cell rescue in the irradiated hosts. Engagement of IL-7R has been linked to increased expression of prosurvival proteins bcl-2 and Bcl-xL, stimulation of glucose metabolism, and repression of proapoptotic signals (48, 49). We found a selective up-regulation of IL-7R and Bcl-xL on T cells that escape deletion (Fig. 6, A–C). Indeed, no deletion of IL-7R-expressing cells was apparent in irradiated mice, whereas IL-7R-negative cells in irradiated mice underwent deletion at a similar rate to that of both IL-7R-expressing and IL-7R-negative cells in normal hosts (Fig. 6B, right panel). Thus, expression of IL-7R is strongly linked to the survival of peptide-specific cells after i.v. immunization of irradiated mice. The antiapoptotic function of IL-7 in lymphopenia is further supported by the demonstration that overexpression of Bcl-2, or inactivation of the proapoptotic protein bim can rescue T cell numbers and function in severely lymphopenic CD127−/− (IL-7R−) knockout mice (50, 51). Importantly, LIP and Ag exposure differentially affect expression of CD127. Thus, T cells undergoing homeostatic expansion never lose CD127 expression, while in our study a higher affinity TCR interaction with exogenous peptide: MHC resulted in the initial loss of CD127 followed by its re-acquisition on a proportion of surviving T cells (Fig. 6B). Our data are consistent with several reports describing high expression of CD127 on a selected subset of effector cells predestined to undergo effector-to-memory transition in both CD4+ (36, 37) and CD8+ compartments (15, 34). Clonal persistence of this type would skew the TCR repertoire and contribute to oligoclonality after recovery from lymphopenia.

CD127 can form a heteromeric complex with either γc chain or the γc-related thymic stromal lymphopoietin (TSLP) receptor (52), resulting in a high-affinity receptor for IL-7 or TSLP, respectively. The concomitant expression of IL-7R and γc chain (Fig. 6A) argues against a role for TSLP in the survival of Ag-experienced T cells in irradiated hosts. Moreover, TSLP is known to be dispensable for development and function of conventional T cells (53). In addition, TSLP has been associated with Th2 responses (54, 55), rather than highly Th1 polarized response observed here. In addition to expression of CD127, survival of expanded clones in irradiated hosts could be due to increased availability of IL-7 secondary to reduced T cell numbers (56). Indeed, increased availability of IL-7 in IL-7-transgenic mice has been shown to support basal homeostasis of a 10- to 20-fold larger population of T cells than in normal animals (57).

The transient nature of the Th1 effector response may be a consequence of the transient nature of the lymphopenia that follows low-dose irradiation. Although recovery from lymphopenia in humans predisposes to development of autoimmune disease in a subset of individuals (21, 22), a genetic defect is required in animal models for transient lymphopenia to precipitate to development of self-perpetuating autoimmune disease (58). In contrast, constitutive lymphopenia is strongly associated with autoimmunity in mice (18), rats (59) and humans (1, 2). It will be interesting to test whether the response to i.v. peptide in constitutively T cell deficient mice can generate long-lived Th1 effector memory.

Besides their implications for our understanding of lymphopenia-associated autoimmune disease, the studies presented here
demonstrate a mechanism whereby recovery from lymphopenia would increase the skewing of the T cell repertoire toward clones reactive with environmental Ags present during the recovery phase, in addition to the self-reactive clones that expand as a result of LIP.

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Disclosures

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References


