

# IL-2 Secretion by CD4<sup>+</sup> T Cells In Vivo Is Rapid, Transient, and Influenced by TCR-Specific Competition

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The secretion of IL-2 is a critical and early landmark in the activation program of CD4<sup>+</sup> T cells in vitro, but the lack of sensitive assays has limited its application for studying T cell activation in vivo. Using a mouse cytokine capture assay we were able to detect the rapid secretion of IL-2 after an in vivo stimulus by 1–2 h in naive T cells and as early as 30 min in memory T cells. Maximal secretion was achieved within 1–2 h for memory cells or 6–8 h for naive T cells. Surprisingly IL-2 production terminated quickly in vivo and secretion was undetectable by 20–24 h in either cell type. We further demonstrated that this short duration of secretion can be influenced by cellular competition between Ag-specific CD4<sup>+</sup> T cells. The consequences of competition were mimicked by reducing the strength of the antigenic stimulus. These data argue that early competition between T cells influences both the eventual frequency of IL-2 producers in the population and also the duration of their secretion, potentially by altering the strength or duration of the stimulus available to each T cell. *The Journal of Immunology*, 2004, 172: 6136–6143.

The rapidity of an early T cell response to antigenic stimulation can significantly affect the outcome of an adaptive immune response in vivo (1). This kinetic parameter is a critical difference between memory and naive T cell responses to an identical antigenic challenge (2, 3). Antigenic stimulation of individual T cells triggers multiple intracellular signaling pathways that are integrated over time until the threshold required for eliciting each functional response is reached (4). The production of the cytokine IL-2 marks a critical landmark in this process and suggests a commitment of the T cell to further programs of proliferation and differentiation (5). It has therefore often been used as a reliable and early readout of potent T cell activation. Early availability of IL-2, especially from Th cells, influences the subsequent expansion, survival, and maturation of both cytotoxic and Th cells in vivo (reviewed in Ref. 6). The production of this critical cytokine is subject to multifactorial regulation at the level of chromatin remodeling, transcription, message stability, and possibly even translation (7–9). The transcript can be detected in naive T cells as early as 1 h after a strong antigenic stimulation in vivo and peaks at 4–6 h (9). The secreted protein is extremely short lived in the serum and therefore difficult to quantitate (10). However, maximal intracellular accumulation of the protein in vivo has been reported to take as long as 12–14 h (11) and even then in <50% of the cells. This implies a potential delay between the appearance of message and protein for this cytokine and is surprising because T cells have been shown to be capable of rapid secretion of other cytokines in vivo (12, 13). The discrepancy between transcription and translation could indicate another level of control during translation of IL-2 or a limitation in the detection of the protein. This makes it difficult, using existing techniques, to estimate the biologically relevant quantities of IL-2 locally available during an immune re-

sponse in vivo. The incomplete description of the dynamics of IL-2 availability is therefore a critical limitation in understanding models of T cell activation and differentiation in vivo.

The regulation of IL-2 expression at the level of TCR signaling and nuclear events have been extensively studied using in vitro model systems (5). The micro environmental conditions that influence T cell function in vivo are likely to be more complex than those observed under in vitro culture conditions (14). A reliable readout of IL-2 secretion in vivo is therefore required to study the cellular interactions that can regulate IL-2 synthesis as well as early T cell responses. To deal with this issue, we used a recently available cytokine “capture” assay to follow single cell IL-2 secretion in vivo under optimal stimulation conditions. This assay is more sensitive than a GFP knockin reporter assay and does not require restimulation of the T cell in vitro. Interestingly, we could detect maximal IL-2 secretion by naive Th cells within 5–6 h of in vivo stimulation. Memory T cells not only made more IL-2 per cell, but also responded faster, reaching peak production within 1–2 h. This rapid kinetics correlated very closely with the induction of the IL-2 message, demonstrating a tight synchrony between transcription, translation, and secretion of this protein. Surprisingly, the secretion of IL-2 was almost completely down-regulated within 14–16 h of reaching maximal production in contrast to what was observed in vitro. Using adoptive transfer strategies we demonstrated that these dynamics are strongly influenced by the density of Ag-specific T cells in the environment. This argues for a hitherto undescribed role for TCR-specific cellular competition in the regulation of early IL-2 production in vivo.

## Materials and Methods

### *Mice and cell preparations*

All the mice used in our experiments were obtained from the National Institute of Allergy and Infectious Diseases contract facility at Taconic Farms (Germantown, NY), an American Association for the Accreditation of Laboratory Animal Care accredited specific pathogen-free barrier and housed in sterile caging at the National Institutes of Health. Recombinant activating gene (Rag)<sup>2</sup> B10.A.5C.C7.Rag2<sup>-/-</sup> TCR transgenic mice (referred to as T-sufficient naive mice), specific for the moth cytochrome *c*

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<sup>2</sup> Abbreviations used in this paper: RAG, recombinant activating gene; SEA, staphylococcal enterotoxin A; MCC, moth cytochrome *c*; MFI, mean fluorescence intensity; RPA, RNase protection assay; 7-AAD, 7-aminoactinomycin D.

(MCC) 88–103 and pigeon cytochrome *c* 81–104 peptides, typically yielded 15–20 million naive CD4<sup>+</sup> T cells (from the lymph nodes and spleen combined). Memory T cells were generated by transferring 3–5 million lymph node cells from the B10.A.5C.C7.Rag2<sup>-/-</sup> mice into T-deficient (B10.A.CD3ε<sup>-/-</sup>) syngeneic animals followed by an i.p. challenge with 100 μg of pigeon cytochrome *c* 81–104 peptide (Bachem, Mount Olive, NJ) plus 10 μg of LPS (Sigma-Aldrich, St Louis, MO). The T cells expanded in this host and maintained 2–5 million CD44<sup>high</sup> memory T cells 48–100 days after the transfer. Such mice are referred to as “expanded memory T cell” hosts in this report. Memory T cells were purified away from B cells and other class II-positive host cells by negative selection. Briefly, cells were labeled with a mixture of Abs to I-E<sup>k</sup> (17.3.3S), B220 (RA3/6B2), CD11b (5C6), and CD16/32 (2.4G2) (all purified at the National Institute of Allergy and Infectious Diseases flow cytometry facility, National Institutes of Health) before binding to a mixture of anti-mouse and anti-rat dynabeads (DynaL Biotech, Lake Success, NY). Unbound cells typically contained 40–70% Vβ3<sup>+</sup> CD4<sup>+</sup> T cells. The IL-2-GFP knockin mouse generated by Naramura et al. (15) was received on a C57BL/6 (N11) Rag1<sup>-/-</sup> (N2) background. These mice were crossed twice onto the B10.A.5C.C7.Rag2<sup>-/-</sup> background before selecting for homozygosity of the 5C.C7 TCR transgene, the GFP allele, and the B10.A MHC. For our experiments, such B10.A.5C.C7<sup>+/+</sup>.Rag2<sup>-/-</sup>.IL-2-GFP<sup>+/+</sup> mice were crossed once more to the B10.A.5C.C7<sup>+/+</sup>.Rag2<sup>-/-</sup> transgenic mouse and used at F<sub>1</sub>. The IL-2 knockout mouse on the 5C.C7.TCR transgenic Rag2<sup>-/-</sup> background has been previously described (16). T deficient mice used as transfer recipients lacked either T cells (B10.A.CD3ε<sup>-/-</sup>) or both T and B cells (B10.A.Rag2<sup>-/-</sup>). The two hosts behaved similarly in the *in vivo* assays described in this study. B10.A mice with a normal repertoire of T and B cells are referred to as polyclonal recipients.

#### *In vivo stimulations*

Antigenic challenge *in vivo* was administered as 300 μg of MCC 88–103 peptide (AnaSpec, San Jose, CA) mixed with 10 μg of LPS in PBS and injected i.p. unless otherwise specified. T cells were recovered from the spleen and lymph nodes (inguinal, axillary, brachial, cervical, mesenteric, and periaortic). Staphylococcal enterotoxin A (SEA; Toxin Technology, Sarasota, FL) was also diluted in PBS, filter sterilized, and 1 μg injected i.p.

#### *RNase protection assay (RPA)*

RPA were performed with the mCK-1 multiprobe template set (BD PharMingen, San Diego, CA) essentially as described by the manufacturer using total RNA from lymph node or spleen cells. The radioactivity was detected using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantified by Gel-Pro Analyzer 3.1 software (Media Cybernetics, Silver Spring, MD). Each sample was normalized to the intensity of the signal from the housekeeping gene L32.

#### *IL-2 secretion assay and FACS analysis*

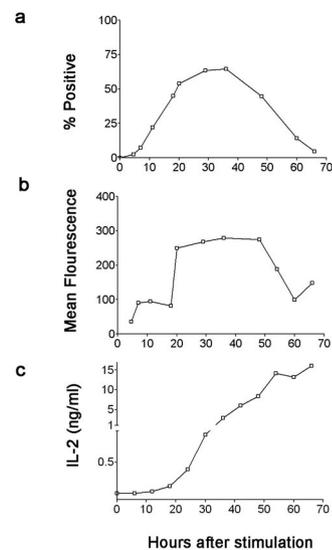
Isolated lymph nodes and spleen were crushed in PBS containing 10% FCS and the single cell suspension was washed to remove cell debris and fat. The secretion assay (Miltenyi Biotec, Auburn, CA) uses a bifunctional Ab capable of binding CD45 on one arm and IL-2 on the other arm. T cells isolated after *in vitro* or *in vivo* stimulation can bind the reagent in the cold via CD45. Subsequently the cell is warmed to 37°C to allow secretion of IL-2. The secreted IL-2 is captured on the cell surface and then detected with a second Ab coupled to a fluorochrome. Finally the cells are analyzed by flow cytometry. The assay does not require a secondary stimulation *in vitro* or incubation with golgi/endoplasmic reticulum disruption agents. Although this assay could reliably measure IL-2 secretion from as few as 2,500 T cells, we routinely used samples containing between 10,000 and 0.5 million cells for each experiment. The IL-2 PE cytokine secretion assay (Miltenyi Biotec) was performed according to the manufacturer's recommendations, except for the following modifications: 0.1 U/ml DNase1 was added during the capture step to reduce cell clumping, and FCS was used instead of mouse serum in some experiments (with no appreciable difference in the results). FACS analysis was performed using 7-aminoactinomycin D (7-AAD), and CD4-FITC (GK1.5; BD PharMingen) and Vβ3-allophycocyanin (clone KJ25; American Type Culture Collection, Manassas VA; purified and labeled from hybridoma supernatants by the National Institute of Allergy and Infectious Diseases flow cytometry facility) and analyzed on a FACSCalibur (BD Immunocytometry Systems, Mountain View, CA).

In the absence of significant published usage of the IL-2 secretion assay, we tested the reliability of this method by comparing it to the accumulated IL-2 measured using a standard sandwich ELISA (Quantikine-M IL-2 immunoassay kit; R&D Systems, Minneapolis, MN). A population of CFSE-

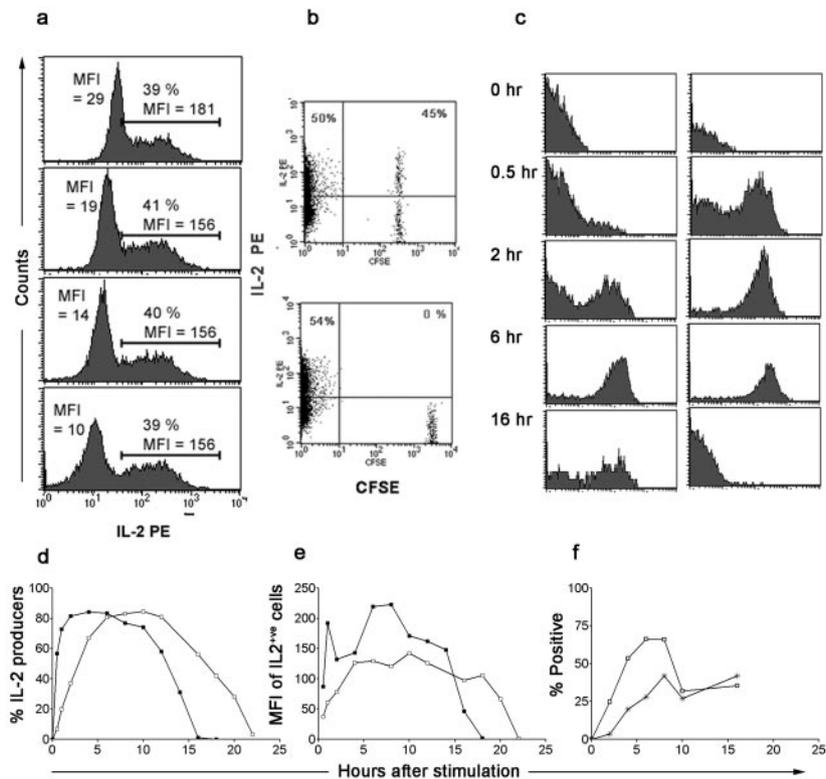
labeled naive 5C.C7 Rag2<sup>-/-</sup> T cells was stimulated with a 50-fold excess number of irradiated B10.A.CD3ε<sup>-/-</sup> splenocytes and 10 μM MCC 88–103 peptide *in vitro*. The secretion of IL-2 at various times after initiation of culture was quantitated in terms of the number of live, CFSE<sup>+</sup> IL-2 producers (Fig. 1*a*) and the per cell production (measured by the mean fluorescence intensity, MFI) of the IL-2-producing population (Fig. 1*b*). The IL-2 in the culture supernatant was measured by ELISA (Fig. 1*c*).

Approximately 25% of the naive T cells initiated secretion within the first 12 h of stimulation (Fig. 1*a*). The maximum number of secretors (~60%) was recruited between 30 and 40 h after initiation of culture. Beyond 42 h, there was a decrease in the number of producers and no significant production was detected after 60 h. The per-cell production was low between 8 and 18 h (Fig. 1*b*), and then increased sharply afterward to stabilize at a 3-fold higher level for up to 48 h. Beyond this time, the MFI slowly declined. The accumulation of IL-2 as measured by ELISA (Fig. 1*c*) steadily increased until 54 h and then plateaued. The initial increase in IL-2 in the supernatants between 12 and 30 h (Fig. 1*c*) can be accounted for by the increased recruitment of secretors between 8 and 30 h (Fig. 1*a*) at the rate of ~2.3% per hour. Subsequently, IL-2 in the supernatant continues to increase (a further 15-fold) until 54 h. This is likely due to continued secretion by already recruited T cells because neither the number of secretors nor the MFI changes significantly during this time. At 54 h, the accumulation reaches a plateau. This correlates well with the decrease in both the number (Fig. 1*a*) and MFI (Fig. 1*b*) of IL-2 secretion starting at around 48 h. The capture assay therefore reliably correlates with the standard ELISA measurements and is a sensitive single cell readout of IL-2 secretion.

A caveat that needs to be addressed using the capture assay is the potential for *trans*-capture whereby cells bearing the bifunctional Ab could capture IL-2 made by close neighbors without making IL-2 themselves. In the presence of large numbers of IL-2-producing cells, we did often obtain a greater background in this assay, consistent with such a *trans*-capture phenomenon (Fig. 2*a*, top panel; assay performed with 3 million T cells shows an enhancement of the MFI in the IL-2-negative population). We addressed this issue by reducing the number of cells used in the assay and diluting the secretion medium. Reduction of the input cell number by 8-fold (Fig. 2*a*, bottom panel; 0.38 million input T cells) reduced the shift due to *trans*-capture without significantly affecting the MFI of the IL-2 producing population. We also confirmed that under our conditions, there was no significant *trans*-capture effect, by mixing in T cells deficient in the IL-2 gene and showing that they did not score as positives in the assay (Fig. 2*b*). The secretion of IL-2 after a strong *in vivo* stimulation could not be



**FIGURE 1.** *In vitro* secretion and accumulation of IL-2. *a*, A total of 100,000 naive T cells were stimulated with 10 μM MCC and 5 × 10<sup>6</sup> syngeneic APC in 24-well plates for different periods of time before measuring the percentage of live T cells positive for IL-2 secretion. Data points are pooled from two separate experiments. *b*, MFI of IL-2 secretion at the same time points of sampling. *c*, A total of 10,000 naive T cells were stimulated with 10 μM MCC and 5 × 10<sup>5</sup> syngeneic APC in 96-well plates. Supernatants from cultures were assayed for total IL-2 production (□) at each time point using an IL-2 ELISA.



**FIGURE 2.** Kinetics of IL-2 secretion by naive and memory T cells. *a*, Increased cell numbers resulted in significant increase in the background staining of IL-2 nonproducers with the detection Ab (top). Naive 5C.C7 TCR transgenic mice were stimulated in vivo with 300  $\mu$ g of MCC plus 10  $\mu$ g of LPS. The secretion assay was set up ex vivo with 3 million (top), 1.5 million (second), 0.75 million (third), or 0.375 million (bottom) T cells. Numbers to the right of the histogram indicate the MFI of the IL-2 secretors (and the percentage of secretors). The MFIs to the left of the histogram indicate the staining intensity of the IL-2 nonproducers. *b*, Under assay conditions using 0.1 million T cells, the *trans*-capture effect does not influence the labeling of IL-2 nonproducers. A representative assay on naive T cells stimulated in vivo for 6 h with an i.p. injection of 300  $\mu$ g of MCC plus 10  $\mu$ g of LPS is shown. CFSE-labeled 5C.C7 TCR transgenic T cells (top) or 5C.C7 TCR transgenic IL-2 knockout T cells (bottom) were transferred into 5C.C7 TCR transgenic hosts. The production of IL-2 by the resident T cells (upper left quadrant) does not influence the labeling of IL-2 nonproducers (upper right quadrant, top). Numbers in quadrants indicate the percentage of the CFSE<sup>+</sup> (right quadrant) or CFSE<sup>-</sup> (left quadrant) cells that secrete IL-2. *c*, Histogram profiles of IL-2 labeling in the TCR<sup>+</sup> CFSE<sup>+</sup> 7-AAD<sup>-</sup> population from representative time points. Naive T cells (left) and memory T cells (right) recovered from lymph nodes and spleen. *d*, Percentage of IL-2-positive cells in naive ( $\square$ ) or memory ( $\blacksquare$ ) T cells derived from the plots in *c* at different time points after in vivo stimulation with 300  $\mu$ g of MCC plus LPS. A representative experiment of three is shown. *e*, MFI of the IL-2<sup>+</sup> population from *c*. *f*, Percentage of IL-2-GFP wild-type IL-2 heterozygous naive T cells secreting IL-2 ( $\square$ ) or expressing GFP ( $\blacksquare$ ) at different times after stimulation.

blocked by actinomycin D or cycloheximide addition during the ex vivo incubation steps (data not shown). Although brefeldin and monensin addition during this step affected the per cell production (MFI), the number of secretors were unaffected by these drugs as well. These results suggest that the assay is detecting the exocytosis of proteins already synthesized in vivo. In further support of this possibility is our observation that maximal ex vivo secretion of IL-2 did not significantly change with 20 min to 4 h of incubation during the ex vivo secretion step (data not shown).

## Results

### Kinetics of IL-2 secretion in vivo

The maximal production of IL-2 in vitro is critically dependent on the robustness of the stimulation condition (17). We therefore engineered an in vivo model to optimally stimulate a small number of Ag-specific naive or memory T cells in vivo, by adoptively transferring 0.5–1 million T cells into a lymphopenic host. Typically 0.03–0.1 million T cells seeded the peripheral lymphoid organs of the recipients within 2 to 6 h of this transfer. One day after transfer, the recipients were challenged with Ag and T cells from the lymph nodes and spleen isolated at various times afterward. The percentage of IL-2 secretors (Fig. 2*d*) and the average per cell production (MFI) of the IL-2<sup>+</sup> population (Fig. 2*e*) were calculated from the histogram profiles (Fig. 2*c*).

Of the transferred naive T cells, ~20% could be seen secreting IL-2 within an hour of this strong in vivo stimulation (Fig. 2*d*,

open square). Over the next 6 h, the number increased to ~80%. This level of IL-2 secretion was maintained for another 4–5 h and then declined over the next 10 h such that secretion was barely detectable at 22 h after stimulation. In the case of transferred memory T cells, the kinetics of IL-2 induction was more rapid (Fig. 2*d*,  $\blacksquare$ ). Over 55% of the memory T cells secreted IL-2 within 30 min of stimulation and ~85% had gained this ability by 2 h. Despite the rapid onset, the overall kinetic pattern of IL-2 secretion between memory and naive T cells was similar, with a plateau phase of 5–6 h and down-regulation over the next 9–10 h. In addition to the rapid induction, the per-cell secretion of IL-2 was consistently higher in the memory population and showed about a 2-fold increase over naive T cells during maximal secretion (Fig. 2*e*). For three different experiments, 80% ( $\pm 1.7$ ) of naive T cells secreted IL-2 at the 6 h time point poststimulation whereas 87% ( $\pm 8$ ) of the memory T cells secreted IL-2 at the 2 h time point. Interestingly, we always observed a small percentage (7–20%) of T cells of either phenotype, in which IL-2 secretion could not be detected. This was not due to a failure of these cells to encounter Ag because 100% of the T cells had uniformly up-regulated CD69 within 8 h of stimulation (data not shown).

The rapid kinetics and high frequency of IL-2 expression was surprising because earlier studies, following the expression of GFP

placed downstream of the IL-2 promoter or by intracellular staining for IL-2, had suggested that expression of IL-2 is restricted to a small subset of T cells during activation *in vivo* (15, 18, 19). To test whether these observations stemmed from a lower sensitivity of the GFP system and/or a stronger challenge regimen used in our protocol, we performed similar experiments using 5C.C7,Rag2<sup>-/-</sup> T cells from an IL-2/GFP heterozygous mouse, as described in *Materials and Methods*. The number of IL-2 secretors (Fig. 2*f*, □) and GFP<sup>+</sup> T cells (Fig. 2*f*, \*) are plotted. The induction of GFP expression (detectable by FACS) took 2–3 h longer to observe than IL-2 secretion. Furthermore, at the maximum levels, GFP expression was evident in only ~40% of the cells (by 8 h) compared with 66% measured by the secretion assay (at 6 h). These data demonstrate that the GFP readout is less sensitive than the secretion assay for detecting IL-2 synthesized by Th cells *in vivo*. Because the GFP molecules have a half-life of 18–24 h, the accumulation also makes it difficult to follow the loss of the secretor phenotype using that marker. Finally, the reduced number of IL-2 secretors in the IL-2/GFP heterozygous cells (<70% at 6 h) compared with cells with two copies of the IL-2 gene (≥80%, Fig. 2*d*) might be a consequence of competition with the GFP allele.

#### T cell density influences the extent of IL-2 production

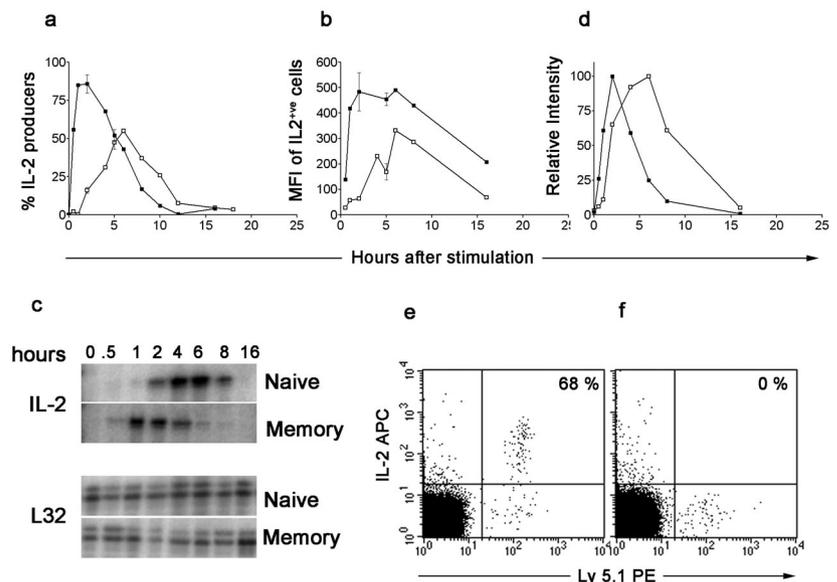
The adoptive transfer of T cells into a lymphopenic environment allows for a large number of resident APCs to pick up the injected Ag and present it to the few injected T cells. Lymphopenia influences the responsiveness of T cells, even leading to proliferation in the absence of cognate Ag (20). This could also impact on the initial activation and the IL-2 production kinetics of the T cells transferred into a T cell-deficient environment. We therefore examined the kinetics of IL-2 production in a T-sufficient host, the naive 5C.C7 TCR transgenic mouse, by directly challenging them with 300 μg of MCC plus 10 μg of LPS. Typically these mice have 15–20 million T cells at the time of challenge. As shown in Fig. 3*a*, IL-2 secretion was again maximally induced within 6 h despite the presence of a 100-fold larger number of T cells. Even at this dose of Ag, however, ~30% fewer naive T cells in the T-sufficient mice secreted IL-2 at the peak time point (49 ± 6%, *n* = 3) compared with the population transferred into a T-deficient environment. In addition, the onset of detectable IL-2 secretion was delayed by 1–2 h. We also examined the response of T cells in the expanded memory T cell hosts, generated as described in

*Materials and Methods* (Fig. 3, *a* and *b*). These T cells maximally secreted IL-2 (82 ± 9%, *n* = 3) within 1–2 h similar to the ones in the T-deficient host, despite the presence of a 30-fold larger number of T cells. The expanded memory T cells similarly made about twice as much IL-2 per cell (Fig. 3*b*) compared with naive T cells.

The major effect observed in both hosts from increasing the T cell density was a truncation in the plateau of the response. The down-regulation of IL-2 in the T-sufficient naive host began immediately after the 6 h maximum and secretion was over by 7–8 h after that point. A similar tendency was observed in the expanded memory T cell hosts with the number of secretors decreasing within an hour of reaching the maximum value. Secretion was again lost 8–9 h later. Therefore, the presence of a greater number of T cells in the environment had a profound effect in reducing the duration of maximal IL-2 secretion from 4 to 6 h to <2 h in both memory and naive T cells. The memory T cells, however, showed slightly more resistance to this effect because they showed no change in the onset and magnitude of the initial response. This could be because the absolute number of T cells (2–5 million) in the expanded memory host is still considerably less than that in the intact naive TCR transgenic (15–20 million).

Considering the possibility that the rapid kinetics of IL-2 secretion observed in memory T cells was a consequence of the method of memory T cell generation we used (Ag challenge in a lymphopenic environment following by extended rest in the same host), we also tested IL-2 secretion by memory T cells generated in a conventional fashion. TCR transgenic 5C.C7 T cells marked by the Ly5.1 congenic marker were transferred into a polyclonal B10.A,Ly5.2<sup>+</sup> host. A day after the transfer the mice were challenged with 100 μg of MCC plus 10 μg of LPS *ip*. Three months after the challenge a small proportion of Ly5.1<sup>+</sup>,5C.C7 T cells persisted in this host and displayed memory markers (CD44<sup>high</sup>, FSC<sup>low</sup>, CD69<sup>-</sup>). We challenged such mice with either MCC plus LPS (Fig. 3*e*) or an irrelevant peptide plus LPS (Fig. 3*f*) and sacrificed them after 1 h or 16 h to assay IL-2 secretion. Over 68% of the resident memory T cells secreted IL-2 at the early time point in an Ag-specific manner. No IL-2 was detected at 16 h (data not shown). These results confirm the rapid onset and termination of IL-2 secretion by CD4<sup>+</sup> memory T cells. This experiment also suggests that the secretion assay might prove to be a valuable tool to quantify Ag-specific memory CD4<sup>+</sup> T cells *in vivo*, similar to the use of tetramers and IFN-γ staining for CD8<sup>+</sup> T cells.

**FIGURE 3.** Density influences the frequency as well as duration of IL-2 secretion. *a*, Frequency of IL-2-secreting T cells after *in vivo* administration of 300 μg of MCC plus 10 μg of LPS to T-sufficient naive (□) or expanded memory (■) hosts. Data shown are pooled from two separate experiments, with overlaps at 2 and 5 h. *b*, MFI of the IL-2 signal on T cells in the IL-2-positive gate of naive (□) and memory (■) cells from *a*. *c*, RPA analysis of total RNA from activated naive and memory T cells stimulated *in vivo* for periods of time (in hours) specified as shown. *d*, Quantitation of IL-2 mRNA relative to the L32 transcript from naive (□) or memory (■) T cells for the blot in *c*. *e* and *f*, The secretion assay can detect IL-2 secretion by 5C.C7 memory T cells generated in a polyclonal B10.A environment within an hour of MCC administration (*e*) and not HY administration (*f*). Dot plots are gated on CD4<sup>+</sup>7-AAD<sup>-</sup> cells.



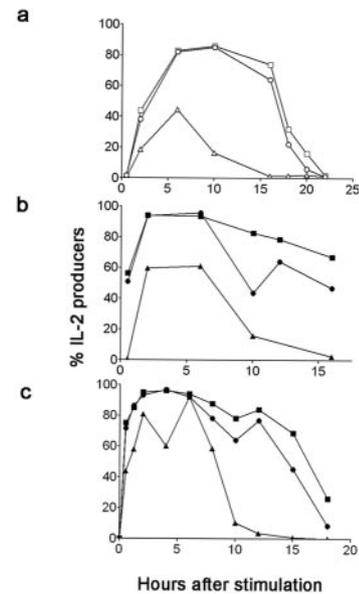
The availability of larger numbers of T cells in our T-sufficient and expanded memory T cell hosts allowed us to examine how the secretion kinetics of memory and naive T cells correlate with steady state mRNA levels measured by RPA (Fig. 3, *c* and *d*). After normalization to L32 levels, the results show that naive T cells transcribed very little IL-2 mRNA for up to 1 h, but this rapidly increased at 2 h and reached maximal expression in 4–6 h. In agreement with the secretion data, IL-2 mRNA levels dramatically decreased after 6 h and were not detectable after 16 h. Memory T cells responded more quickly, producing detectable amounts of IL-2 mRNA as early as 30 min after stimulation. This response peaked by 2 h and then also rapidly declined. Thus, the pattern of RNA expression very closely mirrored the IL-2 secretion profile (Fig. 3, *a* and *b*) arguing for a tight correlation between the appearance of the transcript and secretion of the protein.

*TCR-specific competition and not T cell density per se, regulates the dynamics of early T cell activation*

The increased potency of Ag presentation during lymphopenia could be a result of alterations in APC function or a specific consequence of T cell interactions. The latter has been reported to operate largely through intraclonal competition (21) likely for the surface of APCs or for Ag-specific MHC molecules on the APC (22, 23). We tested the TCR specificity of the interactions we observed by adoptively transferring equal numbers of CFSE-labeled naive or memory T cells into either a T-deficient mouse, a T-sufficient naive TCR transgenic mouse (15–20 million Ag specific naive T cells) or a B10.A mouse with a polyclonal repertoire of T cells (20–25 million CD4<sup>+</sup> T cells of which <1/1000 are likely to be MCC specific). If the differences we observed derived from alterations in the APC, due to the absence of any T cells in the deficient environment, then the enhanced stimulation should not be evident in the B10.A (polyclonal) mouse. In addition, the lack of significant numbers of Ag-specific T cells in the polyclonal recipient should test whether the diminished response in the sufficient host is a result of TCR-specific competition.

Similar to the challenge of a T-sufficient naive mouse (Fig. 3), ~48% of the naive T cells adoptively transferred into a T-sufficient environment secreted IL-2 within 6 h of stimulation (Fig. 4*a*,  $\Delta$ ). There was also no apparent plateau phase and secretion had declined to <3% of the cells by 16 h. By contrast, in a lymphopenic environment (Fig. 4*a*,  $\square$ ) the number of secretors increased to 78% by 6 h and only a small decrease was observed out to 16 h. IL-2 production declined sharply after that and was not detectable after 22 h. Most importantly, naive 5C.C7 T cells transferred into the T-sufficient polyclonal environment mirrored the kinetics of the lymphopenic recipients, with 80% production at 6 h. There was also little or no decrease in producers in this host at 16 h and production was over by 22 h. This extended duration of secretion, despite the presence of high numbers of polyclonal T cells, allows us to conclude that the high density cellular interactions that restrict the duration of IL-2 secretion in vivo are TCR specific.

These conclusions were further confirmed by the response of memory T cells (Fig. 4, *b* and *c*, shown as two independent experiments). The presence of polyclonal T cells did not impact on the early phase of IL-2 production: 95% of the cells expressed IL-2 by 2 h in both the T-deficient (Fig. 4, *b* and *c*,  $\blacksquare$ ) and the polyclonal (Fig. 4, *b* and *c*,  $\bullet$ ) environments. In contrast, the presence of a large number of naive Ag-specific T cells (Fig. 4, *b* and *c*,  $\blacktriangle$ ) reduced the number of IL-2 secretors in the memory population, at all time points. Half an hour after stimulation, 44% ( $\pm 14$ ;  $n = 3$ ) fewer cells expressed IL-2 in the presence of competing TCR transgenic cells (Fig. 4, *b* and *c*,  $\blacktriangle$ ). The maximal percentage of secretors in these recipients (between 2 and 6 h) was also reduced



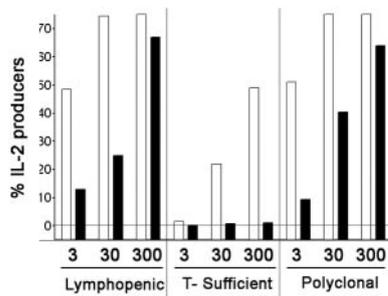
**FIGURE 4.** TCR-specific competition modulates IL-2 secretion in vivo. *a*, One million CFSE-labeled naive T cells adoptively transferred into a T-deficient ( $\square$ ), T-sufficient ( $\Delta$ ), or polyclonal ( $\circ$ ) host were challenged with 300  $\mu\text{g}$  of MCC plus 10  $\mu\text{g}$  of LPS and the IL-2 secretion measured at different times afterward. One of two experiments is shown. *b* and *c*, Two independent experiments showing a profile of IL-2 secretion by 1 million memory T cells adoptively transferred into T-deficient ( $\blacksquare$ ), T-sufficient ( $\blacktriangle$ ), or polyclonal ( $\bullet$ ) host and challenged as shown in *a*.

relative to the polyclonal and T-deficient hosts. The plateau was consistently terminated after 6 h in all hosts, but the rate of decline of secretors was more rapid in the presence of competing TCR transgenic naive T cells. As in the case of naive responding T cells, the presence of polyclonal T cells had only a minor influence on the rate of decline of IL-2 production.

*Lower density of Ag-specific T cells enhances the effective strength of Ag stimulation*

The forces operating in the negative regulation of cytokine production in vivo are poorly understood. The down-regulation of IL-2 after a transient period of secretion could now be exploited to study this process in vivo. The difference in the duration of the plateaus that we observed in the lymphopenic and T-sufficient hosts is unlikely to be related to cell death or cell migration as we could not detect any consistent decrease in cell yields over this time. The most obvious variable distinguishing the two environments is the number of T cells. Because the number of APCs remains relatively constant, the ratio of APCs to T cells would be higher in the lymphopenic environment. This could allow a greater percentage of the T cell population in the lymphopenic host to be stimulated to make IL-2 (e.g., by concentrating more available MHC molecules at the point of TCR engagement, Ref. 24). Thus, the increased number of IL-2 producers in the lymphopenic environment could reflect the response to a greater effective stimulation. If the duration of secretion was also dependent on the strength of signaling, we should be able to mimic the response observed in the T-sufficient environment by reducing the Ag-challenge dose in the lymphopenic environment.

We tested this idea by adoptively transferring naive T cells into the three environments as described in Fig. 4. The transferred cells were tested for their ability to secrete IL-2 at 6 and 16 h in response to 300, 30, and 3  $\mu\text{g}$  challenge doses (Fig. 5). The 300  $\mu\text{g}$



**FIGURE 5.** Ag dose response in the presence or absence of competition. One million CFSE-labeled 5C.C7 TCR transgenic T cells were transferred into environments devoid of T cells (lymphopenic), filled with mono-specific T cells (T-sufficient), or filled with polyclonal T cells (polyclonal). Secretion of IL-2 at 6 h (□) and 16 h (■) are plotted, after stimulation with different doses of Ag in the lymphopenic host (left bars), in the T-sufficient host (middle bars), or polyclonal host (right bars).

dose used in this study induced 77% IL-2 producers in the lymphopenic environment at 6 h (Fig. 5, □) and only a 10% down-regulation was evident at 16 h (Fig. 5, ■). In the T-sufficient host, however (Fig. 5, middle), a similar dose induced only 49% producers and these cells completely lost production by 16 h. A similar profile to that of the T-sufficient host could be observed in the lymphopenic host challenged with 3 μg of MCC (48% at 6 h and significant down-regulation by 16 h). Interestingly the polyclonal environment (Fig. 5, right) mirrored the kinetics in the lymphopenic environment at all doses of Ag, confirming that the competition is Ag specific.

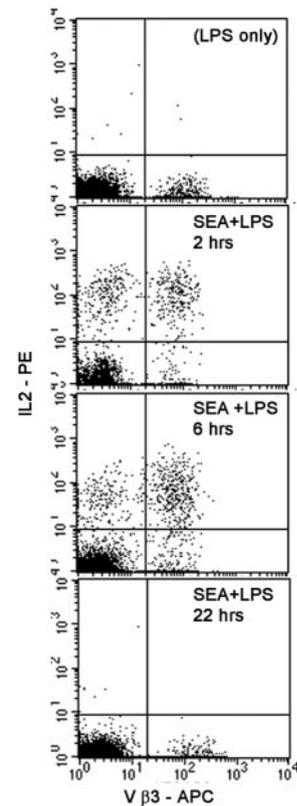
These data suggest that not only the frequency of IL-2 producers, but also the duration of their production is dependent on the dose of the antigenic stimulus. Furthermore, lowering the density of Ag-specific T cells by ~100-fold results in about a 100-fold increase in the effective potency of Ag presentation, at least for the production of IL-2.

#### *In vivo IL-2 secretion by a polyclonal T cell population*

We have used a TCR transgenic system to demonstrate the rapid and transient kinetics of IL-2 secretion in CD4<sup>+</sup> T cells in vivo. To rule out the possibility that this profile reflects a distortion due to the use of a monoclonal population with a high affinity receptor, we confirmed the overall pattern we have observed, by using a polyclonal responding population. Endogenous T cells in the B10.A mouse were challenged with the superantigen SEA and IL-2 secretion analyzed after gating on the live CD4<sup>+</sup> population at key time points (Fig. 6). ~9.5% of the CD4<sup>+</sup> T cells in this host were able to secrete IL-2 as early as 2 h after this stimulation. This included 65% of the CD4<sup>+</sup>Vβ3<sup>+</sup> T cells, which are the dominant SEA reactive T cells in this mouse. The percentage of total secretors decreased to ~5% by 6 h and, again, ~60% of the CD4<sup>+</sup>Vβ3<sup>+</sup> T cells continued to secrete IL-2. No IL-2 secretion was detected 22 h after the stimulus in this host, confirming the rapid up-regulation and transient persistence of IL-2 expression in vivo in a polyclonal repertoire of responders.

## Discussion

The production of IL-2 by CD4<sup>+</sup> T cells is a cellular reporter for the successful integration of multiple intracellular signaling pathways downstream of the TCR, followed by the remodeling of chromatin and transcription (5). This sensitive measure of T cells earliest commitment to proliferate and differentiate has been extensively used in vitro but has been difficult to exploit in vivo for the lack of robust readouts. In these experiments, we have used a



**FIGURE 6.** Polyclonal stimulation of T cells with a superantigen. B10.A mice were stimulated with an i.p. injection of 1 μg of SEA plus 10 μg of LPS (SEA+LPS) for various times in vivo. Secretion of IL-2 from CD4<sup>+</sup>7-AAD<sup>-</sup> T cells is shown.

sensitive cytokine capture assay to quantitatively follow IL-2 secretion at a single cell level and dissect some of the critical cellular interactions that can potentially regulate early T cell activation events in vivo. This assay is a direct readout of what the cells were secreting in vivo, because it does not require restimulation of the T cells. In addition it appears to be more sensitive and physiologic than a GFP knockin reporter assay.

Comparing the capture assay to the measurement of IL-2 in culture supernatants by ELISA (Fig. 1) allowed us to correlate the single cell behavior that results in the accumulation of IL-2 in vitro. Under our stimulation conditions, IL-2 secretion by naive T cells was observed within 12 h after initiation of culture but maximal production took as long as 36 h and continued until ~48 h. Interestingly, the in vivo stimulation of a similar population of naive T cells resulted in observable IL-2 capture as early as 2 h and maximal secretion occurred within 6 h (Fig. 2). This extremely rapid onset of IL-2 secretion also correlated very tightly with the steady state levels of mRNA detected by RPA (Fig. 3). This suggests that a robust program of mRNA synthesis, translation, and protein transport exists even in naive T cells to ensure a coordinated processing of this gene soon after Ag recognition. The sluggishness of the in vitro secretory response implies that the activation of T cells in vitro is either not homogenous or is missing critical environmental variables that are only available in vivo. In either case, this difference calls for a re-examination, using in vivo rather than in vitro model systems, of the molecular processes leading to IL-2 secretion in CD4<sup>+</sup> T cells.

The IL-2 secretion induced in naive T cells in vivo was also surprisingly transient, lasting ~6–10 h after maximal up-regulation in a T-deficient or polyclonal host. Subsequently, the secretion

was rapidly down-regulated and 24 h after Ag administration we could not detect any IL-2 being secreted (Figs. 2 and 4) or transcribed (Fig. 3) by these cells. This transient secretion was also in contrast to the extended (>40 h) duration of secretion observed *in vitro*. Together with the short half-life of IL-2 *in vivo* (10), the transience of IL-2 secretion raises interesting questions regarding the role of IL-2 *in vivo*. The production of IL-2 has been argued to play roles in the augmentation of clonal expansion, contraction (death), and differentiation (25–27) of T cells following activation. The initial recruitment of T cells into proliferation takes as long as 30 h (28) and some of the subsequent differentiation events take several days, requiring multiple rounds of proliferation (29). Therefore, the effect of IL-2 (if any) on these events would have to reflect a cellular memory of early IL-2 exposure or the restimulation of IL-2 production during the actual differentiation process. A memory function could potentially be manifested by altered intracellular biochemical intermediates or changes in chromatin structure.

Not very surprisingly, the lag time to secretion was further shortened in memory T cells, in which secretion could be detected as early as 30 min after stimulation and reached maximal secretion within 1–2 h (Fig. 2). At the maximal time point, we could observe up to 95% of these cells secreting IL-2. The remaining cohort of T cells that does not secrete IL-2 even after a strong stimulus could reflect an intrinsic refractoriness in a small population of T cells *in vivo* (18). However, it can be argued that this is merely a consequence of the asynchrony in the expression and down-regulation kinetics of IL-2. The profile of memory T cell secretion also confirms the transient secretion observed for naive T cells *in vivo*, which last <20 h.

The cellular and molecular parameters regulating the transient expression of IL-2 are poorly understood. It was therefore intriguing that the duration of secretion was greatly reduced in an intact TCR transgenic host relative to T cells stimulated after adoptive transfer into a T-deficient environment, even at high Ag concentration. To control for potential artifacts stemming from adoptive transfer, we transferred CFSE-labeled naive T cells into the T-sufficient host. The behavior of these cells (Fig. 4) mimics the kinetics of the T-sufficient environment (Fig. 3) and not that of adoptive transfer into the T-deficient environment (Fig. 2). This argues for competitive phenomena between T cells as the major influence on the secretion dynamics.

The competition we observed could be attributed to a physical competition for space (due to the presence of a larger number of T cells) including APC surface, a competition for limiting amounts of cytokines such as IL-7, or for specific Ag-MHC complexes. We distinguished the latter from other forms of competition by transferring labeled TCR transgenic T cells into a syngeneic mouse bearing a polyclonal repertoire of T cells (not on a RAG2<sup>-/-</sup> or TCR transgenic background). Despite the presence of 20 million CD4<sup>+</sup> T cells in this mouse, the secretion kinetics of IL-2 by the transferred T cells closely resembled that of the T-deficient host, arguing for TCR specificity of the early competition process. TCR-specific competition for positively selecting ligands has been previously implicated in the “homeostatic” proliferation of naive T cells transferred into lymphopenic hosts (21, 30). Our study suggests that similar rules apply for early T cell activation events during a strong *in vivo* Ag-driven response. The short assay duration (cytokine secretion for 16 h as opposed to proliferation after 7–21 days) should allow us to exploit this system to decipher more efficiently the parameters that regulate these events.

Competition between CD8<sup>+</sup> T cells in other experimental systems is thought to operate through the down-regulation of peptide-

MHC complexes on APCs (31) or the physical interference against sufficient duration of residence on the APC (32). The exact mechanism operating on CD4<sup>+</sup> T cells in our model system is not clear. Still, competition in our system has two consequences: 1) to reduce the number of IL-2 secretors at the maximal time point (6 h for naive T cells) and 2) to reduce the subsequent duration of IL-2 secretion by the stimulated T cells. As discussed earlier, the reduction in the number of producers at 6 h does not reflect a failure to activate these T cells because 100% of the T cells in our system display homogeneous levels of CD69 within 8 h of activation (data not shown). IL-2 production is therefore likely to operate at a different threshold and is more sensitive to the effects of competition. In exploring homeostatic proliferation of CD8<sup>+</sup> T cells (30), it has been suggested that competition lowers the strength of stimulus available to the T cells. A similar mechanism operating in the early phase of *in vivo* stimulation can explain the lower percentage of initial secretors. The lower percentage of T cells that do make IL-2 in the intact host at 6 h, however, have clearly “won” the competition at this time. It is therefore surprising that they do not continue their secretion for a further 10 h as can be seen in the T-deficient host (Fig. 4). This observation is still consistent with competition impacting on IL-2 secretion by modulating the strength of stimulus if either 1) the lowering of the acute (initial) stimulus programmed a reduced duration of the subsequent secretion, or 2) the period of secretion was dependent on the duration of continued Ag engagement in the system. In the latter scenario, competition can either accelerate clearance of Ag from the system (31) or prevent re-engagement of T cells with APC (32). Both of these would prevent the continuation of TCR driven signals. *In vitro* experiments have previously argued that the termination of TCR signals results in the termination of cytokine secretion (33).

If the duration of IL-2 secretion could be “programmed” by the initial strength of the antigenic stimulus itself, then this would be evident even in the subsequent absence of competition. In this model, a strong initial stimulus (before 6 h) not only elicits maximal production by 6 h but also allows for extended production by each responding cell after that time. This could be understood as the consequence of an overshoot in the synthesis of transcription factors required for IL-2 production or the more complete removal of negative regulators. A weaker stimulus, stemming from competition, might only succeed in eliciting enough of these factors to induce IL-2 production, but not to sustain it. We tested the ability of reduced strength of antigenic stimulation to restrict the duration of IL-2 secretion by challenging T cells, in the absence of competition, with different doses of Ag. A 100-fold reduction in the antigenic stimulus yielded almost the same diminished frequency of IL-2 producers as in the intact TCR transgenic host. There were fewer cells making IL-2 at 6 h and more importantly these had stopped production by 16 h (Fig. 5), arguing for a role of stimulation strength in maintaining the duration of secretion. The result with the intermediate (30  $\mu$ g) dose supports this notion as well. At this dose a large percentage of cells were recruited to make IL-2 at 6 h; yet the cells lost production earlier than what was observed at the higher Ag dose (300  $\mu$ g). The response of the memory T cells (to 300  $\mu$ g) produced a similar (“30  $\mu$ g-like”) pattern when faced with competition in the expanded memory host (Fig. 3a). These data, however, do not eliminate the possibility that continued stimulation also contributes to the duration of secretion. Irrespective of the extent of operation of either mechanism, our data demonstrate the ability of interclonal competition to reduce cytokine secretion during early T cell activation, by lowering the effective strength or duration of stimulation in the system.

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