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J Immunol 2003; 171:964-970; doi: 10.4049/jimmunol.171.2.964
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Early Self-Regulatory Mechanisms Control the Magnitude of CD8$^+$ T Cell Responses Against Liver Stages of Murine Malaria

Julius C. R. Hafalla,* Alexandre Morrot, Gen-ichiro Sano,* Genevieve Milon,† Juan J. Lafaille,‡ and Fidel Zavala*‡

Following immunization with Plasmodium yoelii sporozoites, the CD8$^+$ T cell population specific for the SYVPSAEKIQ epitope expressed in sporozoite and liver stages of this malaria parasite revealed the existence of a short term Ag presentation process that translated into a single clonal burst. Further expansion of this CD8$^+$ T cell population in conditions of sustained Ag exposure and additional supply of naive cells was inhibited by regulatory mechanisms that were developed as early as 24–48 h after priming. Studies using mouse models for Plasmodium or influenza virus infections revealed that this mechanism is Ag specific and is mediated by activated CD8$^+$ T cells that inhibit the priming of naive cells. This interference of the priming of naive cells appeared to result from limited access to Ag-presenting dendritic cells, which become disabled or are eliminated after contact with activated cells. Thus, concomitantly with the development of their effector antimicrobial capacity, CD8$^+$ T cells also acquire a self-regulatory role that is likely to represent one of the earliest mechanisms induced in the course of an immune response and that limits the magnitude of the early expansion of CD8$^+$ T lymphocytes reactive to microorganisms. The Journal of Immunology, 2003, 171: 964–970.

The Journal of Immunology

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0022-1767/03/$02.00

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3 Abbreviations used in this paper: Tg, transgenic; DC, dendritic cell.

Received for publication March 31, 2003. Accepted for publication May 16, 2003.

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they may also play a key physiological role by preventing a mas-
sive T cell activation that could overwhelm the immune system
and produce deleterious effects in secondary lymphoid organs and
nonlymphoid tissues infected by microorganisms.

In this report we describe studies using mouse models of infec-
tions by *Plasmodium* and influenza virus that are aimed at char-
acterizing the mechanisms regulating the in vivo expansion of
CD8\(^+\) T cell populations and discuss evidence indicating that this
is a self-regulatory mechanism mediated by activated CD8\(^+\) T
cells.

**Materials and Methods**

**Mice**

Five- to 8-wk old BALB/c (Thy1.2\(^+\)) or CB6F\(_1\) mice were obtained from
the National Cancer Institute (Frederick, MD). The generation of TCR Tg
mice specific for the H2K\(^d\)-restricted epitope SYVPSAEK1 of the circum-
sporozoite protein of *Plasmodium yoelii*, or malTg, has been described
previously (7). Mice were maintained in the BALB/c (Thy1.2
phocytes for 20
24 h. Anti-mouse IFN-

**Parasites and viruses**

*P. yoelii* (17X NL strain) parasites were maintained, and sporozoites were
dissected as previously described (16). Immunizations were performed by i.v.
injection of 3–4 \(\times\) 10\(^7\) radiation-attenuated (gamma source, 20 krad)
parasites or as otherwise specified in the figure legends. Wild-type influen-
za virus hemagglutinin protein (flH1N1) in BALB/c (Thy1.2\(^+\)) backgrounds were
obtained from Dr. L. Sherman (The Scripps Institute, La Jolla, CA). Unless
otherwise specified, the majority of the experiments were performed using
the BALB/c (Thy1.2\(^+\)) background. Normal BALB/c mice expressing
Thyl.1 Ag were obtained from Dr. H. Shen (University of Pennsylvania,
Philadelphia, PA).

**Flow cytometric analysis**

SYVPSAEK1-specific H2K\(^d\) tetramers (subsequently designated Tetrramer)
were either obtained from the National Institute of Allergy and Infectious
Diseases, National Institutes of Health, Tetramer Facility or prepared as
previously described (3, 19). In the absence of a reliable tetramer system to
detect activated flH1Tg CD8\(^+\) T cells, Thy1.2\(^+\) flH1Tg CD8\(^+\) T cells were
used for adoptive transfer into normal Thy1.1\(^+\) mice. These cells were
detected by gating on CD8\(^+\) Thy1.2\(^+\) T cells. FITC-, PE-, and allophyco-
cyanin-labeled mAbs to mouse CD8 (53-6.7), Thy1.1 (OX-7), and Thy1.2
(53-2.1) were obtained from BD Pharmingen. Stained cells were analyzed
using FACSCalibur and CellQuest software (BD Immunocytometry Sys-
tems, San Jose, CA).

**CFSE staining**

Staining of Tg cells with CFSE was performed based on the manufacturer’s
instructions (Molecular Probes, Eugene, OR). The number of CFSE-labeled
CD8\(^+\) T cells undergoing division in the spleen was calculated following the methodology described previously
(20).

**Adoptive transfer**

Spleen cells from Tg mice (malTg or flH1Tg), unlabeled or CFSE-labeled,
containing \(\sim 2 \times 10^6\) naive Ag-specific CD8\(^+\) T cells, were injected i.v.
into syngeneic recipient mice. For experiments in which primed spleen
cells were used for adoptive transfer, *Plasmodium* parasite-primed spleen
cells were obtained from mice that received 2 \(\times\) 10\(^5\) malTg CD8\(^+\) T cells and
3–4 \(\times\) 10\(^4\) sporozoites 48 h earlier. Influenza virus-primed spleen
cells were obtained from mice that received 2 \(\times\) 10\(^6\) flH1Tg CD8\(^+\) T cells and
1–2 \(\times\) 10\(^5\) PFU influenza virus 72 h earlier. For experiments in which a
particular subset was either depleted or purified from primed spleens,
dissociated spleens were stained with FITC-conjugated mAbs (CD8 (53-6.7),
CD4 (RM4-4), NK1.1 (PK136), or CD45R/B220 (RA3-
6B2; all from BD Pharmingen)), followed by anti-FITC-conjugated mag-
etic beads. Depletion by negative selection or enrichment by positive
selection was then performed following the instructions of the manufac-
turer (Miltenyi Biotec, Auburn, CA). Depletion of NK1.1\(^+\) cells was per-
formed using spleens from primed CB6F\(_1\) mice.

**Preparation of DCs**

Mature DCs were prepared as previously described (21). Briefly, bone
marrow was removed from the leg bones of normal mice, and the precur-
sors were cultured in conditioned medium containing 30% of the super-
natant from the myeloma cell line Ag8563 (provided by Dr. A. Rodriguez,
New York University, New York, NY), as a source of murine GM-CSF.
The conditioned medium was replaced every 2 days. Seven or 8 days later,
mature of the DCs was induced by addition of 1 \(\mu\)g/ml *Salmonella
enterica* LPS (Sigma-Aldrich, St. Louis, MO) to the cultures. Twenty-four
hours after LPS addition, DC maturation was verified by flow cytometry
based on the expression and up-regulation of mouse CD11c (HL3), CD40
(3/23), CD80 (16-10A1), CD86 (GL-1), and MHC class I (SFI-1.1; all from
BD Pharmingen) compared with noninduced cells. Mature DCs were then
washed thoroughly and incubated with the SYVPSAEK1 peptide (10
\(\mu\)g/ml) for 2.5 h. After incubation, the SYVPSAEK1-loaded DCs were
washed again before transfer at 2.5 \(\times\) 10\(^5\) cells/mouse.

**Results and Discussion**

Further activation of CD8\(^+\) T cells is inhibited immediately
after priming with malaria sporozoites

Previous studies using Tg CD8\(^+\) T cells specific for an H2K\(^d\)-
restricted epitope (SYVPSAEK1) expressed in sporozoite and liver
stages of *P. yoelii* (malTg CD8\(^+\) T cells) indicated that the CD8\(^+\)
T cell response induced by immunization with sporozoites is the
result of a single clonal burst induced by a short term Ag presen-
tation that lasts \(<\) 96 h. The supply of additional Ag does not en-
hance the magnitude of the CD8\(^+\) T cell response, indicating the
development of a state of refractoriness (8).

That this refractory state is established just a few hours after
priming was indicated when we compared the CD8\(^+\) T cell re-
sponse in groups of normal BALB/c mice that received malTg
CD8\(^+\) T cells and were immunized with 2 \(\times\) 10\(^5\) *P. yoelii* para-
sites. Groups of mice received an additional dose of 8 \(\times\) 10\(^6\) para-
sites 24, 48, 72, or 96 h after priming, and the resulting CD8\(^+\)
T cell response was evaluated by ELISPOT 14 days later. The mag-
nitudes of the responses in mice receiving two immunization doses
was almost identical with that in mice receiving only the initial
2 \(\times\) 10\(^6\) parasite dose (Fig. 1). The response levels in mice recei-
vine two doses were all lower than that in mice that received
1 \(\times\) 10\(^5\) parasites in a single initial dose. Therefore, a regulatory
mechanism that inhibits the additional activation of CD8\(^+\) T cells
is established as early as 24 h after priming, and the magnitude of
the immune response depends on the initial dose. The immuniza-
tions were performed using radiation-attenuated sporozoites, which are capable of invading hepatocytes, but are unable to pro-
liferate and generate blood stage parasites.

**Activation of naive CD8\(^+\) T cells is inhibited in mice previously
primed with sporozoites**

The existence of a mechanism limiting the development of sec-
ondary clonal bursts also became evident in experiments in which
we studied the in vivo proliferative activity of naive CD8\(^+\) T cells.
In these experiments CFSE-labeled naive malTg CD8\(^+\) T cells
were transferred into normal BALB/c mice that were subsequently
immunized i.v. with sporozoites. Seventy-two hours after immu-
nization, flow cytometric analysis was performed to evaluate the
dilution of the CFSE stain in CD8\(^+\) T cells as previously
described (7, 20). As expected, in the absence of sporozoite
delivery, these T cells do not proliferate (Fig. 2Ai), while in mice immunized with sporozoites, they display a strong proliferative response, with at least eight identifiable cell division cycles (Fig. 2Aii). However, CFSE-labeled malTg CD8+ T cells did not proliferate when they were coinfected with parasites into mice already undergoing a T cell response induced 48 h earlier by transfer of naive malTg CD8+ T cells and immunization with sporozoites (Fig. 2Aiii). Quantitative estimation of the proliferative activity of malTg CD8+ T cells under the different experimental conditions (20) confirmed that in sporozoite-primed mice the proliferation of naive CD8+ T cells was severely limited (Fig. 2B). Taken together with the results of the ELISPOT assay (Fig. 1), analysis of the proliferative activity of CFSE-labeled cells indicates that a mechanism that severely inhibits the activation and proliferation of naive CD8+ T cells is developed 24–48 h after immunization. Experiments in which CFSE-labeled malTg CD8+ T cells together with parasites were injected into mice immunized 1 or 2 wk earlier yielded identical results (data not shown).

**Inhibitory process limiting the proliferation of naive CD8+ T cells is mediated by primed CD8+ T cells reactive to the same Ag**

To further characterize the nature of this inhibitory mechanism, we performed adoptive transfer experiments using primed spleen cells obtained from mice that had received malTg CD8+ T cells and sporozoites. In these experiments spleen cells obtained 48 h after priming were transferred into normal mice that subsequently received naive CFSE-labeled malTg CD8+ T cells and were then immunized with parasites. The CFSE dilution profile of the labeled cells, evaluated by flow cytometry 72 h later, revealed a major inhibition of proliferation of naive malTg CD8+ T cells when primed spleen cells were transferred into mice (Fig. 3A). In contrast, no effect was observed when normal spleen cells were transferred into mice. These results clearly indicated that the mechanism inhibiting the activation of naive CD8+ T cells was transferable and mediated by primed spleen cells. Quantification of the CFSE proliferation peaks further confirmed the inhibitory effect of primed spleen cells on the proliferation of labeled CD8+ T cells (Fig. 3B).

In view of these results we next examined whether the regulatory mechanism observed in the plasmodial system might also develop in other microbial systems. For this purpose we performed experiments using influenza virus and Tg CD8+ T cells specific for the H2Kb-restricted IYSTVASSL epitope of the influenza virus hemagglutinin protein (fluTg CD8+ T cells). Immunization with influenza virus was performed i.v., which is known to result in limited or no viral replication (22). CSFE-labeled fluTg CD8+ T cells were transferred into normal mice, and 72 h later the dilution of the CFSE stain was evaluated by flow cytometry. As expected, these CD8+ T cells displayed a strong proliferative response in mice that also received influenza virus (Fig. 4). However, when CFSE-labeled fluTg CD8+ T cells and influenza virus were transferred to mice that had received fluTg CD8+ T cells and virus 72 h
The proliferation inhibitory activity is present in parasite-primed spleen and is transferable. A, The proliferation profile of labeled naive CD8⁺ T cells was assessed 72 h after the transfer of 2 × 10⁶ CFSE-labeled malTg CD8⁺ T cells and 4 × 10⁴ parasites into normal mice (i), normal mice that received parasite primed spleen cells generated as described in Fig. 2 (ii), or normal mice that received nonprimed spleen cells (iii). Flow cytometry CFSE histograms were obtained after gating on CD8⁺Tetramer⁺ T cells. B, The percentage of CFSE-labeled CD8⁺Tetramer⁺ T cells in the spleen undergoing division in each of the above groups was estimated based on previous data (19). Results represent the mean of three mice ± SD.

earlier, the proliferation of labeled naive CD8⁺ cells was strongly inhibited (Fig. 4ii). Furthermore, the adoptive transfer of spleen cells obtained from mice developing an anti-influenza immune response also strongly inhibited the proliferation of naive CFSE-labeled fluTg CD8⁺ T cells administered together with another dose of the virus (Fig. 4iii). These results are strikingly similar to those observed in the malaria system and therefore suggest that this regulatory phenomenon represents a general mechanism that critically constrains the activation and proliferation of naive CD8⁺ T cells against microorganisms.

The existence of similar inhibitory mechanisms in these different infection models allowed us to investigate the Ag specificity of this phenomenon. Specifically, we examined whether Plasmodium sporozoite-primed spleen cells could inhibit the proliferation of naive fluTg CD8⁺ T cells in response to viral immunization and vice versa. Spleen cells obtained from influenza-primed mice did not prevent the proliferation of naive malTg CD8⁺ T cells in response to parasite immunization (Fig. 5A), and conversely, spleen cells from parasite-primed mice did not prevent the proliferation of naive fluTg CD8⁺ T cells in response to influenza virus immunization (Fig. 5B). The inhibitory activity was therefore not only transferable, but also Ag specific. Just as importantly, these results strongly suggested that primed/activated Ag-specific T cells themselves do contribute to the mechanism that prevents activation of additional naive CD8 T lymphocytes sharing the same TCR.

Activated CD8⁺ T cells inhibit the activation of naive CD8⁺ T cells

To identify the inhibitory cell population, spleen cells from parasite-immunized mice were systematically depleted of different T cell populations before transfer into normal mice. The recipient mice then received CFSE-labeled naive malTg CD8⁺ T cells and parasites, and the dilution profile of labeled cells was evaluated at 72 h by flow cytometry. Depletion of NK cells, γδ T cells, or CD4⁺ T cells did not affect the inhibitory capacity of the primed spleen compared with controls (Fig. 6, i–v). However, depletion of CD8⁺ T cells allowed for a normal proliferative profile by the malTg CD8⁺ T cells (Fig. 6vi). Confirmatory evidence that these CD8⁺ T cells were indeed mediating the inhibitory activity was obtained in adoptive transfer experiments. CD8⁺ T cells purified from spleens of parasite-immunized mice inhibited the activation of naive malTg CD8⁺ T cells as efficiently as the whole primed spleen (Fig. 6vii). Similar results were obtained in the influenza system wherein purified activated influenza-specific CD8⁺ T cells inhibited the activation of naive fluTg CD8⁺ T cells (data not shown).

Activated and naive CD8⁺ T cells compete for a limited number of APCs

These results indicated that activated CD8⁺ T cells regulated the activation of naive CD8⁺ T cells, suggesting the possible existence of a competitive relationship between these two cell populations of identical epitope specificity. This possible competitive relationship was further characterized in experiments that measured the in vivo Ag-driven expansion of naive CD8⁺ T cells in the presence of...
increasing numbers of activated cells. For this purpose, mice received a constant number (1.5 × 10⁶) of naive Thy1.1⁺ malTg CD8⁺ T cells plus different numbers of activated Thy1.2⁺ malTg CD8⁺ T cells, ranging from 180–400,000. These mice were then immunized with parasites. Seven days later, the expansion of naive CD8⁺ T cells was assessed by flow cytometry with gating on CD8⁺Tetramer⁺Thy1.1⁺ T cells to measure the expansion of transferred naive cells and not that of transferred activated cells that bear the Thy1.2 marker. The expansion of Thy1.1⁺ CD8⁺ T cells diminished proportionally as the numbers of transferred Thy1.2⁺ activated cells increased (Fig. 7). It is noteworthy that activated cells are highly efficient in competing for APCs, as indicated by the results obtained when transferring equal numbers of naive and activated CD8⁺ T cells. In this situation the proliferation of naive CD8⁺ T cells is inhibited by 90% or more, thus supporting the idea that activated/memory T cells have higher avidity for Ag presented by APCs (23).

The preceding results demonstrating the existence of competition between naive and activated CD8⁺ T cells raised the question of the nature of the mechanism mediating this phenomenon. Recent in vitro and in vivo studies indicate that activated T cells may severely affect the function of professional Ag-presenting DCs by stripping Ag from their surface or by direct killing (24, 25). In view of these observations it has been suggested that competition for DCs could negatively affect the induction of T cell responses (26, 27). To determine whether DCs played a role in the self-regulatory activity of CD8⁺ T cells, we evaluated the effect of introducing large numbers of peptide-loaded DCs on the apparent in vivo competition among CD8⁺ T cells. For this purpose, mature DCs derived from bone marrow precursors were pulsed with the SYVP-SAEOI peptide and transferred into normal mice along with primed spleen cells, CFSE-labeled naive malTg CD8⁺ T cells, and parasites, as described in the preceding experiments. The transfer of peptide-pulsed DCs overcame the inhibitory effect exerted by activated cells on naive CD8⁺ T cells (Fig. 8). These findings...
expressed as total numbers of CD8+ T cells and 180-400,000 purified activated CD8+ T cells and were then immunized with 3 × 10^6 parasites. Seven days later, the expansion of the naive malTg CD8+ T cells was assessed by flow cytometry after gating on CD8+ Tetramer+Thy1.1+ T cells. A. The results of this experiment are expressed as total numbers of CD8+ Tetramer+Thy1.1+ T cells in the spleens of immunized mice, calculated after determining by flow cytometry the percentage of CD8+ Tetramer+Thy1.1+ T cells present in splenocytes of individual mice. The graph shows these results (y-axis) plotted against the number of activated cells (x-axis) transferred into mice. B. Sample of the histograms from which these data were obtained: mice that received 1.5 × 10^5 naive Thy1.1+ CD8+ T cells plus 180 (left panel) or 4 × 10^5 (right panel) activated CD8+ T cells. Numbers indicate the percentage of Thy1.1+ cells present in the CD8+ Tetramer+ T cell population.

FIGURE 7. Competition between activated and naive CD8+ T cells. Groups of mice received 15,000 naive malTg CD8+Thy1.1+ T cells and 180-400,000 purified activated CD8+ T cells and were then immunized with 3 × 10^6 parasites. Seven days later, the expansion of the naive malTg CD8+ T cells was assessed by flow cytometry after gating on CD8+ Tetramer+Thy1.1+ T cells. A. The results of this experiment are expressed as total numbers of CD8+ Tetramer+Thy1.1+ T cells in the spleens of immunized mice, calculated after determining by flow cytometry the percentage of CD8+ Tetramer+Thy1.1+ T cells present in splenocytes of individual mice. The graph shows these results (y-axis) plotted against the number of activated cells (x-axis) transferred into mice. B. Sample of the histograms from which these data were obtained: mice that received 1.5 × 10^5 naive Thy1.1+ CD8+ T cells plus 180 (left panel) or 4 × 10^5 (right panel) activated CD8+ T cells. Numbers indicate the percentage of Thy1.1+ cells present in the CD8+ Tetramer+ T cell population.

indicate that DCs play a key role in mediating this self-regulatory mechanism among activated and naive CD8+ T cells. They also strongly suggest that the limited proliferation and activation of naive cells within an immunogenic environment were not due to a functional disability that was induced upon these T cells, i.e., anergy. Rather, the competition appears to be caused by a restricted access to Ag-presenting DCs, which could be mediated by mechanisms that disable the generation of TCR ligands, such as down-regulation of MHC class I molecules and direct stripping of peptide from DCs, or by a direct cytotoxic effect on DCs.

Self-regulation of CD8+ T cell responses
Taken together, the results of this study revealed the existence of a self-regulatory mechanism controlling the magnitude of CD8+ T cell responses against microbial pathogens before any pathogenic effect of the microbes was detectable. This mechanism could also serve to prevent an excessive activation and expansion of CD8+ T cells that might overwhelm the immune system, leading to undesirable effects and potential pathological sequelae. This self-regulatory activity is likely to represent one of the earliest mechanisms induced in the course of an immune response, developed to control the magnitude of the CD8+ T cell response. It is worth emphasizing that the inability of an established CD8+ T cell response to expand after repeated Ag exposure is not a phenomenon observed only in experimental systems using Tg cells. Indeed, the first evidence indicating the presence of early self-regulatory T cell-mediated mechanisms was strongly suggested in previous studies using normal mice (8). However, the low precursor frequency of Ag-specific T cells in normal mice does not permit the labeling of Ag-specific T cells to perform flow cytometric analyses as described in the present study.

It is striking that the time needed to develop this self-regulatory capacity is quite rapid, i.e., within 24–72 h after delivery of the source of antigenic peptides. However, it is noteworthy that previous studies in mice immunized with Plasmodium sporozoites indicated that CD8+ T cells displayed clear differentiation signs and development of effector mechanisms as early as 24 h after priming (7). In the influenza system there is also evidence indicating that influenza-specific CD8+ T cells become activated 24–72 h after immunization with the virus (28). An important issue that remains to be elucidated is the effect that factors such as virulence, tissue specificity, and ability to replicate may have on the development of these self-regulatory mechanisms.

While the present study establishes that competition between naive and activated CD8+ T cells at the level of APCs is an early regulatory mechanism, it is nevertheless likely that additional regulatory mechanisms are subsequently developed as the immune response evolves. In fact, CD4+ T cells have been shown to be important in sustaining the development of CD8+ T cell responses after clonal expansion. In Plasmodium and influenza systems, mice depleted of or lacking CD4+ cells have very diminished or compromised responses against the microbes (29–31).
Finally, our findings indicating the existence of Ag-specific CD8+ T cells that exert an inhibitory effect on the immune responses raise the issue of whether this represents the activity of a specialized T cell subset, similar to the CD8+ T suppressor cells that many years ago were the subject of extensive studies and were proposed to be regulators of the immune response. While some of the inhibitory phenomena described in those early studies may concur with the regulatory activity of CD8+ T cells described here, our present findings suggest that there is no need to postulate the existence of a specialized class of suppressor CD8+ T cells. Therefore, we favor the idea of a dual function for effector CD8+ T cells, wherein activated CD8+ T cells both perform their antimicrobial functions and also regulate the magnitude of the CD8+ T cell response, using the same or similar effector mechanisms.

Acknowledgments

We thank D. Eichinger for critical review of the manuscript. We also thank B. Waksman and L. Carvalho for discussions. Tetramers were provided in part by the Tetramer Core Facility (National Institutes of Health).

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