A transgenic mouse model to analyze CD8$^+$ effector T cell differentiation *in vivo*

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Antigen-specific effector T cells are prerequisite to immune protection, but because of the lack of effector cell-specific markers, their generation and differentiation has been difficult to study. We report that effector cells are highly enriched in a T cell subset that can be specifically identified in transgenic (T-GFP) mice expressing green fluorescent protein (GFP) under control of the murine CD4 promoter and proximal enhancer. Consistent with previous studies of these transcriptional control elements, GFP was strongly and specifically expressed in nearly all resting and short-term activated CD4$^+$ and CD8$^+$ T cells. However, when T-GFP mice were challenged with vaccinia virus, allogeneic tumor cells, or staphylococcal enterotoxin A, the cytotoxic and IFN-γ-producing T cells lost GFP expression. Upon T cell receptor (TCR) ligation by αCD3, sorted GFP$^+$ cells fluxed calcium and proliferated vigorously. In contrast, GFP$^-$ effector cells showed a diminished calcium flux and did not proliferate. Instead, they underwent apoptosis unless supplied with exogenous IL-2. By reverse transcription–PCR analysis, the GFP$^-$ cells up-regulated the pro-apoptotic molecule, Fas-L, and down-regulated gene expression of the proximal TCR signaling molecule, CD3ζ, and c-jun, a component of the AP-1 transcription factor. Thus, differential regulation of TCR signaling may explain the divergent responses of naïve and effector T cells to antigen stimulation.

A number of phenotypic characteristics differentiate antigen-primed T cells from naïve T cells. The latter express high molecular weight isoforms of CD45 and high levels of L-selectin (CD62L), but relatively low levels of CD44, CD11a (LFA-1), and CD49d (α4 integrin). In contrast, antigen-primed T cells down-regulate L-selectin, up-regulate CD44, CD11a, and CD49d, and express low molecular isoforms of CD45 (1–3). However, most of these changes in surface markers reflect quantitative variations in levels of expression, necessitating assessment of the overall pattern of expression to distinguish between subsets. Moreover, it is not clear if any of them can discriminate between activated (pre-effector) versus effector T cells. Thus, at present there is no simple method to unambiguously differentiate, at the single-cell level, between naïve, activated, and effector T cells (4). Such a method would facilitate cellular and molecular analysis of events involved in effector and memory cell generation.

We have developed a transgenic mouse model (T-GFP) that identifies a T cell population that is highly enriched for effector T cells generated *in vivo*. The green fluorescent protein (GFP) transgene, controlled by the CD4 promoter/proximal enhancer, was expressed in all naïve T cells, but was shut off in a subset of antigen-responsive cells when the animals were challenged with vaccinia virus, superantigen, or allogeneic cells. Analysis of sorted cells revealed that all of the effector cell activity resided in the GFP$^-$ population. T cell receptor (TCR) stimulation elicited distinct responses in GFP$^+$ naïve cells compared with GFP$^-$ effector T cells.

Materials and Methods

Transgene Construct and Microinjection. The murine CD4 promoter and enhancer construct (p37.1) (5) containing a unique *Sal*I cloning site was a gift from Dan Littman, New York University Medical Center. pEGFP-C1 (CLONTECH) was the source of enhanced GFP cDNA. *Sal*I sites at both ends of the GFP coding sequence were introduced by PCR, and the product was cloned into the *Sal*I site of p37.1 to derive the T-GFP construct (Fig. 1*A*). Vector sequences were removed by *Not*I digestion, and the vector-free T-GFP construct was purified on a GeneClean column (Bio-Lab, St. Paul). Pronuclear DNA injection into fertilized FVB oocytes was performed at the Brigham and Women's Hospital Core Transgenic Facility (Boston). Founder pups were identified by PCR analysis of tail DNA and confirmed by flow cytometry. T-GFP animals were crossed to C57 background. Mice were used at 6–8 weeks of age unless stated otherwise.

Antibody Staining, Flow Cytometry, and Cell Sorting. To determine T cell specificity of GFP expression, splenocytes from T-GFP mice were stained with phycoerythrin (PE)-conjugated anti-mouse Thy1.2, CD4, CD8, B220, Mac-1, Gr-1, and NK1.1 mAbs (PharMingen). For activation markers, peritoneal exudate lymphocytes (PELs) and splenocytes were stained with PE-labeled anti-mouse CD62L (L-selectin), CD11a, CD11b, CD44, CD69, and CD25 antibodies (PharMingen). To measure apoptosis, cells were stained with either propidium iodide (PI) or PE-labeled annexin V (PharMingen). All samples were analyzed on a FACScan flow cytometer (Becton Dickinson) following standard procedures. For cell sorting, cells were stained with azide-free PE-labeled antibodies to CD4 or CD8, and the positive subset was sorted on the basis of GFP expression by using an Epics Cell Sorter (Coulter).

Antigen Challenge. For vaccinia virus challenge, T-GFP mice were infected with the WR strain of vaccinia virus (from American Type Culture Collection; 10^5 plaque-forming units/mouse in 0.2 ml of PBS i.p.). To elicit an allo-specific response, mice were injected i.p. with 5×10^6 P815 (H2^d) tumor cells. For superantigen stimulation, mice were injected i.p. with 20 μg/mouse staphylococcal enterotoxin A (SEA) (Sigma).

Cytotoxicity Assays. To test viral-specific cytotoxicity, on day 7 postinfection, PELs were harvested by flushing with 3 ml of PBS. PELs were stained with αCD8, and the GFP$^+$CD8$^+$ and GFP$^-$CD8$^+$ T cells were sorted into separate vials. Sorted cells were tested for lysis of ^{51}Cr-labeled MC57G (H2^b) target cells infected with vaccinia virus in a standard 4-hr chromium release assay. For allo-specific CTL, sorted GFP$^+$CD8$^+$ and GFP$^-$CD8$^+$ T cells obtained 7 days after immunization with P815 cells were tested for lysis of ^{51}Cr-labeled P815 targets. Cytotoxicity was

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Abbreviations: GFP, green fluorescent protein; SEA, staphylococcal enterotoxin A; TCR, T cell receptor; PE, phycoerythrin; PEL, peritoneal exudate lymphocyte; PI, propidium iodide; RT-PCR, reverse transcription–PCR.

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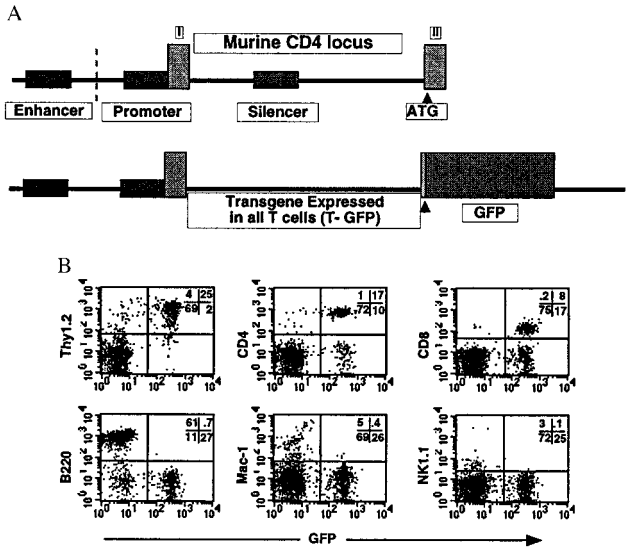

Fig. 1. T cells from T-GFP mice express GFP uniformly and selectively. To generate T-GFP mice, the GFP coding sequence was placed under control of the murine CD4 promoter/proximal enhancer without the intronic silencer and the plasmid construct (*A*) was microinjected into fertilized oocytes. (*B*) Splenocytes from T-GFP mice were stained with mAbs against murine T cells (Thy1.2), CD4, CD8, B cells (B220), macrophages (Mac-1), and NK cells (NK1.1) and examined by flow cytometry. Examination of peripheral blood leukocytes revealed that Gr-1$^+$ granulocytes were also GFP$^-$ (not shown).

defined as: (test release − spontaneous release)/(maximum release − spontaneous release) × 100%.

Intracellular IFN-γ Assay. IFN-γ production was determined at the single cell level by flow cytometry. For vaccinia-specific IFN-γ production, 2×10^6 PELs obtained on day 7 postinfection were restimulated *in vitro* by using vaccinia-infected adherent macrophages as described (6). The cultures were incubated for 6–8 hr in the presence of 1 μM brefeldin A and stained for IFN-γ by using an intracellular staining kit (PharMingen). Briefly, cells were stained with cychrome-labeled αCD8, fixed, permeabilized, and stained with PE-labeled anti-mouse IFN-γ antibody. CD8$^+$ gated cells were analyzed for IFN-γ production versus GFP expression by three-color flow cytometry. For allo-specific and SEA-specific IFN-γ production, PELs from immunized mice were restimulated with either 10^4 P815 cells or 10 μg/ml SEA for 6 hr in the presence of brefeldin A followed by intracellular staining for IFN-γ.

Reverse Transcription–PCR (RT-PCR) Assay. For gene expression studies, mice were immunized with vaccinia virus, and CD4$^+$ and CD8$^+$ T cells from splenocytes obtained on day 7 postinfection were sorted to separate GFP$^+$ and GFP$^-$ subsets. A total of 2×10^5 sorted cells were dissolved in Trizol reagent (BRL), and total cellular RNA was extracted according to the manufacturer's recommendation. RNA was treated with RQ-1 DNase (Promega), extracted with phenol/chloroform, and precipitated with ethanol. One hundred nanograms of RNA was reverse-transcribed by using a Perkin–Elmer RT-PCR core kit. cDNAs were amplified by using the following primer pairs derived from the Genbank database (from 5′ to 3′): GCAAGGGCGAGGAGCTGTTCACCGGGG and TTACTTGTACAGCTCGTCCATGCCGAGAG for enhanced GFP (nucleotides 620–1329); TGGTTCGGCATGACACTCTCAGTGC and CCACTTTCATTCACCACCAGGTTCAC for CD4 (nucleotides 761–1127), CCGAGAGCGGTGCCTACGGCTACAG and GACCGGCTGTGCCGCGGAGGTGAC for c-Jun (nucleotides 676–1027); CCGGGCACCATGAAGGCGGCCGTCGA and GGATTCTGGGTAGGTAGGGTTGGCTC for Ets-1 (nucleotides 501–965); GTGTCTGTTCTCGCCTGCATCCTCC and GGCAGCAGTCGCAGTCTCTGCACTCCTGCTG for CD3ζ (nucleotides 77–253); TCATCTTGGGCTCCTCCAGGGTCAG and GGCTTTGGTTGGTGAACTCACGGAG for Fas-L (nucleotides 185–483); and TGGAATCCTGTGGCATCCATGAAAC and TAAAACGCAGCTCAGTAACAGTCCG for β actin (nucleotides 886–1234). Thermal cycling conditions were: 1 min at 94°C denaturation; 1 min at 55°C annealing; and 2 min at 72°C extension. After amplification for 35 cycles, the PCR products were analyzed on 10% polyacrylamide gels.

Lymphocyte Proliferation Assay. T-GFP mice were immunized with allogeneic P815 cells i.p. Splenic T cells were sorted 7 days later into GFP$^+$ and GFP$^-$ subsets. A total of 5×10^4 sorted cells were cultured in the absence or presence of 1 μg/ml purified αCD3 in triplicate in 96-well culture plates. A total of 10^5 irradiated spleen cells/well were added as feeder cells. In some experiments, 100 units of recombinant IL-2 was added. Two days after stimulation, the cultures were pulsed with ^{3}H thymidine (0.5 μCi/well) for 6 hr, harvested, and counted for ^{3}H incorporation by using a Packard Topcount harvester and microplate reader.

Calcium Mobilization Assay. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined as described (7). Splenocytes from P815-immunized mice obtained on day 7 postimmunization were loaded with 1 μM Indo-1 at 37°C for 1 hr, stained with PE-labeled αCD8, washed, and resuspended to 2×10^6 cells/ml. $[Ca^{2+}]_i$ was determined by using a Coulter Epic V flow cytometer. After running the cells for 1 min to obtain a steady baseline, αCD3ε antibody (5 μg/ml) was added, and after 1 min, goat anti-hamster IgG (1 μg/ml) was added to crosslink CD3. $[Ca^{2+}]_i$ was monitored during the next 10 min.

Results

Production of T-GFP Mice. In an effort to track individual T cells *in vivo*, we generated transgenic mice, termed T-GFP, which express enhanced GFP selectively and uniformly in CD4$^+$ and CD8$^+$ T cells. To generate T-GFP mice, the GFP coding sequence was placed under control of the murine CD4 promoter and proximal enhancer without the intronic silencer (Fig. 1*A*), which suppresses CD4 gene expression in CD8$^+$ cells (8). The T-GFP construct was microinjected into fertilized eggs, and founders were identified by PCR analysis of tail DNA (not shown). Flow cytometric analysis of T-GFP splenocytes confirmed uniform T cell-specific expression of GFP (Fig. 1*B*).

Many Transgenic T Cells Lose GFP Expression After Antigen Challenge. We produced the T-GFP mice in part because we expected that GFP would provide an easy-to-follow marker for use in adoptive transfer experiments to study antigen-specific memory T cells. To test the ability of T-GFP mice to mount a T cell-mediated immune response, we infected T-GFP mice with vaccinia virus and examined their splenocytes and PELs on day 7 postinfection. Surprisingly, many T cells in both the CD4$^+$ and CD8$^+$ compartments had lost GFP expression (Fig. 2*A*). Similar results were obtained from three different founders, excluding the possibility that this finding was the result of positional effects of transgene integration (not shown). GFP$^-$ T cells were not apoptotic because they did not stain with PI or annexin V (Fig. 2*A*). GFP$^-$ T cells were more frequent among PELs than among splenocytes and in both populations the CD8$^+$ subset contained more GFP$^-$ cells than the CD4$^+$ subset. Because the immune response to vaccinia infection is dominated by CD8$^+$ T cells (9), we speculated that primarily the activated virus-specific T cells had lost GFP expression. To test this possibility, we stained PELs and splenocytes for activation markers and analyzed GFP coexpression by flow cytometry after gating on CD8$^+$ T cells. Indeed, most GFP$^-$ CD8$^+$ cells were activated, i.e., they were L-selectin^{low}, LFA-1^{high}, and CD44^{high}. A subset of these cells also expressed CD69, CD11b (Mac-1), and CD25. These

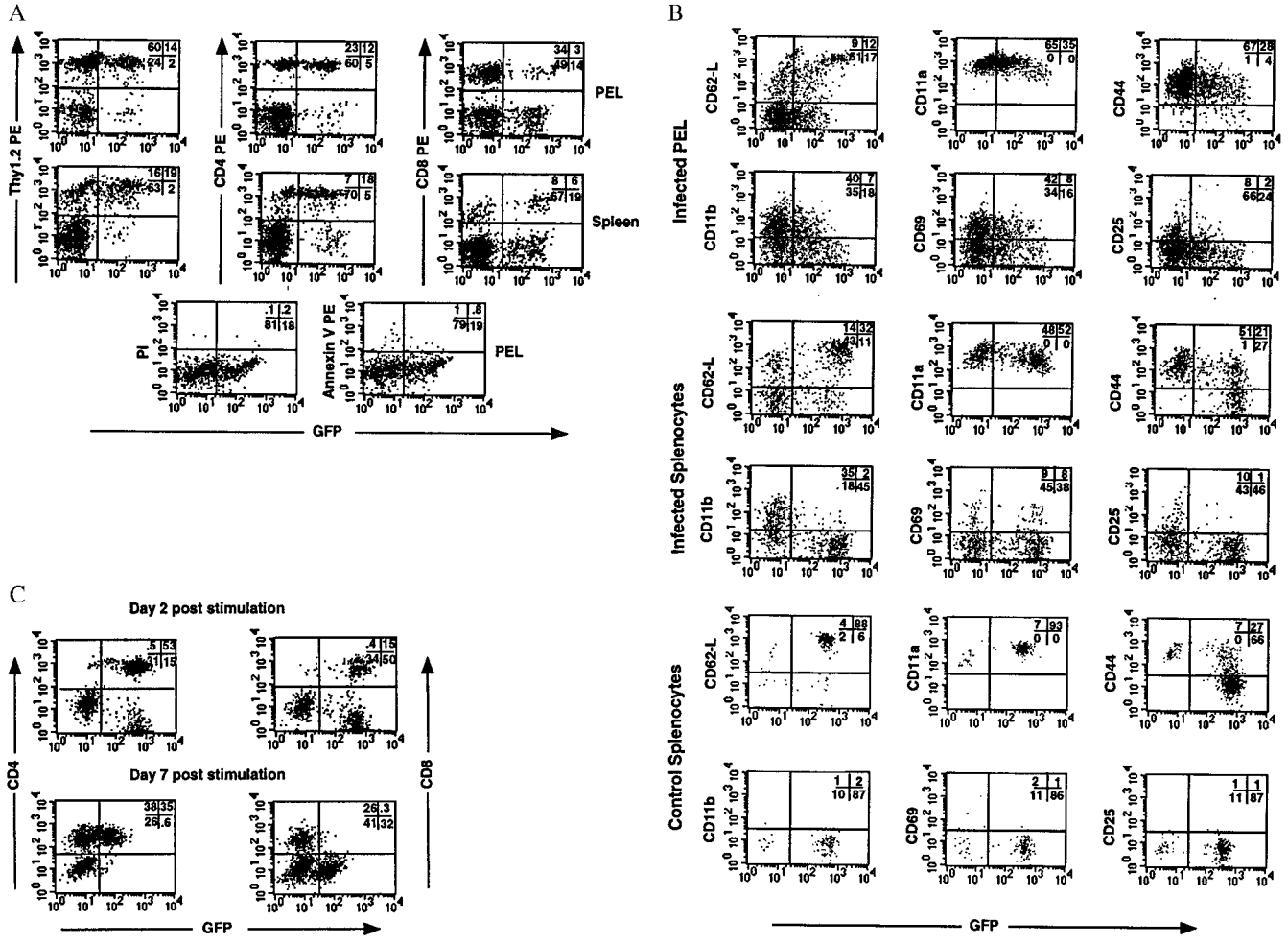

Fig. 2. A subset of activated T cells loses GFP expression after vaccinia infection *in vivo* or after αCD3 stimulation *in vitro*. (*A*) T-GFP mice were infected with vaccinia virus, and on day 7 postinfection, T cells in PELs (*Top*) and splenocytes (*Middle*) were tested for GFP expression. PELs also were tested for cell viability (*Bottom*). Representative results from one infected mouse (of >10 mice analyzed) are shown. (*B*) Six days postvaccinia infection, PELs and splenocytes were analyzed for expression of GFP versus activation markers by using three-color flow cytometry. Splenocytes from uninfected mice are shown as a control. Representative results from one mouse each (of four analyzed) are shown after gating on the $CD8^+$ subset. (*C*) Transgenic splenocytes were stimulated *in vitro* with αCD3, and GFP expression in the $CD4^+$ and $CD8^+$ subset was monitored over time.

changes apparently were caused by the vaccinia infection because nearly all $CD8^+$ T cells from uninfected control mice remained GFP^+ and were L-selectin^{high}, LFA-1^{low}, and CD44$^{low/-}$ (Fig. 2*B*).

A closer examination of Fig. 2*B* revealed that a substantial fraction of GFP^+ $CD8^+$ T cells in infected mice also expressed some of these activation markers. This finding suggests that loss of GFP occurred independent of or perhaps subsequent to changes associated with an activation phenotype. To distinguish between these two possibilities and to determine the kinetics of GFP down-regulation, we stimulated splenocytes from naïve animals *in vitro* with αCD3 and monitored GFP expression at various time points thereafter. Initially, proliferating T cells continued to express GFP, but starting from day 4, a GFP^- population became apparent, and by day 7 poststimulation, the majority of $CD8^+$ and about half of the $CD4^+$ T cells were GFP^- (Fig. 2*C*). Similar kinetics also were seen after infection with vaccinia virus *in vivo* (not shown). These results indicate that loss of GFP expression is associated with events occurring late after activation.

$CD8^+$ T Cells That Lose GFP Expression Are Exclusive Mediators of Effector Functions. During an immune response to viral infection or allo-stimulation *in vivo*, phenotypic signs of T cell activation become evident within 3–4 days, but CTL activity becomes first demonstrable only after 5–6 days, suggesting that a period of maturation is required to generate functional effector CTL (10). Because loss of GFP was also a late event after activation, we examined whether GFP down-regulation was associated with the acquisition of effector functions. For these studies, $CD8^+$ T cells from vaccinia-infected mice were sorted on the basis of GFP expression and tested for cytotoxic function. Only GFP^- cells, but not GFP^+ cells, were capable of virus-specific cytotoxicity (Fig. 3*A*, *Left*). Similar results also were obtained in an allo-specific response after challenge with P815 ($H2^d$) cells (Fig. 3*A*, *Right*), suggesting that loss of GFP may be a marker for effector T cells. To examine this hypothesis further, we also tested GFP^+ and GFP^- cells from vaccinia-infected mice for antigen-induced production of the major $CD8^+$ cell effector cytokine, IFN-γ (Fig. 3*B*). Using three-color flow cytometry to study selectively the $CD8^+$ subset, we detected virus-specific IFN-γ production in GFP^-, but not in GFP^+, cells. These results also held true when effector cell differentiation was induced by other stimuli such as allogeneic P815 tumor cells or SEA (Fig. 3*B*).

Several Molecular Changes Are Associated with Loss of GFP Expression in T Cells. Although T-GFP effector cells had lost CD4 promoter/proximal enhancer-driven GFP expression, the GFP^- $CD4^+$ T cells

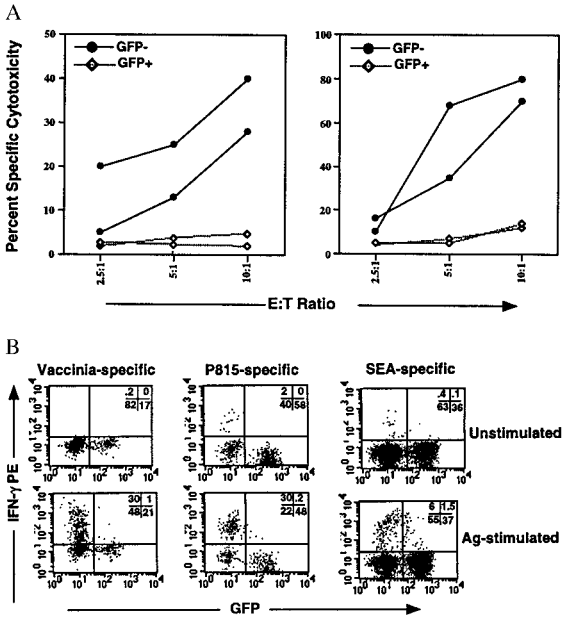

Fig. 3. Loss of GFP expression discriminates a subset of differentiated T cells that are cytotoxic and produce IFN-γ. (*A*) Seven days postvaccinia infection or postimmunization with allogeneic P815 cells, $CD8^+$ T cells from PELs were sorted into GFP^+ and GFP^- cells and tested for lysis of vaccinia-infected MC57G (*Left*) or allogeneic P815 (*Right*) target cells. Results from two T-GFP mice for each method of immunization are shown. E:T ratio, effector-to-target ratio. (*B*) Mice were challenged i.p. with vaccinia virus, P815 cells, or SEA. After 7 days, PELs were tested for antigen (Ag)-specific intracellular IFN-γ production as described in *Materials and Methods*. Gated $CD8^+$ cells from one representative mouse (of three tested with similar results) for each method of immunization are shown.

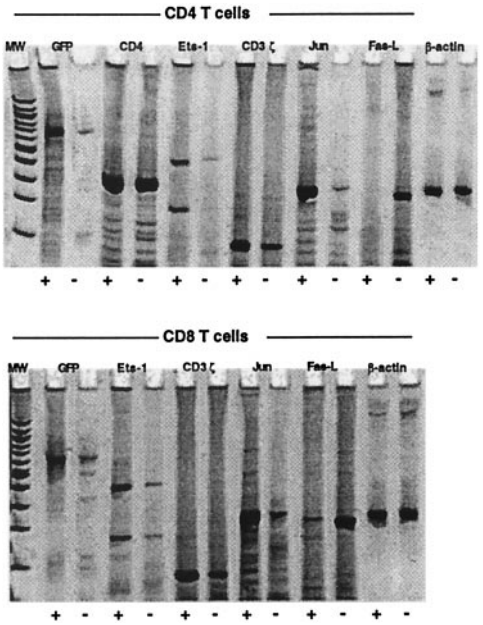

Fig. 4. Differential gene expression in GFP^+ and GFP^- T cells after immunization. Seven days postvaccinia infection, splenocytes were stained with αCD4 or αCD8 antibodies and sorted based on GFP expression. RT-PCR was performed as detailed in *Materials and Methods*, and the PCR products were resolved by PAGE. The MW lane shows a 100-bp ladder; the lowermost band represents 200 bp. The larger of the two bands in lanes Ets-1 represents the predicted full-length product.

continued to express CD4 on their surface. To determine whether this finding was reflected at the transcriptional level, we sorted $CD4^+$ T cells from spleens of P815-challenged T-GFP mice and tested the GFP^+ and GFP^- $CD4^+$ cells for GFP and CD4 gene expression by RT-PCR. Fig. 4 shows that while the GFP transgene was turned off, endogenous CD4 gene expression continued in the GFP^- cells. We interpret these results to mean that the promoter/proximal enhancer used in the T-GFP construct is turned off in the GFP^- cells, but cellular CD4 expression continues presumably because alternate enhancer(s) can drive CD4 gene expression. In fact, in addition to the proximal enhancer contained in our transgene, one or more other CD4 enhancers may exist (11, 12).

Because effector T cells in T-GFP mice lost GFP expression, we reasoned that analysis of transcription factors that are thought to control CD4 expression might reveal molecular events that are distinctly associated with effector T cells. Transcription factors implicated in the control of CD4 expression include the Ets family member Ets-1 and the AP-1 complex (12). Gene expression of these elements was down-regulated in GFP^- effector T cells compared with GFP^+ cells in both $CD4^+$ and $CD8^+$ T cells.

We have observed that the cytotoxic and IFN-γ producing $CD8^+$ T cells from human adults infected with Epstein–Barr virus down-regulate CD3ζ (J.L., unpublished data). Thus, we assessed CD3ζ expression in GFP^+ and GFP^- T cells and found that it, too, was significantly reduced in the GFP^- subset in both $CD4^+$ and $CD8^+$ T cells.

One of the mechanisms of effector T cell-induced target cell killing is induction of apoptosis via the Fas pathway (13). Fas–Fas-L interactions also have been thought to regulate immune homeostasis by autocrine and paracrine interactions (14). It seems likely that this important function also is associated with effector cells. Indeed, gene expression of Fas-L was dramatically up-regulated in sorted GFP^- cells when compared with GFP^+ cells.

GFP^- T Cells Differ from GFP^+ T Cells in Their Response to TCR Engagement. The results in Fig. 4 suggest that the GFP^- population down-regulated CD3ζ and the transcription factor c-jun. This finding was of particular interest because these proteins constitute the most proximal and most distal molecules involved in the induction of IL-2 synthesis after T cell activation via the TCR (15). However, because our data were obtained by RT-PCR that may not be truly quantitative, we sought to characterize directly the response of GFP^- T cells to TCR ligation. We tested whether calcium mobilization in GFP^- T cells is different from GFP^+ T cells. $CD8^+$ splenocytes from P815-stimulated T-GFP mice were analyzed for calcium flux in response to αCD3. GFP^+CD8^+ T cells mobilized calcium rapidly and at a high frequency, whereas the calcium flux appeared delayed and involved a smaller fraction of GFP^-CD8^+ T cells (Fig. 5*A*). This finding is surprising considering that antigen-specific cytotoxicity is thought to be calcium dependent (16). It is possible that a small or transient calcium flux in effector cells is enough for effector functions, whereas a much larger and sustained signal may be needed for naïve cells to proliferate (17).

We also compared sorted GFP^- T cells with their GFP^+ counterparts for proliferation after αCD3 stimulation. As shown in Fig. 5*B*, the GFP^+ T cells proliferated vigorously, whereas the GFP^- T cells did not. However, despite their inability to proliferate, many GFP^-, but almost no GFP^+, T cells responded to αCD3 by producing IFN-γ (Fig. 5*C*).

To determine the ultimate fate of αCD3-stimulated GFP^- T cells, we used annexin V staining to assess whether these cells underwent apoptosis. Indeed, apoptosis was induced in GFP^-, but not in GFP^+, T cells under these conditions (Fig. 5*D*). Thus, in contrast to naïve and pre-effector (GFP^+) T cells, TCR crosslinking on effector (GFP^-) T cells resulted in reduced calcium mobilization and lack of proliferation, but sustained

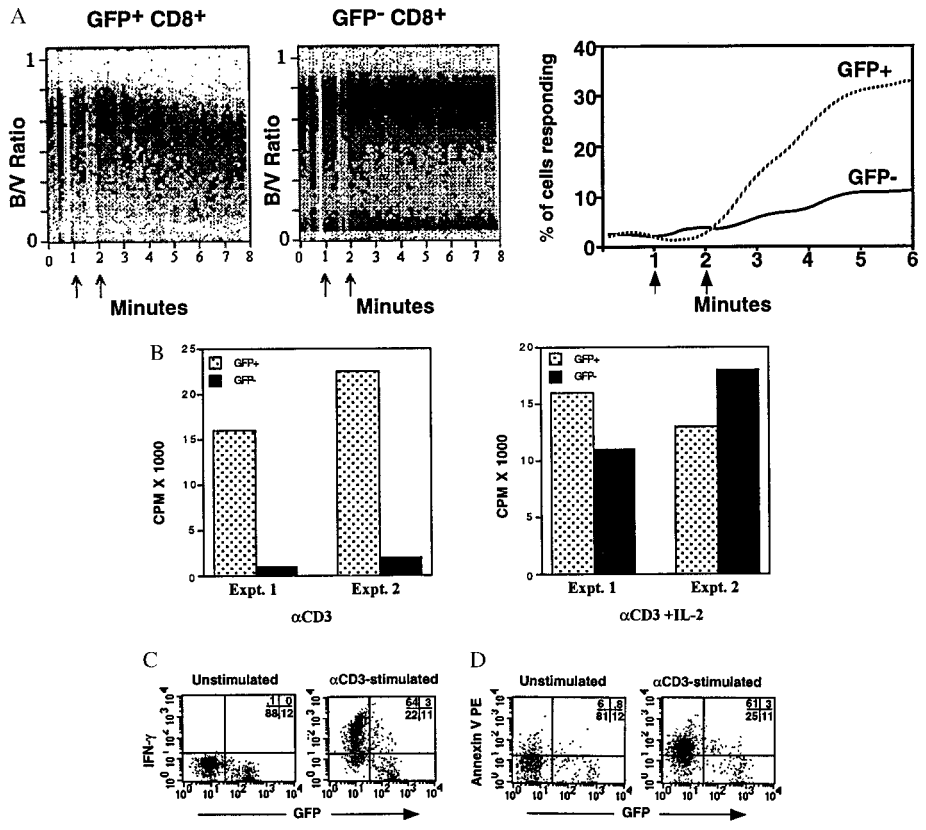

Fig. 5. GFP$^-$ effector T cells show diminished Ca^{2+} mobilization, do not proliferate, and undergo apoptosis after stimulation with αCD3, but they exert effector functions and can be rescued by exogenous IL-2. (*A*) Splenocytes from P815-immunized T-GFP mice were loaded with Indo-1 and stained with αCD8. After gating on the CD8$^+$ subset, the $[Ca^{2+}]_i$ flux response to CD3 crosslinking was monitored in the GFP$^+$ and GFP$^-$ subsets as described in *Materials and Methods*. Ratio of blue to violet (B/V) median channel fluorescence intensity over time (*Left* and *Middle*) as well as % cells responding (*Right*) are shown. A decrease in the blue/violet fluorescence ratio indicates calcium flux. Representative results from one mouse (of three mice tested) are shown. Arrows at 1 min and 2 min indicate addition of hamster anti-mouse CD3 and goat anti-hamster IgG, respectively. (*B*) Sorted GFP$^+$ or GFP$^-$ CD8$^+$ T cells obtained from P815-primed T-GFP mice were cultured with αCD3 in the absence (*Left*) or presence (*Right*) of 100 units of recombinant IL-2 and tested for proliferation by ^{3}H thymidine incorporation after 48 hr. Results from two independent experiments are shown. (*C*) PELs obtained 7 days after challenge with P815 cells were stimulated with αCD3 and tested for intracellular IFN-γ production. Results from one mouse (of three tested with similar results) are shown. (*D*) PELs from P815-immunized mice were tested for annexin V staining without further restimulation (*Left*) or 8 hr after stimulation with αCD3 (*Right*). Results from one mouse (of three tested with similar results) are shown.

effector activity and, eventually, apoptosis. However, it seems unlikely that effector T cells always undergo apoptosis upon antigen stimulation *in vivo*. It is more likely that their survival after antigen recognition is tightly controlled, e.g., through dependence on survival or growth factors that may be released by other cells. To examine this possibility, we tested whether GFP$^-$ T cells could be rescued from apoptosis and induced to proliferate by exogenously supplied IL-2. Indeed, upon addition of IL-2 GFP$^-$ T cells proliferated as well as their GFP$^+$ counterparts. (Fig. 5*B*). Thus, dependence on exogenous IL-2 for survival appears to characterize effector T cells.

Discussion

The ability to better distinguish between naive, activated, and effector T cells would be of help in furthering our understanding of effector T cell differentiation and protective immunity. We have developed a transgenic mouse model in which selective and uniform expression of GFP was seen in naive and early activated (pre-effector) T cells. Surprisingly, the transgene was turned off in a T cell population that contained essentially all of the differentiated CD8$^+$ effector T cells. These mice may provide a valuable tool to isolate mature effector T cells as a discrete subpopulation that can be readily identified by loss of GFP expression.

After recognizing cognate antigen, activated T cells down-regulate L-selectin, up-regulate CD44, CD11a, and CD49d, and express low molecular weight isoforms of CD45 (1–3). Increased expression of CD44 frequently is used as a marker to identify and isolate murine effector T cells because CD44 up-regulation occurs uniformly after activation (18, 19). However, CD44 ligation by antibodies or natural ligands can affect T cell trafficking and function (20, 21). Thus, antibodies against CD44 may not be ideal tools to select T cells for functional studies or in adoptive transfer experiments. Moreover, because CD44 up-regulation occurs within 2 days of antigen recognition (10), it appears to be a marker of T cell activation rather than effector differentiation.

In contrast to most other activation markers, loss of GFP expression is detected only at 4–5 days after activation. This time course of GFP extinction parallels the reported induction of effector cytotoxic molecules like perforin, granzymes, and FasL in CD8$^+$ T cells (22). Thus, the loss of GFP expression is closely linked to and perhaps specifically triggered by effector differentiation. However, it cannot be excluded that down-regulation of GFP transcription occurred somewhat earlier, and GFP$^-$ T cells became detectable only after some delay because GFP protein might have a relatively long half-life in T cells. In either case, T-GFP mice appear to provide a tool to identify and select differentiated effector T cells that are clearly distinct from naïve and early activated (pre-effector) T cells. Antigen-specific CTL activity and IFN-γ production was seen exclusively in the GFP$^-$ population. Moreover, although most of the GFP$^+$CD8$^+$ PEL were CD44^{high}, and the majority expressed little or no L-selectin (Fig. 2*B*), these cells were incapable of killing vaccinia-infected target cells (Fig. 3*A*) or secreting IFN-γ upon antigen challenge (Fig. 3*B*). This population probably represented activated pre-effector T cells.

By day 7 after vaccinia infection, approximately 60% of CD4$^+$ T cells and over 80% of CD8$^+$ T cells accumulating at the site of infection (i.e., the peritoneal cavity) had lost GFP expression (Fig. 1*B*). In the spleen, nearly 30% of the CD4$^+$ T cells and 60% of the CD8$^+$ T cells were GFP$^-$. It is not clear whether all GFP$^-$ T cells specifically recognize vaccinia-associated antigens. However, the presence of large numbers of GFP$^-$-activated T cells in our experiments agrees with recent studies using TCR transgenic mice and MHC-peptide tetramers to detect antigen-specific T cells in other models of viral infection. These studies have shown that there is a massive expansion of such T cells during immune responses *in vivo* (4, 23, 24). It is unlikely that the extinction of GFP represented a nonspecific inflammatory stress response,

because a clearly discernable subset (5–8%) of GFP^-CD8^+ T cells (memory cells) capable of viral-specific IFN-γ production persisted in the spleens 3 months after vaccinia infection (unpublished data). Moreover, GFP^-CD8^+ T cells showed a tendency to increase in spleens of uninfected animals as they aged ($6.9 \pm 3\%$ in 4-week-old mice versus $10.4 \pm 4\%$ in mice older than 1 year ($n = 3$, data not shown). These GFP^-CD8^+ T cells probably represent memory cells to environmental antigens, exposure to which increases over time.

Although antigen-specific effector functions such as cytotoxicity and IFN-γ production resided only in the GFP^- population, not all GFP^- cells produced IFN-γ upon antigen-specific stimulation (Fig. 3*B*). Thus, it cannot be excluded that the GFP^- population contained additional T cell subsets that may not exert effector functions or perform other functions (e.g., regulatory) that were not assayed here. However, it is likely that the method used for antigen-specific stimulation was not optimal to ensure stimulation of all antigen-specific T cells within the 6-hr assay period. Alternatively, some GFP^- cells may have represented activated bystander (antigen-nonspecific) cells. However, even after exposure to αCD3, which should stimulate all T cells, a substantial number of GFP^- cells failed to produce IFN-γ (Fig. 5*C*). These results raise the possibility that not all effector T cells acquired all of the effector functions tested (i.e., cytotoxicity as well as IFN-γ production). In fact, the existence of antigen-specific T cells identifiable by MHC-peptide tetramers *in vivo*, which do not secrete IFN-γ has been recognized recently (25). Further studies with T-GFP mice that will be crossed with TCR transgenic mice might provide insights into these issues.

It has been reported that CD4 expression is up-regulated in antigen-activated $CD4^+$ T cells both *in vitro* and *in vivo* (26). However, upon activation of cells *in vitro* with lectins or phorbol 12-myristate 13-acetate, CD4 expression decreases (26, 27). Our results indicate that while the proximal CD4 enhancer-driven GFP expression is turned off, endogenous CD4 protein continues to be expressed. These differences suggest that the transcriptional regulation of the murine CD4 locus is even more complex than previously thought.

Interestingly, many GFP^- cells, but not naïve GFP^+ T cells, underwent apoptosis after αCD3 stimulation *in vitro* (Fig. 5*D*). However, it is difficult to imagine that antigen recognition would induce apoptosis of e.g., viral-specific effector T cells at a site of viral infection. Accordingly, apoptosis was not evident in effector cells that were generated *in vivo* because GFP^- PELs from vaccinia-infected T-GFP mice did not stain with annexin V or PI (Fig. 2*A*). Moreover, the GFP^- T cells could be rescued from αCD3-induced apoptosis *in vitro* by addition of IL-2 (Fig. 5*B*). Thus, effector T cell survival *in vivo* may depend on growth factors such as IL-2 that must be produced by other T cells. Indeed, it has been reported recently that effector $CD8^+$ T cells are incapable of endogenous IL-2 synthesis (28). Our data suggest a plausible explanation for this observation. Both the most proximal (CD3ζ) and the most distal (AP-1) component in the antigen-triggered signaling cascade that induces IL-2 synthesis were down-regulated in GFP^- T cells. It should be cautioned that the RT-PCR technique that was used to compare the transcription of these molecules is at best semiquantitative. Nevertheless, our findings raise the possibility that differential transcription of key molecules involved in TCR signaling in naive versus effector T cells is responsible for the observed divergent response to antigen in terms of survival and proliferation.

Our findings suggest that effector differentiation may be associated with down-modulation of signaling components that control T cell proliferation. Similar molecular and functional changes have been reported previously in situations of chronic antigen exposure and have been interpreted as a deficiency or failure of the immune system. For instance, in situations of chronic antigenic stimulation like cancer, autoimmune diseases, and HIV infection, T cells down-regulate CD3ζ and may be hyporesponsive to TCR engagement (29–31). Lack of proliferation upon TCR stimulation is also a hallmark of anergic T cells. Anergic T cells also down-regulate the expression of the AP-1 transcription factor (32). Our data indicate that normal effector T cells generated during an ongoing immune response *in vivo* also share these features. Further molecular comparison of isolated effector, anergic, and memory T cells may provide insights into the generation of these T cell subsets. T-GFP mice may provide a valuable tool to such studies.

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