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Aporotic Programs Are Determined during Lineage Commitment of CD4+ T Effectors: Selective Regulation of T Effector-Memory Apoptosis by Inducible Nitric Oxide Synthase

Divya Purushothaman,*†,1 Nimi Marcel,*†,1 Megha Garg,*†,1 Rasika Venkataraman,* and Apurva Sarin*

Lineage-committed T effectors generated in response to Ag during the inflammatory phase are destined to die during termination of the immune response. We present evidence to suggest that molecular signatures of lineage commitment are reflected in apoptotic cascades activated in CD4+ T effectors. Exemplifying this, ablation of inducible NO synthase (iNOS) protected effector-memory T (TEM) cells, but not T naive or central-memory T cells, activated in vitro, from apoptosis triggered by cytokine deprivation. Furthermore, attrition of T effectors generated in the secondary, but not the primary, response to Ag was substantially reduced in mice, which received iNOS inhibitors. Distinct patterns of iNOS expression were revealed in wild-type TEM effectors undergoing apoptosis, and ablation of iNOS protein in primary and TEM wild-type effectors confirmed observations made in iNOS−/− cells. Describing molecular correlates of this dependence, mitochondrial damage, activation of the protein Bax, and release from mitochondria of the apoptosis-inducing factor were selectively abrogated in iNOS−/− TEM effectors. Suggesting that iNOS dependence was linked to the functional identity of T cell subsets, both iNOS induction and apoptosis were compromised in TEM effectors, which mirrored the response patterns of iNOS−/− TEM. Collectively, these observations suggest that programs regulating deletion and differentiation are closely integrated and likely encoded during lineage commitment of T effectors. The Journal of Immunology, 2013, 190: 000–000.

The inflammatory phase of the immune response, marked by the differentiation of lineage-committed T effectors, terminates with their deletion and the concomitant emergence of cells, which escape death to differentiate further into memory subsets (1). Memory cells form a heterogeneous pool marked by differing phenotypic and functional identities and anatomical locations (2–4). Differences in the kinetics of contraction and susceptibilities to apoptotic stimuli between naive and memory cells and effectors derived from them have been demonstrated (5–8); however, underlying mechanisms are not completely understood. In

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The use of RNA interference approaches in wild-type (WT) effectors confirmed observations made in T cells from iNOS−/− mice. iNOS regulated molecular events underlying mitochondrial dysfunction and propagation of the apoptotic cascade, exemplified by the activation of the proapoptotic Bax and release of apoptosis-inducing factor (AIF), respectively. Finally, evidence is presented for a role for IFN-γ in regulating subset-specific iNOS activity. Collectively, the data suggest that naïve and memory T cells may be distinguished by distinctive apoptotic programs regulating postactivation apoptosis of their progeny.

Materials and Methods

Animals

C57BL/6J, B6/SJL, and mice with targeted deletions of iNOS or gp91phox (B6.129S6-Cybb−/−) or IFN-γ were from the Jackson Laboratory (Bar Harbor, ME) and housed in the National Centre for Biological Sciences animal house facility in individually vented cage units (Tecniplast). Animals (both males and females) were used between 6 and 8 wk of age for all analysis in vitro, and experiments in vivo initiated with animals (donors) at 6 wk of age. The Institutional Animal Ethics Committee at National Centre for Biological Sciences, Bangalore, approved all experiments involving animals.

Reagents

Abs were procured from the following sources: CD3, CD62L, and CD44 (BD Pharmingen); CD25 and CD69 (eBioscience); α-tubulin (Novus Biologicals, Littleton, CO); β-actin (Abcam); β-2m (MP Biomedicals); β-galactosidase (Sigma-Aldrich); GAPDH (Thermo Fisher Scientific); IFN-γ (GenScript); IL-2 (Invivogen); IL-4 (R&D Systems); IL-7 (Miltenyi); IL-12 (Peprotech); iNOS (mouse monoclonal: 1:1500; BD Biosciences); AIF (1:1000; Chemicon); HPl-α (1:250; Upstate); and Cox- 4 (1:250; Cell Signaling Technology). Short hairpin RNA (shRNA) to murine iNOS and the scrambled control was from OriGene Technologies. L-NIL and 1400W were from Calbiochem (EMD Biosciences). All other reagents were from Sigma-Aldrich, Calbiochem, or Molecular Probes (Invitrogen Life Technologies). The cytokines IL-2 and IL-7 were from R&D Systems. X-tremeGENE HP was from Roche, and Retronectin from Takara.

Isolation and activation of T cell subsets

T cell subsets were purified from mouse spleens using separate Mag Celect CD4+ T cell isolation kit to isolate naive (CD44+CD62Lhigh) and TEM (CD44highCD62L−) subsets (R&D Systems). Input populations were single-cell suspensions generated from spleens subject to ACK lysis to reduce RBC burden. Purity of isolated populations, ascertained by staining isolated cells with Abs and analysis by flow cytometry, ranged between 90 and 95%. Cell recoveries of CD4+ naïve and memory T cell subsets ranged between 8–10 × 10^6 and 6–8 × 10^6, respectively, per 100 × 10^6 input from WT mice. Recovery of naïve T cells was consistently lower, and TEM cells correspondingly elevated from IFN-γ−/− mice. However, there were no differences in cell surface markers in cells from these mice, and responses to activation were comparable to those of cells derived from other genotypes. To isolate subsets by flow sorting, CD4+ T cells isolated from mouse spleens using the Mag Celect Reagent (R&D Systems) were stained with fluorescent-tagged Abs to CD44 and CD62L on ice. Subsequently, CD44highCD62Llow (TEM) and CD44highCD62Lhigh (central-memory T (TCM)) cells were separated on the BD FACs-Vantage SE Cell Sorter with sorted cells collected into media supplemented with serum and 10 mM HEPES, as described.

T cell subsets isolated as described above were stimulated (2 × 10^6/ml) in vitro for 48 h with paramagnetic anti-CD3–anti-CD28-coated beads (Dynal, Invitrogen) in serum and antibiotics-supplemented RPMI 1640 medium (complete medium (CM)) to generate T effectors. At the end of this period, T effectors were separated from beads using magnetic columns and activated cells, continued in culture with the cytokine IL-2, or used in experiments immediately. Cells were stained with PE-conjugated CD25 and CD69 Abs on ice for 30 min, washed, resuspended in FACS buffer, and analyzed by flow cytometry.

Retroviral delivery of shRNA was performed in cells, which have been stimulated for 24 h with anti-CD3–anti-CD28, as described above. Retronectin (20 μg/ml in PBS) was coated (250 μl/well) on 24-well plates at 4°C. Plates were left overnight, excess Retronectin was removed, and the wells were blocked with sterile PBS containing 2% BSA (fraction V) for 30 min at ambient temperature. This was followed with a final rinse of the wells with sterile PBS before use. Retroviruses were generated using the HEK393T cell line for packaging. HEK cells plated the previous day in 6-well dishes were transfected with 1 μg pCL-Eco (packaging vector) and 1 μg vector carrying the target shRNA sequence using X-tremeGENE. After 48 h, supernatants were harvested by centrifugation at 18,000 × g at 4°C for 90 min and used in assays. For the spinfection protocol, supernatants were mixed with T cells, which had been activated for 24 h and centrifuged (600 × g for 90 min, ambient temperature) in retinonectin-coated wells. At the end of this process, supernatants were replaced with fresh CM supplemented with IL-2 (500 ng/ml), and cells continued in culture at 37°C for another 24 h. Infected T cells were expanded in media supplemented with IL-2 (1 μg/ml) and IL-7 (2 ng/ml), and selected in puromycin (1 μg/ml) for 2–3 d. Live cells were isolated by centrifugation over Ficoll (Sigma-Aldrich) and used in experimental protocols.

shRNA plasmids were assessed for the knockdown of protein of interest following transfections using standard protocols and immunoblot analysis in NIH3T3 fibroblast cell lines. One or two plasmids that resulted in 80–90% reduction in protein expression were taken forward for retroviral packaging and infection of T cells.

Induction and assays of apoptotic damage

To trigger apoptosis in response to cytokine withdrawal, T effectors were washed three times in 5–10 ml CM and continued in culture (0.3 × 10^6/ml) without cytokine in CM for 18–24 h. To assess nuclear damage, cells were stained for 1–2 min with Hoechst 33342 (1 μg/ml) in saline buffer at ambient temperature, and nuclear morphology scored in blinded samples by fluorescence microscopy (13). Intact cells were stained with annexin V FITC per the manufacturer’s instructions (Invitrogen), washed, resuspended in a fixative-free buffer, and analyzed by flow cytometry. For staining with propidium iodide, dye was added to cells suspended in phenol red and fixative-free cold buffer and immediately analyzed by flow cytometry. Immunofluorescence analysis for 6A7 staining was performed, as described (13). To trigger Fas-FasL–induced apoptosis (activation-induced cell death [AICD]), T effectors cultured for 24 h with IL-2 were restimulated with plate-bound anti-CD3 (500 ng/ml), and nuclear damage was assessed after 14–16 h. IL-2–cultured activated T cells were treated with 5 μg/ml etoposide for 12–14 h before being assessed for apoptotic damage.

Responses in vivo

To assess survival following Ag challenge, WT and iNOS−/− mice (CD45.2+) were immunized s.c. with 100 μg maleylated OVA (mOVA) in CFA and TEM isolated from draining lymph nodes of immunized mice 2 wk later. A total of 0.5 × 10^6 cells each was adoptively transferred into congenic B6.SJL (CD45.1) mice. Likewise, naïve T cells isolated from unimmunized WT or iNOS−/− mice were adoptively transferred into congenic hosts. Twenty-four hours later, all host mice were challenged with s.c. injection of 100 μg mOVA in CFA. Numbers of transferred donor cells (CD45.2+) in host spleens were assessed after 30 d by surface staining for CD45.2 marker and analyzed by flow cytometry.

In experiments involving inhibitors, the following protocol was adopted: OT-II CD4+ naïve T cells (0.4 × 10^6/mouse) were injected into congeneric host mice and challenged with mOVA (as above) on day 1 after adoptive transfer, and inhibitors or vehicle control given i.p. daily for the duration of the experiment. Concentrations of inhibitors used in this protocol were 10 and 3 mg/kg body mass (per day) for 1400W and L-NIL, respectively. The recovery of OT-II donor cells relative to CD4+ cells was estimated by flow cytometry from blood at specific days after rechallenge. In experiments in which the recall response was analyzed, mice were rested for 21 d after the primary challenge. After this period (when mice were not given the inhibitors), hosts were rechallenged with mOVA-CFA, and the inhibitors or vehicle control administered daily by the i.p. route for the course of the experiment. Inhibitors were used at the same concentration for the primary and secondary challenge. Recovery of OT-II donor cells relative to CD4+ cells was estimated by flow cytometry from blood or lymphoid organs at indicated days before rechallenge.

Cell fractionations and Western blot analysis

Cell fractionations used a commercial kit to separate the nuclear and cytoplasmic fractions (NE-PER; Thermo-Scientific). A total of 10 × 10^6 T effectors was used as input for fractionation following the manufacturer’s instructions with the following modification. The nuclear pellet was resuspended in 200 μl in buffer C (provided in the kit) to equate the volume of the cytoplasmic fraction. A total of 0.5 × 10^6 cells was used for the nuclear pellet lysis. A quantity amounting to 25 μl of g(w) fraction was processed in loading buffer and used in the Western blot analysis, as described before (13). Samples were analyzed using 12% SDS-PAGE gels and probed with Abs indicated in the figures.
CFSE labeling and cell cycle analysis

A total of 1 × 10^6 cells was loaded with 0.5 μM CFSE in 1 ml PBS at ambient temperature for 10 min, followed by three washes in chilled medium to remove excess dye. Dilution of dye following culture of cell populations in vitro was assessed by flow cytometry. To assess the cell cycle status of primary and secondary effectors, 1 × 10^6 T effector cells were incubated with 10 μM Vybrant DyeCycle Green in 1 ml media (5% serum) at 37°C for 30 min. At the end of the incubation period, cells were analyzed by flow cytometry.

Mitochondrial transmembrane potential

T effector cells were resuspended in 0.5 ml DiOC6 (Molecular Probes) staining solution (PBS containing 40 nM DiOC6) and incubated for 20 min in the dark at 37°C. Cells were washed twice in excess PBS, resuspended in 0.5 ml PBS, and analyzed by flow cytometry.

Immunofluorescence analysis

A total of 0.5 × 10^6 T effectors was used in each condition, fixed with 1% paraformaldehyde (10 min at ambient condition), and permeabilized using 0.2% CHAPS for 30 min at 4°C. Samples were blocked with 5% BSA for 1 h at ambient temperature. Cells were stained with the 6A7 Ab (1:50; Neo-markers) for 2 h at 4°C, followed by a secondary Ab, and finally, samples were counterstained with Hoechst 33342 to visualize the nucleus. Confocal imaging of cells was performed using a Zeiss 510 Meta or Zeiss LCM5 LIVE, ×63 with a 1.4 NA objective lens, and Z-stacks were taken at 1 μm thickness with zoom 2.4.

Statistical analysis and data presentation

Data are presented as mean ± SD derived from a minimum of three to five independent experiments, unless stated otherwise. Statistical significance was calculated using the two-population Student t test.

Results

iNOS selectively regulates apoptosis of TEM effectors

Primary and secondary effectors generated by the polyclonal activation of TNaive (CD4+CD44+CD62L+) and TEM (CD4+CD44−CD62L−) cells, respectively, undergo apoptosis to comparable extents if cultured in the absence of exogenous cytokine (Fig. 1A, 1B; WT condition). The apoptotic assay recapitulates several features of T effector apoptosis in vivo, permitting analysis of the molecular mechanisms underlying this process (12, 13). Thus, TNaive or TEM precursors derived from either gp91phox−/− or iNOS−/− mice were activated using surrogate Ags, and the apoptotic response to cytokine deprivation was compared in resultant effectors. In contrast to WT cells, cytokine withdrawal-induced apoptosis was blunted in both gp91phox−/− effector subsets (Fig. 1A). Intriguingly, when precursors were derived from iNOS−/− mice, only the TEM effectors and not primary effectors were protected, in multiple readouts of apoptotic damage (Fig. 1B–D).

Reduced apoptosis may reflect impaired T cell activation; however, activation-induced expression of CD25 and CD69 Ags or progression into cell cycle was comparable in iNOS−/− effectors generated from TNaive or TEM cells (Supplemental Fig. 1A, 1B).

In related experiments, WT or iNOS−/− TEM effectors (donors) generated in vitro were injected into the tail veins of congenic (CD45.1+) hosts, and recoveries of CD45.2+ donors were assessed after an interval of 7 d. The recovery of iNOS−/− effectors from host mice was consistently higher than WT effectors (Fig. 1E), although migration to distinct locations within the animal could account for these differences observed in this analysis. Comparable dilution profiles of the dye CFSE in WT and iNOS−/− TEM effectors indicated that increased proliferation did not underlie protection from apoptosis in iNOS−/− cells (Supplemental Fig. 1C). To ascertain whether iNOS dependence was a general feature conserved in memory subsets, apoptotic responses were compared in effectors derived from TEM and TCM subsets (15). TCM (CD4+CD62Lhigh) and TEM effectors from WT and iNOS−/− mice were separated by cell sorting from the same starting populations and activated in vitro. Strikingly, in contrast to TEM-derived effectors, TCM effectors were not protected from cytokine withdrawal-induced apoptosis indicated by the analysis of nuclear damage or annexin V binding in the different conditions (Fig. 1F, 1G).

To assess whether dependence on iNOS for induction of apoptosis was an outcome of the development and differentiation of TEM in iNOS−/− mice, we attempted to derive CD44+CD62L− TEM-like cells in vitro using iNOS−/− TNaive cells as the starting population. TNaive cells from WT or iNOS−/− mice express high levels of CD44 and continue to express CD62L following their activation in vitro (Fig. 1H, inset, upper panel). As already

FIGURE 1. iNOS regulates TEM effector apoptosis. (A and B) Apoptotic damage (relative to T0) in WT and gp91phox−/− (A) or iNOS−/− (B) CD4+ effector cells cultured without IL-2 for 18–24 h. (C) Representative analysis of propidium iodide uptake (upper right quadrant) in iNOS−/− primary and secondary effectors cultured without IL-2. Propidium iodide uptake was equivalent and low in the IL-2 condition and not shown. (D) Representative plots of annexin V binding in iNOS−/− primary and secondary effectors cultured with (open) and without (filled) cytokine. (E) Recovery (×10^6) of WT and iNOS−/− SE from host spleen 7 d post-adaptative transfer. The mean ± SD of two trials using a total of five mice is shown. (F) Nuclear damage in WT or iNOS−/− TEM or TCM effectors in different culture conditions. (G) Representative plots of annexin V binding in iNOS−/− TEM and TCM effectors cultured with (open) or without (filled) IL-2. The values above the bar indicate annexin V+ cells in cultures without IL-2 and those below, the IL-2 cultures. (H) Apoptotic damage in response to cytokine deprivation in effectors derived from the activation of iNOS−/− TNaive cells or in effectors resulting from two rounds of rest and restimulation of primary effectors. Inset shows expression of CD44 and CD62L on the effectors used in functional assays. (A, B, and F–H) show the mean ± SD calculated from three independent experiments.
demonstrated, iNOS−/− primary effectors are not protected from apoptosis (Fig. 1H). Restimulating these cultures with anti-CD3–anti-CD28 after 5- to 7-d culture (rest) in cytokines results in the gradual emergence of CD44+CD62Llow populations. A minimum of two rounds of this rest and restimulation protocol yielded cultures enriched for CD44+CD62Llow TEM-like effectors (Fig. 1H, inset, lower panel). This population was separated by cell sorting, and, unlike primary effectors, was protected from apoptosis triggered by cytokine deprivation (Fig. 1H). Thus, CD44+CD62L− cells derived from multiple rounds of rest stimulation of iNOS−/− TNaive cells recapitulated the responses of iNOS−/− TEM effectors isolated from mice.

The analysis of effectors generated in vitro, although providing insight into cellular machinery regulating apoptotic responses, does not incorporate modulation by APCs or other immune cells associated with Ag-induced activation of T cells. In an attempt to address this, we tracked cell recoveries of effectors derived from WT and iNOS−/− mice, and, in a second approach, tested the outcome of pharmacological inhibitors of iNOS on WT cell survival following Ag challenge in congenic hosts.

iNOS limits survival of Ag-reactive TEM effectors

In the first set of experiments, WT and iNOS−/− mice were challenged with mOVA, and, 21 d later (CD44highCD62Llow), TEM pools, which include mOVA-primed memory cells (14), were isolated from these mice. These cells and, in parallel, WT and iNOS−/− TNaive cells from unimmunized mice were adoptively transferred into nonlymphopenic, unmanipulated congenic hosts. All four groups of recipient mice were challenged with mOVA 1 d after cell transfers. Thirty days later, recoveries of residual donor CD45.2+ iNOS−/− or WT cells were scored in each of the four experimental groups. In the analysis involving the primary response, in which TNaive had been transferred and then challenged, the recovery of cells from WT and iNOS−/− mice was comparable (Fig. 2A; primary). However, higher numbers of cells were recovered from hosts that had received Ag-primed iNOS−/− TEM (Fig. 2A; TEM). Again, as was seen in effectors generated in response to polyclonal stimuli, innate differences in proliferation did not account for this increase, as the number of donor cells recovered following primary immunization with mOVA estimated 21 d after transfer was comparable in the two genotypes (Fig. 2B).

Next, to address the possibility that development and differentiation in iNOS−/− environments modulate T cell responses, TCR transgenic TNaive cells were used as input and tracked through primary and secondary responses to Ag in the presence or absence of two NOS inhibitors tested in separate groups. Naive OT-II transgenic T cells, transferred to congenic hosts, were challenged with mOVA, accompanied by continued (daily) injections of the inhibitors 1400W (16) or L-NIL (17) or the vehicle control. Cell recoveries were assessed in circulation of immunized mice on days 4 and 14 after the first challenge. In mice injected with 1400W or L-NIL, cell recoveries were not different from mice injected with the vehicle control at either time point (Fig. 2C), which suggested that inhibiting iNOS did not modulate the persistence of the T cell pool activated in the primary response. To follow the secondary response, mice that had received the primary challenge with mOVA were rested for 30 d and then rechallenged in three groups, two of which received daily injections of 1400W or L-NIL, and the third, which received the vehicle control through the course of the experiments. It should be noted that these mice had not been injected with inhibitors during the course of the primary response. In a notable difference from the primary response, from day 4 onward, higher cell recoveries were noted in the group injected with 1400W relative to the vehicle- or L-NIL–injected control (Fig. 2D). However, in the L-NIL group, differences in cell recoveries relative to control were apparent on day 9 and substantially enhanced by day 14 (Fig. 2D). The difference in cell recoveries persisted till day 21 and was confirmed in cells recovered from lymph nodes and spleens of the different groups (Fig. 2E, 2F). Experiments performed with the inhibitor amino-guanidine (18) yielded comparable results (Supplemental Fig. 2). These results assessing the effect of iNOS on T cell contraction in vivo were consistent with the analysis in vitro and supported a requirement for iNOS in postactivation deletion of TEM effectors.
We reasoned that if T_{Naive} and TEM-derived effectors are indeed distinguished by iNOS dependence, this must be demonstrable in WT effectors in vitro. For this purpose, iNOS levels in primary or TEM effectors derived from WT mice were manipulated using RNA interference and assessed for susceptibility to apoptosis.

**iNOS is required for apoptosis in TEM and not T_{Naive}-derived effectors**

T cells were infected using retroviruses with commercially available shRNA that were confirmed to abrogate iNOS expression in the NIH3T3 fibroblast cell line (data not shown). WT activated T_{Naive} or TEM cells were infected with shRNA to iNOS or a scrambled control packaged in retroviruses and cultured in puromycin for 4 d to select for infected cells, as described in Materials and Methods. These cells were then tested for the response to cytokine withdrawal. As seen in iNOS^{−/−} cells, the ablation of iNOS selectively protected TEM effectors, but not T_{Naive}-derived effectors from apoptotic damage (Fig. 3A, 3B). The ablation of iNOS protein, relative to cells infected with scrambled shRNA, was near complete and comparable in primary and TEM effectors (Fig. 3C). A similar selective protection in TEM effectors was also confirmed using annexin V to measure apoptosis (Fig. 3D). The amelioration of the apoptotic response in TEM effectors following the disruption of iNOS matched observations made in the comparison of primary or TEM effectors from iNOS^{−/−} mice. We extended these observations to assess whether the differential requirement for iNOS was associated with distinct patterns of iNOS protein expression in T cell subsets. iNOS protein levels were comparable in effectors generated from T_{Naive} or TEM cells. However, cytokine withdrawal triggered a consistent increase in levels of iNOS expression in activated TEM relative to primary effectors (Fig. 3E).

**FIGURE 3.** iNOS regulation is restricted to WT TEM effectors. Apoptotic damage in TEM (A) and (B) primary effectors infected, as described in Materials and Methods, with scrambled or iNOS shRNA assessed at onset of assay (T0) and 15 h after culture without cytokine. (C) Representative immunoblots for iNOS and tubulin in cells generated in (A) and (B) are shown. (D) Flow cytometry plots of annexin V binding in primary effectors (upper panels) and TEM effectors (lower panels) cultured with and without IL-2, as indicated. Profiles from cells infected with scrambled (open) or iNOS (filled) shRNA are shown. (E) Primary effectors and TEM effectors were cultured without cytokine for 6 h and iNOS protein levels at onset of assay (T0) and T6 assessed by Western blot analysis. STAT-3 was used to assess the parity of loading.

The protection from apoptosis in WT-TEM effectors, following ablation of iNOS, indicates a pivotal role for iNOS in the regulation of TEM effector apoptosis. iNOS is not a generalized regulator of apoptosis in TEM effectors

To test whether the effect of iNOS was stimulus specific or more general, we assessed apoptotic responses to other stimuli. Interactions between the Fas receptor and its ligand are thought to regulate the deletion of activated autoreactive T cells (8, 9). Differences in response to Fas engagement have also been reported in T cell subsets (6). However, TCR-dependent restimulation of freshly generated T effectors triggered AICD in WT and iNOS^{−/−} TEM effectors (Fig. 4A), which we confirmed was mediated by Fas–Fas ligand interactions in iNOS^{−/−} cells (Supplemental Fig. 3). The apoptotic response to the DNA-damaging drug etoposide, mediated via mitochondrial events, was also equivalent in WT and iNOS^{−/−} TEM effectors (Fig. 4B). We also assessed cell death or contraction following challenge with the bacterial superantigen staphylococcal enterotoxin-A (SEA) in WT and iNOS^{−/−} mice. Superantigens activate T cell subsets (both naive and memory) bearing specific Vβ elements (19). The number of SEA-responsive CD4^{+}Vβ3^{+} cells, 48 h after challenge with SEA, was comparable in WT and iNOS^{−/−} mice (Fig. 4C). The subsequent decline on day 4 postchallenge, indicative of deletion of the SEA-reactive subset, was equivalent in both genetic backgrounds (Fig. 4C). These data suggest that molecular interactions during TCR engagement may regulate iNOS-dependent apoptotic signaling in TEM effectors, because earlier experiments have shown that the loss of gp91^{phox} delayed the deletion of cells in this paradigm (13). Recoveries of the Vβ6^{+} subset, which is not responsive to SEA, at either time point were unchanged in the same mice, establishing the specificity of the response measured in this analysis (Fig. 4C).
To elucidate the molecular intermediates regulated by iNOS in the apoptotic cascade activated by cytokine deprivation, we mapped events that mediate organelle damage or are outputs of mitochondrial dysfunction in T effector. Cytokine deprivation initiates a multistep apoptotic cascade in T effectors, with the mitochondrial serving as a key site of integration of the cell death pathway.

**iNOS activity impinges on mitochondrial function**

Mitochondrial trans-membrane potential (MTP) assessed using potentiometric dyes is an effective measure of mitochondrial integrity and function. MTP was unchanged in TEM effectors cultured in the presence or absence of cytokine, consistent with the observed protection from apoptotic damage. Primary effectors showed an expected loss of MTP in cells cultured without cytokine (Supplemental Fig. 4). These data indicated that iNOS controlled events that regulate mitochondrial integrity in TEM effectors.

Cytokine withdrawal triggers a conformational change in the Bcl-2 family protein Bax, which forms hetero-oligomeric complexes at the mitochondrial outer membrane, marking irrevocable commitment to death. The change in conformation reveals an epitope in the N terminus, which is otherwise masked in healthy cells and is recognized by the 6A7 Ab clone (20). The 6A7 reactivity (green in all images) is readily detected in WT TEM effectors cultured without cytokine for 6–8 h (Fig. 5A; WT), whereas very few cells were positive for 6A7 in iNOS−/− TEM effectors cultured with or without cytokine (Fig. 5A; 5B). However, it should be noted that, despite the low reactivity with 6A7, Bax protein levels were comparable in T effectors from different genetic backgrounds (Fig. 5C). These experiments also assessed iNOS−/− primary effectors, and, as shown, induction of 6A7 reactivity was readily apparent in iNOS−/− primary effectors cultured without cytokine (Fig. 5A; PE). Reactivity to this Ab was undetected in cells cultured with cytokine (data not shown).

To confirm that mitochondrial outputs associated with dying cells were regulated by iNOS in TEM effectors, we used cell fractionation approaches to follow the distribution of the flavoprotein AIF, a mitochondrial intermembrane space resident. AIF is released from damaged mitochondria during the apoptotic response and localizes in the nucleus to trigger DNA damage (21). Apoptosis of activated T cells in this context is independent of caspase function, and reduced levels of AIF correlate with protection from apoptosis in activated T cells (22). In contrast to its expected presence in the cytoplasmic fraction alone in live cells, AIF was detected in both the nuclear (H2A-e-positive) and cytoplasmic (Cox-4-positive) fractions in WT TEM effectors following cytokine deprivation (Fig. 6A, 6B). In iNOS−/− TEM effectors, fractionated following a matched period of cytokine deprivation, AIF was predominantly detected in the cytoplasmic fraction (Fig. 6C), consistent with intact mitochondrial outer-membrane integrity. Again, in iNOS−/− primary effectors cultured without cytokine, AIF was detected in both the nucleus and cytoplasm (Fig. 6D), indicating progression to apoptosis in this subset when cultured without cytokine. Thus, both the activation of the proapoptotic Bax and release of AIF were mediated via an iNOS-independent signaling cascade in primary effectors.

Whereas TEM and TCM may arise from IFN-γ-producing precursors (23), the production of IFN-γ is a signature output of Ag-induced activation of TEM (3, 24). Therefore, to test whether dependence on iNOS is linked to differentiation of TEM, apoptosis of TEM effectors was assessed using mice with a targeted deletion of IFN-γ (25).

**IFN-γ−/− TEM effectors are selectively protected from apoptosis**

TEM and TNaive cells isolated from IFN-γ−/− mice were activated to generate T effectors and assayed for the induction of apoptosis triggered by cytokine withdrawal. IFN-γ−/− primary effectors cultured without exogenous cytokine divided rapidly in the initial phase and were also protected from apoptosis (Fig. 7A; 24-h time point). Subsequently, apoptotic damage was comparable in WT and IFN-γ−/− primary effectors, whereas IFN-γ−/− TEM effectors remained protected from death at this time (Fig. 7A). Like others, we noted that the loss of IFN-γ conferred a proliferative advantage relative to WT cells, which was demonstrated by the increase in cell number and was equivalent in primary and TEM IFN-γ−/− effectors (Fig. 7B). As seen for iNOS−/− TEM, IFN-γ−/− TEM effectors undergo apoptosis in response to the genotoxic drug etoposide (Fig. 7C), an apoptotic response not ameliorated by cytokines such as IL-2.

**FIGURE 5.** iNOS regulates mitochondrial damage in TEM effectors. (A) A67 and H33342 staining in WT and iNOS−/− TEM effectors and iNOS−/− primary effectors (PE) cultured for 8 h without IL-2. (B) As in (A), in TEM effectors cultured with IL-2. (C) Immunoblot for Bax in WT and iNOS−/− TEM effectors. In (A) and (B), representative of two experiments that examined >100 cells each are shown. Scale bars, 5 μm.
IL-2 or IL-7. Providing evidence that IFN-γ was a key regulator of iNOS activity during TEM effector apoptosis, the induction of iNOS observed in WT TEM effectors, in response to cytokine withdrawal, was compromised in cells derived from IFN-γ2/2 mice (Fig. 7D). Again, as seen in the iNOS−/− TEM effectors, 6A7 reactivity was abrogated in IFN-γ−/− TEM effectors and was equivalent (and low) in cells cultured with or without cytokine (Fig. 7E). Furthermore, subfractionation of IFN-γ−/− TEM effectors, following cytokine deprivation, established that AIF was predominantly detected in the cytoplasmic fraction (Fig. 7F), consistent with protection from apoptosis and recapitulating observations made in iNOS−/− TEM effectors.

Discussion

The programmed deletion of TEM effectors generated in an inflammatory response is necessary for immune homeostasis, resetting the immune system for subsequent encounters with infectious agents. The observations in this study demonstrate that molecular configurations of apoptotic cascades activated in response to cytokine deprivation are intrinsically distinct in effector subsets. The data suggest that T cell subset identity influences postactivation apoptosis, exemplified by IFN-γ–iNOS signaling distinguishing the death of activated TEM from that of T naïve or TCM-derived effectors.

Supporting the observations made with T cells activated in culture, differences in the regulation exercised by iNOS in T effector subsets were also apparent, when cell survival was tracked following a primary or secondary challenge with Ag. Thus, effectors generated in mice injected with iNOS inhibitors during the recall response to Ag persisted for longer durations, as opposed to effectors in the primary challenge. Elevated cell recoveries in mice injected with inhibitors, relative to those injected with the vehicle control, were noted in circulation and in lymphoid organs, indicating that differences were not attributable to sequestration in lymphoid tissues. The difference between primary and secondary effectors vis-à-vis iNOS dependence was also corroborated in experiments wherein iNOS expression was disrupted, which selectively protected TEM effectors from apoptosis in response to cytokine deprivation. Taken together, the data indicate that following matched stimulation conditions, T naïve and TEM CD4+ cells, isolated from the same starting population, present distinct outcomes, vis-à-vis the requirement for iNOS in the apoptotic response to cytokine deprivation in culture and deletion of cells following Ag challenge.

iNOS signaling integrated events that converge on mitochondrial damage in TEM effectors. The mitochondrion is a key site for the integration of apoptosis in T cells as in several other cell types.

**FIGURE 6.** iNOS signaling regulates mitochondrial outer-membrane integrity. (A and B) Representative immunoblots of cytoplasmic and nuclear fractions of WT (cultured with and without IL-2 [8 h]), and (C, D) iNOS−/− TEM and primary effectors (cultured without IL-2 [8 h]), probed for AIF, HP1-α, and Cox-4.

**FIGURE 7.** iNOS induction and apoptosis are impaired in IFN-γ−/− TEM effectors. (A) Apoptotic damage (mean ± SD) in IFN-γ−/− and WT effectors, resulting from cytokine deprivation for the indicated periods. (B) Cell recoveries of WT and IFN-γ−/− effectors initiated in culture (~48 h) at 0.3 × 10⁶ cells/ml. (C) Apoptotic damage in cells from different backgrounds in response to 18-h treatment with etoposide. (D) Immunoblots of WT and IFN-γ−/− effectors cultured without IL-2 for 6 h. T0, indicates onset of assay. The graph below (mean ± SD) shows the quantification of iNOS protein intensity with respect to the loading control, quantified by Image J software. (E) H33342 staining in TEM effectors also counterstained for 6A7 (not positive and hence not seen), cultured for 8 h with or without cytokine. (F) Representative immunoblots of cytoplasmic and nuclear fractions of IFN-γ−/− TEM effectors cultured for 8 h without cytokine, probed for AIF, HP1-α, and Cox-4. Scale bar, 5 μm.
Although caspase-9 activity is reportedly reduced in iNOS−/− CD4+ T cells (26), T effector apoptosis is independent of caspase activity and regulated dominantly by Bcl-2 family proteins and redox complexes (10, 12). We followed two events associated with mitochondrial dysfunction during T cell apoptosis: the activation of the Bcl2 protein Bax, which triggers mitochondrial damage, and the nuclear translocation of the mitochondrial intramembrane space resident AIF. Both Bax activation and the consequent release of AIF from mitochondria with compromised outer membrane integrity were abrogated in iNOS−/− and IFN-γ−/− TEM effectors. We have previously shown that Bax activation is regulated by gp91phox activity (13), a conserved intermediate in the apoptosis of T effectors generated from naive and memory subsets (this study). Bax activation and the release of AIF were not compromised in iNOS−/− primary effectors, suggesting that iNOS integrates with the gp91phox-activated cascade in TEM effectors. In this context, BH3-domain-only proteins are primary sensors of apoptotic stressors (27–29), and ongoing experiments are investigating the possible differential recruitment of these in response to the distinct intracellular redox environments generated in T effector subsets.

Protection from apoptosis in iNOS−/− TEM did not result in the accumulation of CD44+ cells or a consistent change in the representation of naive and effector or central memory subsets in T cells in iNOS−/− mice. A possible explanation for maintenance of homeostasis in these mice is the eventual deletion of iNOS−/− TEM effectors by the activation of gp91phox (13). This oxidative complex regulates apoptosis in both primary and TEM effectors (Fig. 1) and may be critical for TEM apoptosis when Ag burden is substantially reduced. Increased T cell number, attributed to impaired apoptosis, has been reported in iNOS−/− mice in the context of chronic infection (30). In addition, the increased generation of induced T regulatory cells reported in iNOS−/− mice (31) indicates that the perturbation of iNOS influences multiple aspects of immune function and homeostasis.

Several studies have documented differences in the kinetics and patterns of contraction in effectors generated in primary and secondary responses (5–7, 32, 33). Studies also support a role for IFN-γ in the regulation of CD4+ T cell apoptosis (26, 34, 35) via interactions between IFN-γ and iNOS (34) or IFN-γ-dependent signaling that may not involve iNOS (35). Distinct from this literature, we report qualitative differences in the molecular machinery of apoptosis in T effector subsets, reminiscent of the functional heterogeneity of T cells (3, 15). In this analysis, employing purified murine T cell subsets, we describe a specific requirement for IFN-γ-regulated iNOS-dependent activation of a cell-intrinsic apoptotic program in CD4+ TEM effectors. We propose that IFN-γ may regulate the apoptosis of IFN-γ-producing TEM effectors via a negative feedback loop incorporating iNOS, which collaborates with gp91phox activity to initiate temporally regulated apoptotic programs (Bax and AIF mediated) in this subset. How these molecular complexes integrate with the cell death machinery in TEM effectors in response to TCR engagement remains to be established.

Whereas IFN-γ regulates T cell number in many Ag-based systems, these studies have not always addressed whether these outcomes result from its effects on T cell differentiation, on APCs, or other immune cells, and are in some instances confounded by its effect on T cell proliferation (23, 34–37). Indeed, the deficiency of IFN-γ also compromises AICD (38), a pathway by which activated T cells generated in response to high concentrations of Ag or in situations of chronic infection or autoreactive cells are deleted (9). Notably, iNOS was dispensable for apoptosis triggered by Fas–Fas ligand, which agrees with an earlier study establishing a role for the neuronal isoform of NOS in TCR-induced Fas–Fas ligand-mediated apoptosis (39).

Human and murine T cells are reported to differ in the dependence on iNOS activity (40), and whether the regulatory features described in this study in murine T cells are indeed conserved across species, as suggested in other contexts (14, 41), remains to be established. The emergence of selective programs of apoptosis in differentiated populations is an intriguing aspect of homeostatic mechanisms in the T cell lineage, reflecting the stringent regulation of cell number necessary for the maintenance of functional space in the immune system. Collectively, the data suggest that chemical signatures of TCR-induced differentiation in CD4+ memory subsets are reflected in the molecular configuration of cell death cascades following their reactivation.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Information

Apoptotic programs are determined during lineage commitment of CD4⁺ T-effectors: iNOS selectively regulates T-effector-memory apoptosis

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Figure Legends

Supplemental Figure 1: Expression of activation markers on SE.

(A) Status of cell cycle progression in iNOS⁻/⁻ primary and secondary effectors post-activation. (B) Representative flow cytometry profiles of CD25 and CD69 markers in iNOS⁻/⁻ primary (open) and secondary effectors (filled). US, indicates the unstained condition. A and B are representative data of two experiments each. (C) CFSE dilution profiles in WT and iNOS⁻/⁻ cells following CD3-CD28 stimulation in vitro.

Supplemental Figure 2: Inhibiting iNOS does not affect cell recovery on primary antigenic challenge.

A, Recovery of OT-II CD4⁺ T-cells adoptively transferred into congenic hosts after primary challenge with maleyl OVA (mOVA) and continued daily administration of aminoguanidine (AG) or saline. Thus, CD4⁺ OVA transgenic T-cells were isolated from OT-II (CD45.2⁺) mice and adoptively transferred into immune-competent B6/SJL (CD45.1⁺) hosts. 24 hours after transfer, host mice were challenged with 100μg of mOVA subcutaneously (D0). AG (2mg/mouse) was injected by the intra-peritoneal route everyday for 21 days from D1. Control mice were injected with saline. Recovery of donor cells was estimated by flow cytometry on day 21. B, Following adoptive transfer of OT-II naïve T-cells and primary challenge with mOVA, congenic hosts were re-challenged with mOVA after 30 days rest and AG or saline given intra-peritoneally for 29 days. Recovery of OT-II donor cells was estimated by flow cytometry.
**Supplemental Figure 3  Inhibiting Fas signaling abrogates AICD in iNOS⁻/⁻ T-effectors.**

CD4⁺T-effectors from iNOS⁻/⁻ mice were cultured overnight in wells pre-coated with anti-CD3 (clone 2C11, 10μg/ml) in CM with IL-2. One set of duplicate cultures included the addition of soluble Mouse Fas-Fc Chimera (300ng/ml; R&D Sytems). Cells cultured in IL-2 with and without Fas-Fc Chimera were the controls in these experiments. 18 hours later cells were harvested and apoptotic damage scored using Hoechst 33342.

**Supplemental Figure 4  Loss of MTP is attenuated in iNOS⁻/⁻ TEM effectors.**

Primary and TEM effectors were cultured with and without cytokine. 15 hours later, cells were stained with DiOC₆ and mitochondrial transmembrane potential measured by flowcytometry. Change in mitochondrial transmembrane potential relative to cells cultured in cytokine is plotted.