Cancer Dormancy. VII. A Regulatory Role for CD8$^+$ T Cells and IFN-γ in Establishing and Maintaining the Tumor-Dormant State[1]

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Dormant tumor cells resistant to ablative cancer therapy represent a significant clinical obstacle due to later relapse. Experimentally, the murine B cell lymphoma (BCL_1) is used as a model of tumor dormancy in mice vaccinated with the BCL_1 Ig. Here, we used this model to explore the cellular mechanisms underlying dormancy. Our previous studies have demonstrated that T cell-mediated immunity is an important component in the regulation of tumor dormancy because Id-immune T cells adoptively transferred into passively immunized SCID mice challenged with BCL_1 cells significantly increased the incidence and duration of the dormant state. We have extended these observations and demonstrate that CD8$^+$, but not CD4$^+$, T cells are required for the maintenance of dormancy in BCL_1 Ig-immunized BALB/c mice. In parallel studies, the transfer of Id-immune CD8$^+$ cells, but not Id-immune CD4$^+$ cells, conferred significant protection to SCID mice passively immunized with nonprotective levels of polyclonal anti-Id and then challenged with BCL_1 cells. Furthermore, the ability of CD8$^+$ T cells to induce a state of dormancy in passively immunized SCID mice was completely abrogated by treatment with neutralizing α-IFN-γ mAbs in vivo. In vitro studies demonstrated that IFN-γ alone or in combination with reagents to cross-link the surface Ig induced both cell cycle arrest and apoptosis in a BCL_1 cell line. Collectively, these data demonstrate a role for CD8$^+$ T cells via endogenous production of IFN-γ in collaboration with humoral immunity to both induce and maintain a state of tumor dormancy. *The Journal of Immunology,* **1999, 162: 2842–2849.**

A functional immune system is important in controlling cancer growth, since immunocompromised individuals are at increased risk of developing certain kinds of cancer. The prevailing theory to explain the importance of a functional immune system in controlling cancer growth is that the immune system recognizes tumor-specific Ags expressed by the cancer cells as foreign and kills the tumor cell population by classical cytotoxic immune effector mechanisms. However, the immune system also has the potential to control tumor growth through the expression of soluble factors, e.g., Abs and cytokines, that can have cytostatic effects on cancer cell growth. These inhibitory effects could potentially induce a state of cancer dormancy, in which tumor cells would be present, but progressive tumor growth would not be clinically apparent.

In fact, cancer dormancy is well established for many tumor types (1–4). However, little is known about the importance of the immune system in establishing and maintaining dormancy and the possibility of using passive and active immunization to induce long lasting dormancy. To investigate the interplay between the immune system and cancer growth, a mouse model of dormancy has been established in which an aggressive B cell lymphoma, BCL_1, can be induced into a dormant state by prior immunization with the BCL_1-derived Ig to generate an anti-Id immune response.

The BCL_1 model represents a bona fide paradigm for tumor dormancy by several criteria (5–8): 1) mice can remain clinically well for long periods of time (1–2 yr) while harboring a potentially aggressive lymphoma; 2) the number of tumor cells in the spleens of dormant mice (the primary site of tumor growth) remains relatively constant ($0.5–1 \times 10^6$ BCL_1 cells) for the 1.5 yr of observation; 3) the dormant population shows partial cell cycle arrest, and there is evidence that the Id immune response is responsible for inducing cell cycle arrest and apoptosis by signal transduction mechanisms; and 4) escape from dormancy is usually caused by loss of signaling capacity via mouse Ig, presumably due to mutation or aberrant expression of a molecule in the signaling cascade (5–8).

A significant body of literature has implicated humoral immunity in mediating the induction and maintenance of dormancy in Id-immune mice: 1) Id vaccination results in high serum α-BCL_1-Ig Ab titers (5, 9); 2) sera from Id-immune mice can induce dormancy in passively immunized recipients challenged with BCL_1 cells (10); 3) Abs that hypercross-link the surface MIg act directly on BCL_1 cells to induce cell cycle arrest and apoptosis both in vitro and in vivo (6, 7, 11, 12); and 4) in our studies there has been a good correlation between an Ab's ability to negatively signal BCL_1 cells in vitro and its ability to induce dormancy in passively immunized SCID mice in vivo (12). In many instances, however, Id-immune mice harboring dormant BCL_1 tumor cells

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[3] Abbreviations used in this paper: α-BCL_1-Ig, anti-BCL_1-immunoglobulin Ab; KLH, keyhole limpet hemocyanin; rIg, rat immunoglobulin; Gt-αMIgM, goat anti-mouse immunoglobulin M; 7-AAD, 7-amino actinomycin D.

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have very low serum α-BCL_1-Ig titers (5). Also, reports by others have failed to correlate the induction of dormancy with the relative serum Ab concentration (9). In summary, despite low anti-Id titers, Ab as a signaling ligand appears to play a major role in the induction of dormancy in the BCL_1 model.

In contrast to the well-characterized role of humoral immunity, early studies suggested that cellular immunity might not have a role in this dormancy model. George et al. demonstrated that the transfer of Id-immune splenocytes to naive recipients had no effect in providing protection against a subsequent challenge with BCL_1 cells (10). Moreover, in combination with passive immunization with Id-immune sera, the transfer of Id-immune splenocytes did not significantly increase protection compared with that in animals receiving only the passive immunization (10).

However, later studies demonstrated that Id-specific T cells can be readily detected following Id vaccination (1), and both Id-specific $CD4^+$ and $CD8^+$ cells have been shown to regulate dormancy in vivo in a variety of other murine tumor models, including the MOPC 103 plasmacytoma (3, 13, 14), a methylcholanthrene-induced sarcoma (15), and the L5178 lymphoma (16–18). Indeed, more recent studies have demonstrated a role for T cells in regulating the dormancy of the BCL_1 lymphoma. For example, when BALB/c mice were immunized with irradiated BCL_1 cells, a dormant state ensues following challenge with live tumor cells (19). In this situation, very little α-BCL_1-Ig Ab is produced in response to tumor cell vaccination, and T cells transferred from these animals to naive recipients provided significant antitumor immunity. In addition, our own studies have demonstrated that the transfer of enriched populations of Id-immune T cells can increase the incidence and duration of dormancy in SCID mice receiving suboptimal passive immunization (12). These recent studies implicate the involvement of Id-specific T cells in regulating the dormancy of BCL_1 cells.

The goals of the present study were to determine which T cell subpopulations, i.e., $CD4^+$ or $CD8^+$ cells, are required to maintain an established state of dormancy in immunocompetent mice. Additionally, we sought to determine whether adoptively transferred Id-immune T cell subpopulations could act independently or in collaboration with each other to induce and maintain a state of dormancy in passively immunized SCID mice.

Materials and Methods

Materials

T cell enrichment columns were purchased from R&D Systems (Minneapolis, MN), and baby rabbit complement was purchased from Pel Freeze (Rogers, AR). Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem (La Jolla, CA), and Con A was purchased from Sigma (St. Louis, MO). [^{3}H]TdR was purchased from Amersham (Arlington Heights, IL), and FCS was supplied by JRH Biosciences (Lenexa, KS). All chemicals used were reagent grade or better.

Mice

Six- to eight-week-old female BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed under specific pathogen-free conditions. Also, 6- to 8-wk-old, nonspecific pathogen-free, female BALB/c mice were obtained from the Microbiology Colony at the University of Texas Southwestern Medical Center (Dallas, TX). Six-week-old female CB-17$^{scid/scid}$ (SCID) mice were purchased from the University of Wisconsin (Madison, WI) and housed under specific pathogen-free conditions.

Antibodies

Rat IgG1-κ isotype control (clone R3-34), rat IgG2a-κ isotype control (clone R35-95), mouse α-rat IgG1/IgG2a (clone G28-5), α-CD4 (L3T4, clone RM4-5), α-CD8α (Ly-2, clone 53-6.7), and α-CD45R/B220 (clone RA3-6B2) were purchased from PharMingen (San Diego, CA). A monoclonal rat α-BCL_1-Id (clone 6A5) was a gift from Dr. F. Stevenson (Tenovus Laboratory, Southampton, U.K.). The mAbs used for in vivo therapy, α-CD4 (GK1.5), α-CD8 (YTS169.4), and α-IFN-γ (XMG1.2), were prepared by affinity purification on protein G-Sepharose (Pharmacia, Piscataway, NJ) from hybridoma culture supernatants. Rat IgG for in vivo therapy was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Any resultant endotoxin contamination was removed by filtration through endotoxin binding columns (Sepracor, Marlborough, MA), and the Ab solution was sterilized by filtration and stored at -80°C before in vivo administration.

Preparation of BCL_1-αMIg

BALB/c mice were immunized with a BCL_1-KLH conjugate emulsified with CFA as previously described (10). Seven days following the last of three immunizations, mice were injected i.p. with 0.5 ml of Pristane (Sigma, St. Louis, MO). Seven days following Pristane priming, the mice were injected with 1×10^6 SP2/0 myeloma cells, and ascites formation was monitored by visual inspection of the abdominal cavity. Upon abdominal distention, the mice were euthanized, and ascites fluid was collected and placed on ice. Cells and debris were removed by centrifugation, and whole Ig and other proteins were precipitated from the clarified ascites fluid by precipitation in 50% saturated ammonium sulfate. The total protein concentration was determined by the Bradford Coomassie blue assay (Bio-Rad, Hercules, CA), and the total α-BCL_1-Ig titer was determined by ELISA. The Ab was sequentially filtered to remove endotoxin and bacterial contamination.

Id vaccination and dormancy in BALB/c mice

The BCL_1 lymphoma was passaged in syngeneic BALB/c mice by transferring 1×10^6 BCL_1 splenocytes from an animal harboring $>10^9$ BCL_1 cells in the spleen. Generation of dormant mice was performed by first immunizing BALB/c mice with the BCL_1-Ig-KLH conjugate as described above. Seven days following the last immunization, animals received 1×10^6 BCL_1 splenocytes on day 0 of tumor transfer. Mice were then monitored for splenomegaly by palpation every 7–10 days. The average length of time for naive animals given 1×10^6 BCL_1 splenocytes to display splenomegaly was approximately 25–30 days post-tumor transfer, and day 60 has historically been the time point at which animals were categorized as achieving a state of tumor dormancy (5). Therefore, animals that displayed overt splenomegaly before day 60 post-tumor transfer were considered nondormant, whereas animals that remained free of splenomegaly by day 60 were considered dormant. Furthermore, those dormant animals that displayed splenomegaly after day 60 were classified as escapees because they relapsed from the dormant state. On the average, approximately 70% of Id-immune mice achieve a state of dormancy by day 60, and previous studies have demonstrated that the relative concentration of α-BCL_1-Id correlates with induction of the dormant state (8). Once overt splenomegaly was detected, the animal was euthanized.

For T cell depletion studies, dormant mice were treated on day 60 with 0.5 mg/dose of GK1.5 (α-CD4), YTS169.4 (α-CD8), or a control rat IgG (rIgG; Jackson ImmunoResearch Laboratories) Ab and then every 14 days thereafter for the duration of the experiment. This protocol was effective in depleting >90% of the targeted peripheral T cell population as determined by monitoring the expression of CD4, CD8, and CD3 on splenocytes from control treated animals by FACS analysis. These animals were monitored for depletion of T cell subpopulations 4 days following the first treatment and subsequently when animals presented with splenomegaly.

Quantification of serum α-BCL_1-Ig titers

Ninety-six-well microtiter plates were coated with 100 μl of 2.5 mg/ml BCL_1-Ig (IgM, λ light chain) (10) followed by a blocking step with 20% FCS/PBS. Serially diluted serum samples as well as a reference α-BCL_1-Ig (described above) were incubated at room temperature for 1 h, and then Ig isotypes were detected with biotin-conjugated secondary Abs specific for MIgG1 or MIgG2a (PharMingen). The plates were then incubated with streptavidin-conjugated horseradish peroxidase and developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). Ab titers were determined by applying linear regression analysis to both the internal standard and the serum serial dilutions. The titer values were expressed as the midpoint of the sample titration curve relative to the midpoint of the standard titration curve as previously described (19).

Statistical analysis

Each group represented in the survival curve analyses was subjected to pairwise comparisons using the log rank test for statistical variance. Student's *t* test was applied to all other groupwise comparisons. A group was considered significantly different from the corresponding control if

$p < 0.05$. Only groups that were significantly different were identified as such in the results.

Preparation of enriched T cell subpopulations

Spleen cell suspensions were prepared from Id-immune mice by lysis of erythrocytes with a hemolytic solution containing 37 mM ammonium chloride, 0.7 mM EDTA, and 0.2 mM KH_2PO_4. The cells were washed three times in 10 ml of HBSS and were resuspended in HBSS with 20% FCS.

T cell enrichment columns were prepared by equilibrating the column medium (RatαMIg-coated glass beads) in HBSS/20% FCS, and the flow rate was adjusted to approximately 1 ml/min with a lure-lock stopcock. Whole spleen cells were added to the column (1×10^7 cells/ml of bed resin volume) and allowed to enter the resin followed by the addition of 2 ml of HBSS/20% FCS. Elution media (HBSS/2% FCS) was continually added thereafter, and enriched T cells were eluted by collecting 30–40 ml of flow-through volume. The cells were then pelleted and resuspended in RPMI 1640 containing 10% (v/v) FCS, 10 U/ml penicillin, 10 μg/ml streptomycin solution (Life Technologies, Grand Island, NY), 10 mM HEPES (pH 7.55), and 50 μM 2-ME (cRPMI).

To enrich for T cell subpopulations, aliquots of enriched T cells were depleted of CD4$^+$ or CD8$^+$ cells with Ab and complement. Depletion was performed by the addition of 100 μg/ml GK1.4 (α-CD4, to enrich for CD8$^+$ cells) or YTS169.4 (α-CD8, to enrich for CD4$^+$ cells) to 1×10^7 cells/ml in cRPMI. Following incubation on ice for 30 min, cells were washed twice with cRPMI followed by the addition of 100 μg/ml M RαIgG. The secondary Ab was allowed to bind for 30 min on ice followed by two washes in cRPMI. In vitro lysis was performed by incubating the Ab-bound cells in a 1/8 dilution of baby rabbit complement in cRPMI at 37°C for 30 min. The remaining cells were then washed twice and resuspended in cRPMI. T cell purity was >95% as determined by monitoring the percentages of CD3$^+$, CD4$^+$, CD8$^+$, and Thy-1$^+$ populations by flow cytometry. Purified T cell populations were routinely assayed for proliferative activity in response to Con A as a measure of viability.

Passive immunization and T cell supplementation of SCID mice

Previous studies have demonstrated that SCID mice challenged with BCL_1 cells can achieve dormancy when passively immunized with BCL_1-αMIg as well as other Abs directed at the surface Ig (12). In this series of experiments, SCID mice were passively immunized i.v. with 50 μg (12) of BCL_1-αMIg 1 day before receiving BCL_1 tumor cells and then every 7 days thereafter for 7 wk. On day 0 of tumor transfer, SCID mice received a one-time inoculation of 3×10^4 BCL_1 splenocytes by i.p. injection as described above. Those animals that were supplemented with enriched populations of T cells were given 5×10^6 T cells by i.p. injection beginning on day −2 of tumor transfer and then every 2 wk thereafter throughout the experiment. In some cases SCID mice were treated with either α-IFN-γ (XMG1.2) mAb or a control rIgG at 0.5 mg/dose, two doses per week, for the duration of the experiment.

In vitro proliferation assay

BCL_1.3B3 cells (3×10^4) were plated in triplicate in 96-well tissue culture plates and incubated in the presence of culture medium alone or with cytokines. LPS or Ab as described in the text. After 24 h of culture, 1 μCi of [^{3}H]TdR was added to each well, and the plates were incubated for an additional 24 h. Macromolecular material was harvested from each well onto glass-fiber filters using a Skatron plate harvester (Skatron Instruments, Lier, Norway). [^{3}H]TdR incorporation was measured by scintillation counting.

Cytofluorometry

In vitro enriched populations of T cells as well as treated animals were routinely monitored for cellular composition by flow cytometry to determine percentages of lymphocytes and BCL_1 lymphoma cells. For animals receiving treatment, splenocytes were stained with dilutions of specific FITC- and/or phycoerythrin-conjugated mAbs (L3T4, Lyt-2, α-BCL_1-Ig (6A5), α-Thy-1, α-CD3ϵ, or α-B220) or FITC- and/or phycoerythrin-conjugated isotype control mAbs. In some cases biotinylated mAbs were used followed by incubation with streptavidin-phycoerythrin. Stained samples were analyzed on a single-laser FACScan (Becton Dickinson, San Jose, CA) with emission wavelength compensation adjustment.

For cell cycle analysis, BCL_1.3B3 cells were treated with cytokines or Ab as described in the text, then harvested and stained with 400 μM 7-amino actinomycin D (7-AAD; Molecular Probes, Eugene, OR) in PBS for 30 min followed by fixation in 0.5% paraformaldehyde. The cells were then stained with 15 μM Hoechst (Sigma) in 5% PBS at 4°C overnight. Fluorescence intensity was measured with a FACStar Plus (Becton Dickinson) at 320 and 488 nm excitation, and the data were analyzed with the Paint-a-Gait software (Becton Dickinson) as described previously (12).

Results

Involvement of T cell subpopulations in the maintenance of dormancy in immunocompetent mice

Id-specific T cells have been demonstrated to arise following Id vaccination (1, 3, 20). However, the role of Id-specific T cell subpopulations in either inducing and/or maintaining dormancy has not been thoroughly investigated. Although unfractionated Id-immune splenocytes alone do not appear to provide significant protection against BCL_1 cells when transferred to naive animals in the induction phase (10), it was of interest to determine whether T cell subpopulations play a role in the maintenance phase of an on-going antitumor response in mice with tumor dormancy. In these experiments, either CD4$^+$ or CD8$^+$ T cell subpopulations were depleted in vivo by specific mAbs in dormant mice. Mice were treated with α-CD4, α-CD8, or a control rIgG Ab on day 60 (i.e., after establishment of dormancy) and every 2 wk thereafter. Since BCL_1 grows primarily in the spleen, splenomegaly was monitored by palpation every 3–4 days to measure tumor cell expansion.

As shown in Fig. 1*A*, depletion of the CD8$^+$ T cell subpopulation significantly decreased the duration of dormancy compared with that in control rIgG-treated animals ($p = 0.001$). Depletion of CD8$^+$ T cells also decreased the average time at which splenomegaly developed in those mice that relapsed (Fig. 1*B*). Depletion of the CD4$^+$ subpopulation had no statistically significant effect on the duration of dormancy ($p = 0.144$). Thus, the CD8$^+$ population appears to be playing an important role in the maintenance of an established state of dormancy in immunocompetent mice.

Synergy between anti-Ig and CD8$^+$ T cells in establishing a tumor-dormant state

SCID mice that are passively immunized with Abs specific to BCL_1-Ig achieve a state of dormancy when challenged with 3×10^4 BCL_1 splenocytes, and adoptive transfer of Id-immune, but not naive, spleen cells can increase the incidence and duration of dormancy in passively immunized SCID mice (12). Based on our findings of a CD8$^+$ T cell subpopulation bias in the regulation of dormancy in immunocompetent mice (described above), it was of interest to determine whether this same bias was also operative in the SCID model of dormancy. T cells from Id-immune mice were enriched by T cell column purification followed by subpopulation depletion with Ab and complement. For in vivo therapy, SCID mice received weekly doses of a suboptimal amount (50 μg) of α-BCL_1-Ig beginning on day −1 of tumor transfer. This dose was not effective at inducing dormancy by itself and thereby would potentially be a sensitive indicator of an effect of transferred T cells. Groups of mice received enriched T cells every 14 days beginning on day −2. All animals were then challenged with 3×10^4 BCL_1 on day 0. As shown in Fig. 2*A*, the suboptimal dose of α-BCL_1-Ig did not significantly delay tumor growth compared with that in mice receiving BCL_1 treated with a control rIg (data not shown) or BCL_1 alone. However, as shown previously (12), the combined treatment with α-BCL_1-Ig and Id-immune T cells significantly delayed the onset of splenomegaly compared with that in both untreated controls and groups receiving only the passive immunization. The detectable effect of Id-immune T cells was dependent upon the inclusion of α-BCL_1-Ig, as the transfer of Id-immune T cells alone had no statistically significant affect.

To determine which T cell subpopulation(s) was mediating the delay of tumor growth, mice were treated with α-BCL_1-Ig alone or

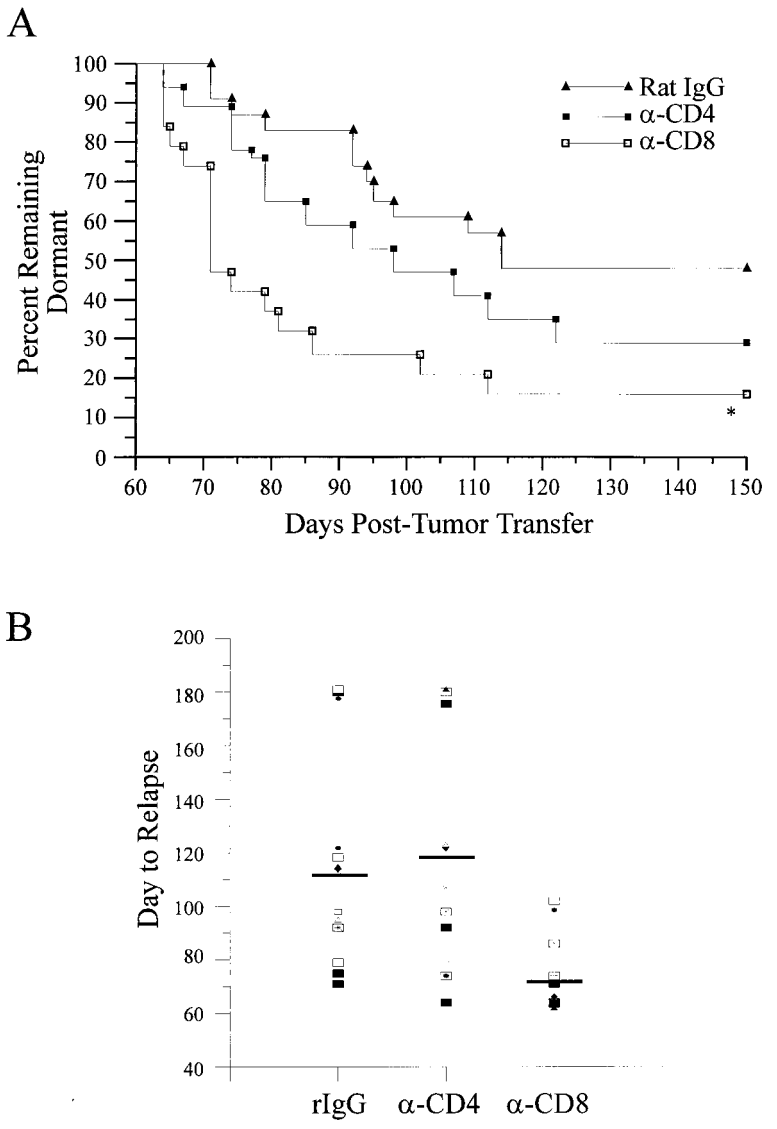


FIGURE 1. Dependence of T cell subpopulations on the maintenance of dormancy. BALB/c mice were immunized with the BCL_1-Ig and challenged with tumor on day 0 as described in *Materials and Methods*. Those animals that displayed splenomegaly before day 60 were disregarded, and only the animals that achieved dormancy by day 60 were used in these depletion studies. *A*, Dormant mice treated with rat IgG ($n = 26$), α-CD4 ($n = 21$), or α-CD8 ($n = 23$). *, Significantly different from the rIgG control ($p = 0.001$). *B*, The day on which each treated animal displayed splenomegaly is plotted on the *y*-axis. Each symbol represents an individual animal. The average number of days to relapse was 111.8 days for rIgG, 118.5 days for α-CD4, and 72.8 days for α-CD8.

in combination with enriched $CD4^+$ or $CD8^+$ T cells from Id-immune mice. The results demonstrate that the $CD8^+$ population could synergize with α-BCL_1-Ig in establishing a tumor-dormant state; in contrast, there was no significant effect contributed by $CD4^+$ T cells (Fig. 2*B*). The trend toward delayed onset of splenomegaly in animals receiving $CD4^+$ T cells, although not significantly different from that in control animals, was reproducible. This effect cannot be readily explained by contamination with $CD8^+$ cells during purification because there was consistently $<0.1\%$ contamination of enriched $CD4^+$ cells with $CD8^+$ cells. Dormancy induced by transferred $CD8^+$ T cells required passive immunization, as growth of tumor cells in nonimmunized (rIg-treated) mice receiving only $CD8^+$ T cells was indistinguishable from that in animals receiving BCL_1 alone (data not shown). Provocatively, the incidence of dormancy induction by day 60 was higher in animals given only $CD8^+$ T cells than that in animals given unfractionated Id-immune T cells (55 vs 17%, respectively), suggesting that the $CD4^+$ T cells might even be counteracting the dormancy-induced role of $CD8^+$ T cells.

Historically, CTL were thought to function in an immune response through direct cell-cell contact. However, more recently, $CD8^+$ T cells have been found to also influence an immune response through the secretion of soluble cytokines, especially IFN-γ (21). We explored the possibility that the activity of transferred Id-immune $CD8^+$ T cells was dependent upon the in vivo production of IFN-γ. Thus, α-BCL_1-Ig-treated SCID mice receiving Id-immune $CD8^+$ T cells were simultaneously treated with an α-IFN-γ mAb to deplete IFN-γ. As shown in Fig. 2*C*, the activity of $CD8^+$ T cells to induce and maintain dormancy was completely abrogated by treatment with α-IFN-γ mAb, and this effect was specific to the depleting mAb, as transferred $CD8^+$ T cells were unaffected by an irrelevant rIg Ab (data not shown). Taken together, these data demonstrate that in combination with α-BCL_1-Ig, Id-immune $CD8^+$ T cells contribute to the induction and maintenance of dormancy in SCID mice. Furthermore, the in vivo activity of $CD8^+$ T cells is dependent upon IFN-γ.

Increased α-BCL_1-IgG2a concentrations in dormant mice

Cytokine expression strongly influences the production of specific Ig isotypes. For example, IFN-γ induces class switching to the IgG2a isotype (22), and IL-4 strongly influences class switching to IgG1 and IgE (22–24). In addition, IFN-$\gamma^{-/-}$ (25) and IFN-$\gamma R\alpha^{-/-}$ (26) animals show severe reductions in the amount of IgG2a produced after immunizations, further supporting a critical

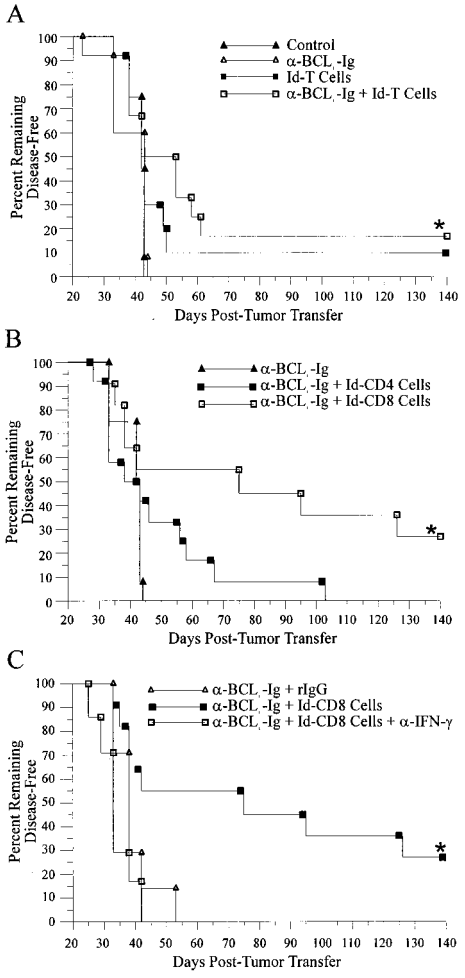


FIGURE 2. Effect of T cell subpopulations on the incidence and duration of dormancy in CB.17 SCID mice. CB.17 SCID mice received 3×10^4 BCL_1 spleen cells on day 0 of tumor transfer. In addition, SCID mice received, either alone or in combination, no additional treatment (control), passive immunization (α-BCL_1-Ig, 50 μg), column-purified Id-immune whole T cells (Id-T Cells), enriched Id-immune CD4$^+$ T cells (Id-CD4 Cells), enriched Id-immune CD8$^+$ T cells (Id-CD8 Cells), a control rat IgG Ab (rIgG), and an α-IFN-γ neutralizing mAb (α-IFN-γ) as described in *Materials and Methods*. The data are pooled from three separate experiments ($n = 12$/group). *A*, Mice receiving α-BCL_1-Ig and Id-T cells were significantly different from both the control and α-BCL_1-Ig groups (*, $p = 0.022$; and, $p = 0.029$, respectively). *B*, Mice receiving α-BCL_1-Ig and Id-CD8$^+$ cells were significantly different from the α-BCL_1-Ig group (*, $p = 0.033$). *C*, Mice receiving α-BCL_1-Ig and Id-CD8$^+$ cells were significantly different from the group receiving α-BCL_1-Ig and rIgG or the group receiving α-BCL_1-Ig, Id-CD8$^+$ cells, and α-IFN-γ (*, $p = 0.028$; and $p = 0.003$, respectively).

role for IFN-γ in IgG2a production. Hence, in some cases the relative concentrations of serum IgG1 and IgG2a reflect cytokine production in vivo (27–31). To investigate a possible connection between cytokine production and the induction of dormancy, serum was harvested from Id-immune animals before tumor transfer, and the α-BCL_1-IgG1 and α-BCL_1-IgG2a titers were measured. As shown in Fig. 3, animals that later became tumor dormant displayed a 4.8-fold higher level of IgG2a Ab titers than animals that did not become dormant. These data further support the importance of increased IFN-γ production in establishing the tumor-dormant state.

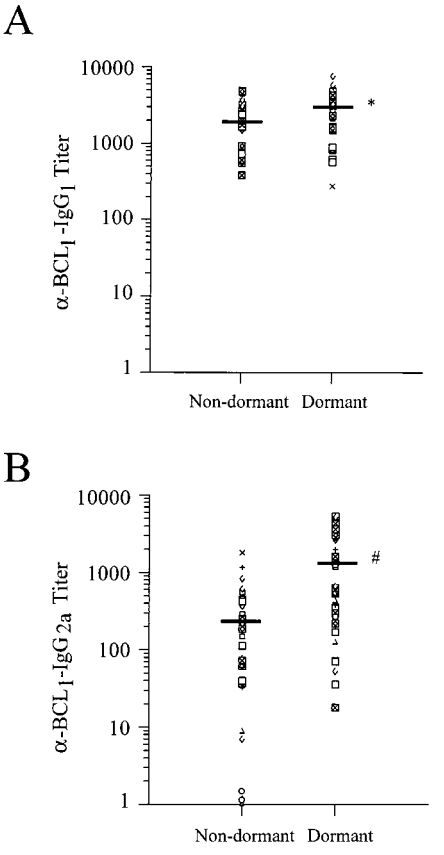


FIGURE 3. Serum α-BCL_1-IgG1 and IgG2a titers in non-dormant and dormant animals. Serum α-BCL_1-Ig titers were measured by ELISA from Id-immune tumor-challenged mice on day 0 before tumor transfer. Following tumor challenge, mice were monitored for splenomegaly until day 60 post-tumor transfer, at which time the mice were categorized as either dormant or nondormant based on splenic palpation. Serum α-BCL_1-Ig titers specific for the IgG1 (*A*) and IgG2a (*B*) isotypes from individual non-dormant and dormant animals are plotted on the ordinate ($n = 35$/group). *, $p = 0.011$; #, $p < 0.001$).

Induction of cell death and cell cycle arrest in IFN-γ- and α-IgM-treated BCL_1.3B3 cells

Based on our finding that IFN-γ was critical for controlling tumor cell expansion in SCID mice and that IFN-γ production correlated with the induction of dormancy in BALB/c mice, we sought to determine whether IFN-γ was playing a direct role in suppressing the growth of the BCL_1 tumor cells. First, proliferation of a BCL_1 cell line (3B3) in response to various stimuli was analyzed by [^{3}H]TdR incorporation (Fig. 4*A*). As expected, cross-linking the MIg on BCL_1.3B3 cells with Gt-αMIgM (polyclonal Ab) dramatically reduced proliferation; in contrast, LPS acted as a stimulus. IFN-γ alone and in combination with Gt-αMIgM markedly inhibited DNA replication in BCL_1.3B3 cells. The titration of the effects of IFN-γ and Gt-αMIgM are shown in Fig. 4, *B* and *C*. They demonstrate that BCL_1.3B3 responded to both reagents in a concentration-dependent manner.

To extend these observations, we tested the ability of IFN-γ to induce cell cycle arrest and apoptosis. Previous studies have demonstrated that signaling through membrane Ig (MIg) of the BCL_1 lymphoma results in arrest in the G_1 phase of the cell cycle (cell cycle arrest) and the induction of apoptosis both in vitro and in vivo (5, 12). As shown in Fig. 5, treatment of BCL_1.3B3 cells with Gt-αMIgM and recombinant murine IFN-γ reduced the proportion of viable cells in the $S/G_2/M$ phases of the cell cycle and increased

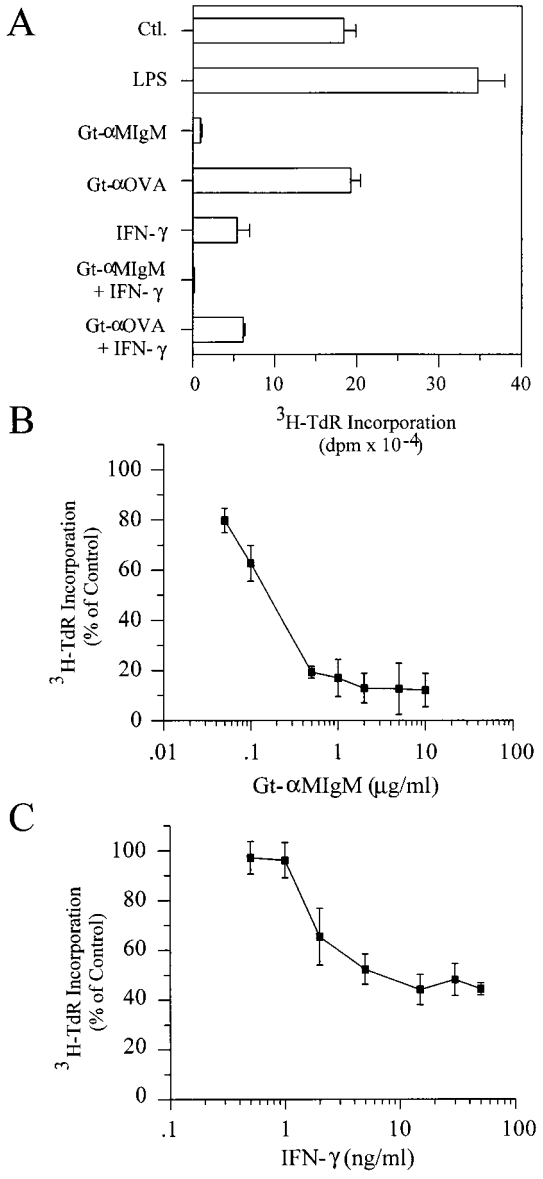
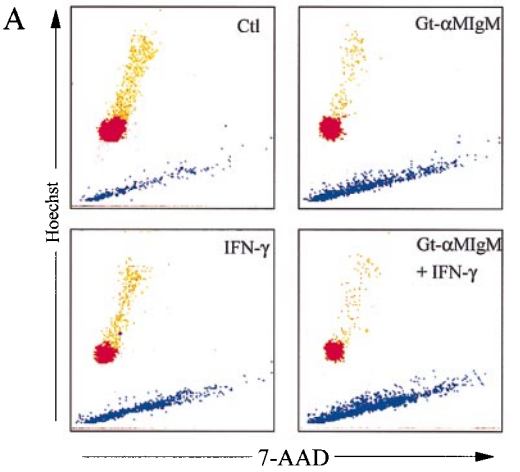


FIGURE 4. IFN-γ inhibits the proliferation of BCL1.3B3. *A*, [^{3}H]TdR incorporation was measured in BCL_1.3B3 cells cultured in the presence of LPS (1 μg/ml), Gt-αMIgM (8 μg/ml), a control Ab Gt-αOVA (8 μg/ml), and recombinant murine IFN-γ (50 ng/ml). *B* and *C*, The dose response of BCL_1.3B3 cells was measured by [^{3}H]TdR incorporation in the presence of Gt-αMIgM (*B*) and IFN-γ (*C*), and the data are expressed as percentage of the untreated control value.



B

	G1	G2/S/M	Apoptotic
Ctl.	62.8	37.2	11.8
Gt-αMIgM	90.3	9.6	41.6
IFN-γ	81.8	18.1	33.2
Gt-αMIgM + IFN-γ	88.7	11.2	64.6
Gt-αOVA	66.8	33.2	10.2
Gt-αOVA + IFN-γ	86.1	13.9	33.4

FIGURE 5. IFN-γ affects cell cycle progression and induces apoptosis of BCL_1.3B3 cells. *A*, BCL_1.3B3 cells were treated with medium alone, Gt-αMIgM (8 μg/ml), IFN-γ (50 ng/ml), a control Gt-αOVA (8 μg/ml), or the combination of Gt-αMIgM and IFN-γ for 24 h followed by staining with 7-AAD and Hoechst dye. Fluorescence was analyzed by flow cytometric analysis using Paint-a-Gait (Becton Dickinson, San Jose, CA) software to quantify the percentages of apoptotic (blue population), G_1 stage (red population), and G_2/S/M stage cells (orange population). *B*, The G_1 and G_2/S/M populations are expressed as a percentage of the total live population, whereas the apoptotic cells are expressed as a percentage of the total number of events.

the percentage of apoptotic cells. Moreover, the combinatorial treatment with Gt-αMIgM and IFN-γ acted synergistically to induce cell cycle arrest and apoptosis. These data implicate IFN-γ in directly regulating the growth of BCL_1 through the induction of cell cycle arrest and apoptosis.

Discussion

With the advent of Id vaccination, cellular anti-Id responses have been clearly documented in both mice (1–3) and humans (4). However, the role of cellular immunity in mediating dormancy has not been well established. Although early studies discounted a cellular involvement in regulating BCL_1 cell dormancy (10, 32), recent data from our group have demonstrated that purified Id-immune T cells can collaborate with α-BCL_1-Ig Ab to increase the incidence and duration of dormancy elicited in SCID mice (12).

In this study T cell subpopulation depletion experiments demonstrated that $CD8^+$ T cells were required for maintaining dormancy in immunocompetent mice. The involvement of $CD8^+$ T cells in regulating dormancy was further supported by the observation that Id-immune $CD8^+$ T cells were sufficient to significantly induce and maintain a dormant state in passively immunized SCID mice.

Previous studies have demonstrated that the transfer of naive BALB/c T cells to C.B-17 SCID mice promotes the development of these cells to a Th1 phenotype by endogenous secretion of IFN-γ (33). In addition, secretion of IFN-γ by NK cells was shown to be required, in some situations, for the induction of CTL activity by $CD8^+$ T cells (34). $CD8^+$ CTLs could act to regulate dormancy by two main mechanisms: 1) direct cytolysis mediated by degranulation and the release of granzyme and perforin, and 2) growth control through a soluble cytokine-mediated mechanism (35). In this regard, it is important to note that IFN-γ is important for this effect, since depletion of IFN-γ abrogates the effect of the $CD8^+$ T cells in the SCID model. Thus, in the SCID model of dormancy, IFN-γ could have two necessary roles; one that involves the development of $CD8^+$ T cells into cytolytic effector cells and another that is directly growth inhibitory to tumor cells. However, it is not clear whether the role of the $CD8^+$ cells is to produce IFN-γ or

whether the role of IFN-γ is to alter the properties of the CD8$^+$ cells.

Surprisingly, we found no requirement for CD4$^+$ T cells to maintain dormancy in immunocompetent mice. Studies designed to determine the efficacy and mechanism of in vivo Ab depletion have demonstrated that depletion of CD4$^+$ T cells with the GK1.5 mAb deletes 90–95% of all resting, naive cells while leaving Ag-activated cells unaffected (36). It was, therefore, possible that the depleting mAb seemingly had no effect due to its inability to delete Ag-specific CD4$^+$ T cells. However, this possibility was unlikely given that the transfer of Id-immune enriched CD4$^+$ T cells had no effect on the induction and maintenance of dormancy in passively immunized SCID mice, while Id-immune whole T cells significantly increased dormancy induction in this model. Thus, we conclude from these results that the CD4$^+$ population is not required to maintain an established state of dormancy, but, rather, may be responsible for the initial production of IFN-γ that we observed as an increase in serum IgG2a levels in animals that were able to achieve the dormant state by day 60. We also observed that animals receiving CD8$^+$ T cells achieved a higher incidence of dormancy than those receiving whole Id-immune T cells, indicating that the CD4$^+$ cells could be suppressing the activity of CD8$^+$ cells. Although both CD4$^+$ and CD8$^+$ cells have the capacity to produce IFN-γ, the transferred CD4$^+$ population could counteract the efficacy of CD8$^+$ cells by the production of other suppressive cytokines such as TGF-β. Further experimentation is warranted to define the interaction between CD4$^+$ and CD8$^+$ T cells in regulating the growth of BCL_1 in vivo.

IFN-γ is a pleiotropic cytokine that displays a variety of both direct and indirect tumoricidal activities. For example, IFN-γ has been shown to activate NK, macrophages, as well as CD8$^+$ T cells, increasing their cytolytic activity on a variety of tumor targets (37–39). IFN-γ can have direct effects on tumor cells by increasing the concentration of class I MHC expressed on the cell surface, thereby increasing their antigenicity (40). Moreover, IFN-γ has been shown to induce cell cycle arrest and apoptosis (41, 42). In this study we demonstrated that IFN-γ can arrest tumor cells in the G_1 phase of the cell cycle and induce apoptosis. Moreover, the pattern of growth arrest comparing IFN-γ with cross-linking surface Ig was indistinguishable as analyzed by flow cytometry. This result indicates that the two unique signals delivered via the IFN-γR and surface Ig act synergistically culminating in a powerful growth arrest response. Taken together, these data support a model in which IFN-γ in collaboration with CD8$^+$ T cells directly mediates growth arrest in vivo, thereby acting synergistically with α-BCL_1-Id Ab to maintain the dormant state.

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References

1. Wilson, A., A. J. T. George, C. A. King, and F. K. Stevenson. 1990. Recognition of a B cell lymphoma by anti-idiotypic T cells. *J. Immunol. 145:3937.*
2. Campbell, M. J., W. Carroll, S. Kon, K. Thielemans, J. B. Rothbard, S. Levy, and R. Levy. 1987. Idiotype vaccination against murine B cell lymphoma: humoral and cellular responses elicited by tumor-derived immunoglobulin M and its molecular subunits. *J. Immunol. 139:2825.*
3. Lauritzsen, G. F., S. Weiss, Z. Dembic, and B. Bogen. 1994. Naive idiotype-specific CD4+ T cells and immunosurveillance of B-cell tumors. *Proc. Natl. Acad. Sci. USA 91:5700.*
4. Kwak, L. W., M. J. Campbell, D. K. Czerwinski, S. Hart, R. A. Miller, and R. Levy. 1992. Induction of immune responses in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumors. *N. Engl. J. Med. 327:1209.*
5. Uhr, J. W., T. Tucker, R. D. May, H. Siu, and E. S. Vitetta. 1991. Cancer dormancy: studies of the murine BCL_1 lymphoma. *Cancer Res. 51:5045.*
6. Yefenof, E., L. J. Picker, R. H. Scheuermann, T. Tucker, E. S. Vitetta, and J. W. Uhr. 1993. Cancer dormancy: isolation and characterization of dormant lymphoma cells. *Proc. Natl. Acad. Sci. USA 90:1829.*
7. Yefenof, E., L. J. Picker, R. H. Scheuermann, E. S. Vitetta, N. E. Street, T. Tucker, and J. W. Uhr. 1993. Induction of B cell tumor dormancy by anti-idiotypic antibodies. *Curr. Opin. Immunol. 5:740.*
8. Vitetta, E. S., T. Tucker, E. Racila, Y.-W. Huang, R. Marches, N. Lane, R. H. Scheuermann, N. E. Street, T. Watanabe, and J. W. Uhr. 1997. Tumor dormancy and cell signaling. V. Regrowth of the BCL_1 after dormancy is established. *Blood 89:4425.*
9. George, A. J. T., S. G. Folkard, T. J. Hamblin, and F. K. Stevenson. 1988. Idiotypic vaccination as a treatment for a B cell lymphoma. *J. Immunol. 141: 2168.*
10. George, A. J. T., A. L. Tutt, and F. K. Stevenson. 1987. Anti-idiotypic mechanisms involved in suppression of a mouse B cell lymphoma, BCL_1. *J. Immunol. 138:628.*
11. Scheuermann, R. H., E. Racila, T. Tucker, E. Yefenof, N. E. Street, E. S. Vitetta, L. J. Picker, and J. W. Uhr. 1994. Lyn tyrosine kinase signals cell cycle arrest but not apoptosis in B-lineage lymphoma cells. *Proc. Natl. Acad. Sci. USA 91:4048.*
12. Racila, E., R. H. Scheuermann, L. J. Picker, E. Yefenof, T. Tucker, W. Chang, R. Marches, N. E. Street, E. S. Vitetta, and J. W. Uhr. 1995. Tumor dormancy and cell signaling. II. Antibody as an agonist in inducing dormancy of a B cell lymphoma in SCID mice. *J. Exp. Med. 181:1539.*
13. Lauritzsen, G. F., and B. Bogen. 1993. The role of idiotype-specific, CD4$^+$ T cells in tumor resistance against major histocompatibility complex class II molecule negative plasmacytoma cells. *Cell. Immunol. 148:177.*
14. Lauritzsen, G. F., and B. Bogen. 1991. Idiotype-specific, major histocompatibility complex restricted T cells are of both Th1 and Th2 type. *Scand. J. Immunol. 33:647.*
15. Shen, Y., and S. Fujimoto. 1996. A tumor-specific Th2 clone initiating tumor rejection via primed CD8$^+$ cytotoxic T-lymphocyte activation in mice. *Cancer Res. 56:5005.*
16. Khazaie, K., S. Prifti, P. Beckhove, A. Griesbach, S. Russel, M. Collins, and V. Schirrmacher. 1994. Persistence of dormant tumor cells in the bone marrow of tumor cell-vaccinated mice correlates with long-term immunological protection. *Proc. Natl. Acad. Sci. USA 91:7430.*
17. Marsili, M. A., M. K. Robinson, G. A. Truitt, and E. F. Wheelock. 1983. Elimination of L5178Y cells from tumor-dormant DBA/2 mice by specific active immunotherapy. *Cancer Res. 43:15.*
18. Marsili, M. A., M. K. Robinson, G. A. Truitt, and E. F. Wheelock. 1983. Cytotoxic T lymphocytes in DBA/2 mice harboring L5178Y cells in a tumor dormant state. *Cancer Immunol. Immunother. 16:59.*
19. Flamand, V., T. Sornasse, K. Thielemans, C. Demanet, M. Bakkus, H. Bazin, O. Tielemans, J. Leo, J. Urbain, and M. Moser. 1994. Murine dendritic cells pulsed in vitro with tumor antigen induce tumor resistance in vivo. *Eur. J. Immunol. 24:605.*
20. King, C. A., M. R. Wills, T. J. Hamblin, and F. K. Stevenson. 1993. Idiotypic IgM on a B-cell surface requires processing for recognition by anti-idiotypic T cells. *Cell. Immunol. 147:411.*
21. Noble, A., P. A. Macary, and M. Kemeny. 1995. IFN-γ and IL-4 regulate the growth and differentiation of CD8$^+$ T cells into subpopulation with distinct cytokine profiles. *J. Immunol. 155:2928.*
22. Snapper, C., and W. E. Paul. 1987. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science 236:944.*
23. Berton, M. T., J. W. Uhr, and E. S. Vitetta. 1989. Synthesis of germ-line γ1 immunoglobulin heavy-chain transcripts in resting B cells: induction by interleukin 4 and inhibition by interferon γ. *Proc. Natl. Acad. Sci. USA 86:2829.*
24. Finkelman, F. D., J. Holmes, I. M. Katona, J. J. Urban, M. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann, and W. E. Paul. 1990. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol. 8:303.*
25. Wang, Z., S. L. Reiner, S. Zheng, D. K. Dalton, and R. M. Locksley. 1994. CD4$^+$ effector cells default to the Th2 pathway in interferon γ-deficient mice infected with *Leishmania major*. *J. Exp. Med. 179:1367.*
26. Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Aguet. 1993. Immune response in mice that lack the IFN-γ receptor. *Science 259:1742.*
27. Agrewala, J. N., G. P. Raghava, and G. C. Mishra. 1993. Measurement and computation of murine interleukin-4 and interferon-γ by exploiting the unique abilities of these lymphokines to induce the secretion of IgG1 and IgG2a. *J. Immunoassay 14:83.*
28. Taylor, C. E., P. J. Baker, T. Hraba, P. Stashak, and M. B. Fauntleroy. 1994. Effects of interferon γ on the antibody response to *Pseudomonas aeruginosa* lipopolysaccharide in mice. *Immunobiology 192:106.*
29. Agrewala, J. N., and G. C. Mishra. 1995. A 38-kDa antigen of *Mycobacterium tuberculosis* predominantly induces the secretion of interleukin-2, interferon-γ and IgG2a antibodies. *Microbiol. Immunol. 39:801.*
30. Waki, S., S. Uehara, K. Kanbe, H. Nariuch, and M. Suzuki. 1995. Interferon-γ and the induction of protective IgG2a antibodies in non-lethal *Plasmodium berghei* infections of mice. *Parasite Immunol. 17:503.*

31. Raz, E., H. Tighe, Y. Sato, M. Corr, J. A. Dudler, M. Roman, S. L. Swain, H. L. Spiegelberg, and D. A. Carson. 1996. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc. Natl. Acad. Sci. USA 93:5141.*
32. Dyke, R. J., H. McBride, A. J. T. George, T. J. Hamblin, and F. K. Stevenson. 1991. Idiotypic vaccination against B-cell lymphoma leads to dormant tumor. *Cell. Immunol. 132:70.*
33. Varkila, K., R. Chatelain, L. M. C. C. Leal, and R. L. Coffman. 1993. Reconstitution of C. *B-17 scid* mice with BALB/c T cells initiates a T helper type-1 response and renders them capable of healing *Leishmania major* infection. *Eur. J. Immunol. 23*:262.
34. Kos, F. J., and E. G. Engelman. 1995. Requirement for natural killer cells in the induction of cytotoxic T cells. *J. Immunol. 155:578.*
35. Kagi, D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science 265:528.*
36. Chace, J. H., J. S. Codery, and E. H. Field. 1994. Effect of anti-CD4 on CD4 subsets. I. Anti-CD4 preferentially deletes resting, naive CD4 cells and spares activated CD4 cells. *J. Immunol. 152:405.*
37. Wigginton, J. M., D. B. Kuhns, T. C. Back, M. J. Brunda, R. H. Wiltrout, and G. W. Cox. 1996. Interleukin 12 primes macrophages for nitric oxide production in vivo and restores depressed nitric oxide production by macrophages from tumor-bearing mice: implications for the antitumor activity of interleukin 12 and/or interleukin 2. *Cancer Res. 56:1131.*
38. Maas, R. A., P. M. A. Roest, M. J. Becker, I. S. Weimar, H. F. J. Dullens, and W. D. Otter. 1992. Effector cells of low-dose IL-2 immunotherapy in tumor bearing mice: tumor cell killing by $CD8^+$ cytotoxic T lymphocytes and macrophages. *Immunobiology 186:214.*
39. Mehrotra, P. T., D. Wu, J. A. Crim, H. S. Mostowski, and J. P. Siegel. 1993. Effects of IL-12 on the generation of cytotoxic activity in human $CD8^+$ T lymphocytes. *J. Immunol. 151:2444.*
40. Kaplan, D. H., V. Shankaran, A. S. Dighe, E. Stockert, M. Aguet, L. J. Old, and R. D. Schreiber. 1998. Demonstration of an interferon γ-dependent tumor surveillance system in immunocompetent mice. *Proc. Natl. Acad. Sci. USA 95:7556.*
41. Bromberg, J. F., C. M. Horvath, Z. Wen, R. D. Schreiber, and J. E. Darnell. 1996. Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon α and interferon γ. *Proc. Natl. Acad. Sci. USA 93:7673.*
42. Trubiani, O., D. Bosco, and R. DiPrimio. 1994. Interferon-γ (IFN-γ) induces programmed cell death in differentiated human leukemic B cell lines. *Exp. Cell Res. 215:23.*