Anti-Gamma Interferon Antibodies Enhance the Immunogenicity of Recombinant Adenovirus Vectors

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Vaccination for eliciting antigen-specific memory CD8+ T cells may be facilitated by manipulating the pleiotropic effects of gamma interferon (IFN-γ). We assessed strategies for modulating the contribution of IFN-γ during the development of antigen-specific cytotoxic T lymphocyte (CTL) populations. We first showed that recombinant IFN-γ suppressed antigen expression in vitro from a recombinant adenovirus (rAd) vector in a dose-dependent manner and that addition of an anti-IFN-γ antibody (Ab) eliminated this suppression. Consistent with these in vitro findings, we found that HIV-1 envelope (Env)-specific CTL responses were higher in IFN-γ-knockout (GKO) mice than in wild-type mice following immunization with rAd. Since these observations suggested that IFN-γ might suppress rAd-induced CTL development, we assessed the ability of anti-IFN-γ Ab administration to augment rAd-elicited CTL in vivo. In fact, blockage of IFN-γ activity by monoclonal Ab administration was associated with elevated levels of interleukin 7 receptor alpha chain-positive (IL-7Rα+) Env-specific CTL populations postboost. These observations illustrate the utility of an anti-IFN-γ Ab for potentiating rAd immunizations to effect quantitative and qualitative changes in the effector and memory CTL populations.

Replication-incompetent recombinant adenovirus serotype 5 (rAd5) vectors have been studied extensively as vaccine candidates for human immunodeficiency virus type 1 (HIV-1) (11, 49, 54) and have been evaluated in gene therapy and T-cell-based vaccine clinical trials (14). However, the usefulness of rAd vectors in human populations may be limited by preexisting anti-Ad5 immunity, which is common in humans (14, 16, 39, 55). In fact, the results of a recent clinical trial of a T-cell-based AIDS vaccine delivered by rAd vectors suggest that preexisting immunity to Ad5 may affect both immunogenicity and vaccine efficacy (14). We are therefore interested in exploring strategies to enhance rAd5 immunogenicity.

Previous studies from our laboratory have shown that rAd expressing a luciferase transgene (rAd-Luc) generates high-level transgene expression for approximately 1 week in wild-type mice (20). In these studies, differences between the clearance of transgene expression in wild-type and athymic mice after rAd-Luc administration suggested that the kinetics of this clearance might be associated with the generation of cellular immune responses. In fact, a relationship between the rate of clearance of transgene expression and the generation of long-term memory cellular immune responses was noted (20). Since vaccine antigen (Ag) clearance can limit the magnitudes of adaptive immune responses, and these responses are associated with the magnitudes of the elicited memory cellular responses, we are evaluating changing the kinetics of rAd-delivered antigen clearance as a strategy for enhancing rAd vaccine-elicited memory responses.

Gamma interferon (IFN-γ) plays a central role in modulating immune responses. During the early phase of the immune response, IFN-γ produced by natural killer (NK) cells acts to initiate acute inflammation. Later, during the antigen-specific phase of the immune response, IFN-γ produced by T lymphocytes regulates antigen presentation and both the proliferation and differentiation of lymphocyte populations (10, 19). IFN-γ is also produced by antigen-presenting cells (APCs), especially interleukin-12 (IL-12)-stimulated macrophages and dendritic cells (DC) (19, 26). IFN-γ may also negatively regulate Ag-specific CD8+ T cell responses by promoting the upregulation of expression of inhibitory NK receptor (iNKR) ligands, resulting in a series of events leading to the restriction of CD8+ T cell proliferation (40). Further, IFN-γ has been shown to suppress IL-7R expression on Ag-specific effector CD8+ T cells, which may be detrimental to the development of memory CD8+ T cells, since IL-7 is an in vivo maintenance factor for memory T cells (45).

The binding of IFN-γ to IFN-γR initiates a cascade of events that includes the activation of kinase janus (JAK) and STAT transcription factor family members to activate the transcription of a variety of IFN-stimulated genes (ISG) (13). IFN-γ increases cellular expression of major histocompatibility complex (MHC) class I and class II molecules, thus augmenting antigen presentation to both CD4+ and CD8+ T cells (10). It also stimulates the exchange of three active-site subunits of the proteasome, which likely favors optimal production of MHC class I-peptide complexes and contributes to the induction of cytotoxic T lymphocytes (CTL) (23, 26). IFN-γ regulates both the expression of certain adhesion molecules, which can influence lymphocyte adhesion and migration to sites of inflammation, and the secretion of chemokines, such as RANTES (10).

It has been suggested that the death phase of antigen-specific T cells following antigen stimulation is regulated by IFN-γ (3, 5, 26). IFN-γ-knockout (GKO) mice and wild-type mice...
injected with attenuated *Listeria monocytogenes* developed similar frequencies and total numbers of epitope-specific CD8+ and CD4+ T cells during the acute phase of the immune response (3, 5, 26). However, in contrast to those in wild-type mice, the frequencies and total numbers of epitope-specific CD8+ or CD4+ T cells in GKO mice did not decrease as the infection was cleared; rather, they remained elevated for >3 months postinfection. Questions have been raised about a central role for IFN-γ in the death of antigen-specific T cells, since increased levels of memory CTL were observed in tumor necrosis factor (TNF)-, TNFR1-, and TNFR2-deficient mice following lymphocytic choriomeningitis virus (LCMV) infection (26). IFN-γ may act directly on antigen-specific CTL or may act indirectly on these CTL via accessory cells (24, 53).

The mechanism of action of IFN-γ in this setting is also unclear. It may act early after infection, suggesting an effect that is not related directly to the generation of antigen-specific cells, or at the onset of the contraction phase of the immune response. It may trigger a programmed contraction of the CTL, enhance the death of these cells, or rescue a subset of these cells from deletion (26).

The *in vivo* neutralization of IFN-γ by anti-IFN-γ antibodies (Ab) inhibits the proliferation and activation of CTL (3, 5, 10). IFN-γ promotes T cell apoptosis, and neutralization of IFN-γ by the administration of an anti-IFN-γ Ab can inhibit the death of effector T cells (3, 5, 10, 50). Moreover, *in vivo* treatment with a blocking anti-IFN-γ Ab has been shown to affect disease pathogenesis in some experimental model systems, suggesting a potential therapeutic use for these antibodies (8–10, 17, 18, 26).

We reasoned that IFN-γ may play an important immunomodulatory role in the generation of immune responses elicited by vaccine vectors, such as live recombinant adenoviral vectors. In fact, it has been shown previously that IFN-γ inhibits transgene expression from rAd vectors by a transcription-related mechanism (46, 52), and reporter gene expression from a rAd-β-galactosidase construct **in vivo** in GKO or anti-IFN-γ Ab-treated mice is greater than that in wild-type mice (52). Extended transgene expression could influence the levels of CTL specific for the transgene product.

The present study was initiated to explore the influences of IFN-γ on the development of CTL following immunization with a rAd5 vector. We show that HIV-1 envelope (Env)-specific CTL responses were higher in GKO mice than wild-type mice following immunization with rAd5 and that administration of an anti-IFN-γ Ab augmented rAd5-elicted CTL **in vivo**. Further, we show by phenotypic analyses that this Ab treatment during a priming immunization changed the effector and memory CTL populations that arose after boosting.

**MATERIALS AND METHODS**

**Mice.** Female BALB/c mice (10 to 12 weeks of age) were obtained from Charles River Laboratories (Cambridge, MA). Female GKO mice (BALB/c background) and age-matched Balb/C mice (49 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal studies were performed in accordance with the Harvard University handbook for the care and use of laboratory animals.

**Vaccine constructs.** The recombinant replication-incompetent, E1-deleted, and E3-inactivated adenovirus (human serotype 5) constructs expressing firefly luciferase (**luc**) (rAd-Luc) or HIV-1 HXB2 **env** (rAd-gp140) and the control rAd expressing no insert were generously provided by Gary Nabel of the National Institutes of Health Vaccine Research Center. The rAd vectors were used in vitro as described below or in vivo by intramuscular (i.m.) inoculation with 105 or 106 particles, half of the particles into each quadriceps.

Recombinant vaccinia virus vABT-271-2-1 containing HIV-1 BH10 **env** (rVacc-gp160) was kindly provided by Dennis Panicali of Therion Biologics Corporation (Cambridge, MA). Mice were inoculated intraperitoneally (i.p.) with 3.3 × 1011 PFU of the virus construct when it was used as a boosting vector or with 2 × 106 PFU when the construct was used as a priming vector.

H-2D* β-p18 tetramer construction. Tetrameric H-2D* major histocompatibility complex (MHC) class I with the p18 peptide RGGPGRAFVTI from the V3 loop of HIV-1 HXB2 Env (48) (H-2D* p18 tetramer) was produced as described previously (1, 37) by using streptavidin coupled to phycoerythrin (PE) (Dako Corporation, Glostrup, Denmark).

**Surface staining of PBMC.** Peripheral blood mononuclear cells (PBMC) from 100 μl blood were prepared as described previously, with red blood cells (RBC) lysed in a solution of NH4Cl-Tris (37, 47). PBMC were washed in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) and were stained with 0.1 to 0.2 μg PE-conjugated H-2D* p18 tetramer (H-2D* p18 tetramer–PE) with anti-CD62L–fluorescein isothiocyanate (FITC) and anti-CD8α-peridinin chlorophyll protein (PerCP) (clones ME1-4 and 53-6-7, respectively; BD Biosciences, San Diego, CA). Anti-CD127 (anti-IL-7Rα–allophycocyanin (APC) (clone A7R34; eBioscience, San Diego, CA) was added to the staining procedure for analyses following boost immunizations. Samples were fixed in 1.5% paraformaldehyde and were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Immunocytometry, San Jose, CA).

**Anti-IFN-γ antibody for in vitro administration.** The National Cell Culture Center produced and purified anti-IFN-γ antibodies from clone XMGl.2, generously provided by Dnax (Palo Alto, CA). XMGl.2, a rat anti-mouse IFN-γ IgGl, was first described by Cherwinski et al. (15) and has been used extensively **in vivo** in mice (41, 59) with various dosing regimens. In these studies, mice were injected i.p. with 200 μg of the anti-IFN-γ antibody or a control purified rat IgG (Antibodies, Inc., Davis, CA) in 250 μl or 400 μl PBS, respectively.

**Measurement of Luc expression in cell culture.** Luciferase expression was assessed using a method adapted from Barouch et al. (6) as follows. Human A549 cells (lung carcinoma epithelia; ATCC) were used. Threefold dilutions of recombinant IFN-γ protein (human; BD Biosciences) and/or the anti-IFN-γ Ab (human, clone B27; BD Biosciences) were performed in 96-well plates in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS (final volume, 50 μl). Then 5 × 104 particles of rAd-Luc were added to each well in 50 μl DMEM–10% FBS. Finally, 105 cells in 100 μl DMEM–10% FBS were added to each well for a total volume of 200 μl, resulting in a multiplicity of infection (MOI) of 500. For experiments with varying MOIs, serial dilutions of rAd-Luc and/or cells were performed as needed. Following incubation at 37°C for 24 h, the medium was replaced with 100 μl PBS, and the plates were frozen at −20°C. Upon thawing, 100 μl of Steady-Glo reagent (Promeza Corporation, Madison, WI) was added to each well. After a 15-min incubation at room temperature, luminescence was analyzed using a microplate luminometer (Turner Biosystems, Inc., Sunnyvale, CA).

**Measurement of Luc expression in muscle homogenates.** Quadriceps muscles were harvested as described previously (22) from mice 1 or 3 days following inoculation with 105 particles of rAd-Luc and were placed in homogenization buffer (40 mM K2HPO4, 0.8 mM EDTA, 0.8 mM dithiothreitol [DTT], 8% glycerol, and 1× reporter lysis buffer, pH 7.2 [Promega]). Muscles were homogenized using a PowerGen model 125 homogenizer (Fisher Scientific) at high speed and were centrifuged. Supernatants in homogenization buffer (total volume, 100 μl) were mixed with 100 μl of Steady-Glo luciferase reagent (Promega). After a 15-min incubation, luminescence was analyzed using a microplate luminometer (Turner Biosystems). Luminescence values are reported as the means for 10 muscles (from 5 mice) ± standard errors (SE).

**Statistical analyses.** Immune cell data are presented as medians with standard errors; luminescence data are reported as means with standard errors. Comparisons among immune cell groups were performed by the Wilcoxon rank-sum test. Comparisons of luminescence were performed by the t test. P values of <0.05 were considered significant.

**RESULTS**

**Effect of IFN-γ on in vitro luciferase expression from rAd-Luc.** To assess the effect of IFN-γ on the immune response elicited by a rAd immunogen, we first evaluated the effect of this cytokine **in vitro** on the expression of a reporter gene by a
rAd construct. We reasoned that levels of reporter gene expression in vitro might be correlated with gene expression in vivo in mice inoculated with a rAd construct carrying a transgene and might predict whether IFN-γ will affect levels of antigen expression from a vaccine vector in vivo.

We first measured the effects of IFN-γ on the expression of luciferase (Luc) in the human A549 (lung carcinoma/epithelium) cell line infected with rAd-Luc for 24 h in the presence of increasing concentrations of recombinant IFN-γ protein. In A549 cells, the addition of recombinant human IFN-γ (rhuIFN-γ) at 50 pg/ml or more decreased the Luc expression measured (in relative light units [RLU]) by approximately two-thirds (4,193 ± 315 RLU with 50 pg/ml rhuIFN-γ versus 12,826 ± 1,145 RLU with no rhuIFN-γ [Fig. 1]). The addition of at least 0.9 µg/ml anti-huIFN-γ Ab to cultures treated with rhuIFN-γ eliminated this effect (14,321 ± 1,889 RLU with 50 pg/ml rhuIFN-γ plus 0.9 µg/ml anti-huIFN-γ Ab versus 4,193 ± 315 RLU with 50 pg/ml rhuIFN-γ alone [Fig. 1]). Moreover, the decrease in Luc light units was dependent on the dose of rhuIFN-γ added to the cultures: cells incubated in the presence of 500 or 1,000 pg/ml rhuIFN-γ produced progressively fewer Luc light units, and progressively higher concentrations of anti-huIFN-γ Ab were required to block these effects.

Development of CTL populations in GKO mice. We next compared the abilities of rAd-gp140 to elicit a dominant epitope-specific CTL response in wild-type and GKO mice in order to evaluate the contribution of IFN-γ to the CTL contraction phase of the vaccine-induced antigen-specific memory CTL. In these experiments, we monitored the H-2Dβ-restricted p18-specific CD8⁺ T lymphocyte responses following vaccination. rAd-gp140 immunization of GKO mice reproducibly elicited higher levels of H-2Dβ/p18⁺ CD8⁺ T cells at peak, throughout the contraction phase, and into the memory phase of the immune response than immunization of wild-type mice (Fig. 2; results of one of two experiments are shown). The H-2Dβ/p18⁺ CD8⁺ T cell population was maximal in GKO mice on day 13 postvaccination, with responses of 19.84% ± 1.42% of peripheral blood CD8⁺ T cells. The CTL responses peaked earlier in wild-type mice, on day 8 postvaccination, with responses of 14.38% ± 1.95%. Eight weeks postvaccination, steady-state levels of H-2Dβ/p18⁺ CD8⁺ T cells were still higher in GKO mice (7.63% ± 0.21% of CD8⁺ T cells) than in wild-type mice (4.81% ± 0.37%). Therefore, although we did not observe the absence of a CTL contraction phase in GKO mice as described by Badovinac et al. (5), we documented consistently higher levels of H-2Dβ/p18⁺ CD8⁺ T cells in GKO mice than in wild-type mice during all phases of the immune response following rAd-gp140 immunization.

Effects of IFN-γ on luciferase expression from rAd-Luc in vivo in muscle. We were interested in determining whether the significant increase in p18-specific CTL levels observed in GKO mice inoculated with rAd-gp140 reflected a specific effect of IFN-γ on CTL development following inoculation with rAd vectors. Since others had shown that IFN-γ inhibits transgene expression from rAd vectors in vitro and in vivo (46, 52), we were interested in determining whether the high levels of p18-specific CTL we had observed in GKO mice were a consequence of high levels of expression of the transgene product in these mice. We therefore compared rAd-transgene expression in inoculated muscle in vivo in GKO mice and wild-type mice in order to monitor the effects of IFN-γ on rAd transgene expression. Following i.m. inoculation with 10⁹ particles of rAd-Luc, Luc luminescence was detected in the muscles of both wild-type and GKO mice. However, we did not detect any significant differences between the measured Luc light units in wild-type versus GKO muscles at 1 (2,528 ± 758 RLU for BALB/c mice versus 1,634 ± 522 RLU for GKO mice; P = 0.34) or 3 (1,226 ± 343 RLU for BALB/c mice versus 1,516 ± 416 RLU for GKO mice; P = 0.60) days following administration of the rAd-Luc construct (data not shown). We considered that this might be the case because the expression levels of IFN-γ mRNA and protein may simply not be high enough in these mice to document differences in expression in their skeletal muscle tissues. Thus, we were unable to determine whether expressed protein levels were higher in the muscles of GKO mice than in those of wild-type mice.
Effects of the anti-IFN-γ Ab on CTL development. We then sought to determine whether the high levels of H-2D<sup>d/p18</sup> CD<sup>8⁺</sup> T cells generated in GKO mice immunized with rAd-gp140 (Fig. 2) would also be observed in wild-type mice treated with anti-IFN-γ monoclonal antibodies in conjunction with rAd-gp140 immunization. We first sought to determine when during the course of CTL development an absence of IFN-γ would contribute the most to increasing levels of H-2D<sup>d/p18</sup> CD<sup>8⁺</sup> T cells. Specifically, we wanted to clarify whether the activities of IFN-γ were most important during the induction, contraction, or memory maintenance phase of the vaccine-elicted CTL response. To investigate these possibilities, we administered an anti-IFN-γ Ab at varying times following rAd-gp140 inoculation of wild-type mice and evaluated the evolution of p18-specific CTL responses.

Initially, we used anti-IFN-γ Ab clone XMG1.2 (rat IgG1) to explore the effects of IFN-γ on CTL responses elicited by rAd-gp140 immunization using 3 administration regimens. Mice were immunized with rAd-gp140 and were then treated with 200 μg anti-IFN-γ Ab or control rat IgG according to one of three regimens: treatment A (days 3, 5, and 7 postinoculation [p.i.]) (pre-CTL peak), treatment B (days 6, 8, 10, and 12 postinoculation) (CTL peak), or treatment C (days 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26 postinoculation) (peak and post-peak CTL). The levels of H-2D<sup>d/p18</sup> CD<sup>8⁺</sup> T cells in peripheral blood samples were monitored for 8 weeks following immunization.

With experimental treatment A, increases in the H-2D<sup>d/p18</sup> CD<sup>8⁺</sup> T cell populations in anti-IFN-γ Ab-treated mice (23.7% ± 2.25% of CD8<sup>+</sup> T cells) over those in control Ab-treated mice (12.35% ± 0.87%) (P < 0.02) (Fig. 3A) were observed only at the day 7 peak time point. Control Ab-treated mice developed responses that peaked on day 10 postinoculation, at 21.1% ± 0.88%. The same early and transient difference was observed in a second, similarly performed experiment (data not shown). We then treated mice with the anti-IFN-γ Ab beginning on day −3 relative to rAd-gp140 inoculation in order to determine whether earlier administration of the anti-IFN-γ Ab would result in an even further increase in the proportion of H-2D<sup>d/p18</sup> CD<sup>8⁺</sup> T cells. With this altered regimen, we observed no further increase in the H-2D<sup>d/p18</sup> CD<sup>8⁺</sup> T cell populations over that seen with treatment A; instead, we saw results strikingly similar to those for treatment A, with mice that received the anti-IFN-γ Ab showing an elevation in the levels of H-2D<sup>d/p18</sup> CD<sup>8⁺</sup> T cells only at the day 7 time point (P < 0.01) (data not shown). We also observed no differences between groups with treatment B (Fig. 3B), in which the Ab was administered on days 6, 8, 10, and 12 post-rAd-gp140 inoculation.

Treatment C, however, increased the populations of H-2D<sup>d/p18</sup> CD<sup>8⁺</sup> T cells in mice treated with the anti-IFN-γ Ab beginning on day 17 post-rAd-gp140 inoculation and continuing for the duration of the experiment (Fig. 3C). On day 17 postinoculation, 20.57% ± 0.91% of CD8<sup>+</sup> T cells in the PBMC of mice treated with the anti-IFN-γ Ab were p18 specific, compared to 13.48% ± 1.11% in mice receiving the control Ab (Fig. 3C). These differences represent increases of approximately 50% in the levels of H-2D<sup>d/p18</sup> CD<sup>8⁺</sup> T cells in experimentally treated mice over those in control mice and were significant (P < 0.01 at day 27 postinoculation). Levels of p18-specific memory CTL were also significantly increased at day 56 postinoculation: 12.94% ± 1.27% of CD8<sup>+</sup> cells in mice treated with the anti-IFN-γ Ab, compared to 6.91% ± 1.18% in control Ab-treated mice (P < 0.02), representing an increase of approximately 90%. We repeated the treatment C regimen and again observed this late and persistent increase in the levels of H-2D<sup>d/p18</sup> CD<sup>8⁺</sup> T cells in anti-IFN-γ Ab-treated mice (data not shown).

Effects of the anti-IFN-γ Ab on the development of memory CTL. In previous studies, we and others have shown that the definition of memory CD8<sup>+</sup> T cell subpopulations in mice on the basis of CD62L expression alone does not allow the delineation of functionally distinct CTL subpopulations (35, 38, 58,
We performed two analyses to determine whether the population elicited by rAd5 vectors in the presence or absence of IFN-\(\gamma\) administration to rAd-gp140-immunized mice. Shown are the percentages of total CD8\(^+\) T cells (left) and H-2D\(^b/p18\) tetramer-positive CD8\(^+\) T cells (right) expressing CD62L in the PBMC of BALB/c mice inoculated i.m. with 10\(^6\) particles rAd-gp140 and treated with 200 \(\mu\)g of anti-IFN-\(\gamma\) Ab clone XMG1.2 or control rat IgG i.p. on days 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26 p.i. Plotted values represent the medians \(\pm\) SE for 6 mice treated with the control Ab or 7 mice treated with an anti-IFN-\(\gamma\) Ab.

Huster et al. (34) showed that the expression of the IL-7 receptor (IL-7R) alpha chain (CD127) can serve as a marker for long-lived memory T cells in mice, its presence distinguishing between memory and effector T cells (see also references 2 and 36). This observation was consistent with data showing that IL-7 can contribute to the maintenance of memory T cells in vivo (45). Moreover, these investigators employed a combination of surface staining for CD127 and CD62L to further distinguish between two functionally distinct memory cell subsets that are similar to central memory T cells (CD127\(^{high}\) and CD62L\(^{low}\)) and peripheral effector memory T cells (CD127\(^{high}\) and CD62L\(^{low}\)) (34).

We built upon this phenotyping strategy for memory T cell analysis to obtain information about the protective immunity elicited by rAd5 vectors in the presence or absence of IFN-\(\gamma\). We performed two analyses to determine whether the populations of H-2D\(^b/p18\) CD8\(^+\) T cells generated by rAd-gp140 inoculation in association with anti-IFN-\(\gamma\) administration had the same characteristics as H-2D\(^b/p18\) CD8\(^+\) T cells generated in the presence of IFN-\(\gamma\). First, we monitored the expression of CD62L on the surfaces of these cells during the 8-week studies, evaluating the kinetics of CD62L reexpression on CD8\(^+\) T cells as a surrogate marker for the emergence of memory CD8\(^+\) T cells. The control mice for this study included a group of mice treated with a control Ab as well as previously studied mice (not shown) evaluated to define the kinetics of CD62L expression on H-2D\(^b/p18\) CD8\(^+\) T cells following rAd-gp140 immunization.

The kinetics of CD62L reexpression by the CTL populations was identical for mice that had received the anti-IFN-\(\gamma\) Ab and those in mice treated with the control Ab. However, for the second 4 weeks, the levels of H-2D\(^b/p18\) CD8\(^+\) T cells in mice previously treated with the anti-IFN-\(\gamma\) Ab remained elevated, while those in mice originally treated with the control Ab began to decline. By 7 weeks postboost, mice previously treated with the anti-IFN-\(\gamma\) Ab had significantly higher p18-specific CD8\(^+\) T lymphocyte levels (35.23\% \(\pm\) 2.73\% of CD8\(^+\) T cells) than mice previously treated with the control Ab (23.47\% \(\pm\) 1.47\%) (\(P < 0.05\)). Similar results were observed for mice previously treated with the anti-IFN-\(\gamma\) Ab according to the experimental treatment C regimen but boosted with 3.3 \(\times\) 10\(^5\) PFU rVac-gp160 at 27 rather than 46 weeks postinoculation (data not shown).

Following rVac-gp160 boost immunization, we also observed no difference in the kinetics of CD62L reexpression in either the bulk CD8\(^+\) T cell population (Fig. 5B, top left) or the H-2D\(^b/p18\) CD8\(^+\) T cell population (Fig. 5B, top right) between the groups of mice treated with the anti-IFN-\(\gamma\) Ab versus the control Ab associated with the priming rAd immunization. However, the CD62L reexpression kinetics following boosting were different from those following priming: surface expression of CD62L was reduced to \(<30\%\) of H-2D\(^b/p18\) CD8\(^+\) T cells in both groups of mice as soon as these cells were generated (day 7 postboost). These cells began to reexpress CD62L almost immediately, ending the 8-week boost immunization study at approximately 55\% CD62L\(^{+}\) cells. In contrast, surface expression of CD62L on the total CD8\(^+\) T cell population in both groups dropped to approximately 60\% by day 7.
postboost and then slowly returned to 70 to 75% by week 8 postboost.

Conversely, we observed that the number of H-2D$d/p18$ CD8$^+$ T cells expressing IL-7R$\alpha$ in response to the rVac-gp160 boost immunization was significantly higher in mice that had received the anti-IFN-\(\gamma\) Ab associated with the priming immunization than in mice that had received the control Ab throughout the first 6 weeks of the boost study (\(P < 0.05\)) (Fig.

**FIG. 5.** Memory response characteristics of total CD8$^+$ T cells and H-2D$d/p18$ tetramer-positive CD8$^+$ T cells following rVac-gp160 boost immunization of mice previously administered an anti-IFN-\(\gamma\) Ab in association with rAd-gp140 prime immunization. (A) H-2D$d/p18$ tetramer-positive CD8$^+$ T cells as a percentage of total CD8$^+$ T cells were monitored in the PBMC of BALB/c mice inoculated i.p. with \(3.3 \times 10^5\) PFU rVac-gp160 after i.m. priming with \(10^9\) particles rAd-gp140 associated with 200 \(\mu\)g of anti-IFN-\(\gamma\) Ab clone XMG1.2 or control rat IgG administered i.p. on days 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26 p.i. (B) Percentages of total CD8$^+$ T cells (left) and H-2D$d/p18$ tetramer-positive CD8$^+$ T cells (right) expressing CD62L (top) or IL-7R$\alpha$ (bottom) in the PBMC of BALB/c mice treated as described for panel A. (C) Percentages of H-2D$d/p18$ tetramer-positive CD8$^+$ T cells expressing IL-7R$\alpha$ but not CD62L in the PBMC of BALB/c mice treated as described for panel A. Plotted values represent the medians ± SE for 6 mice treated with the control Ab or 7 mice treated with the anti-IFN-\(\gamma\) Ab. *, \(P < 0.05\).
5B, lower right). At the time of the boosting immunization, IL-7Rα was expressed on 94.80% ± 1.13% of H-2Dd/p18\(^+\) CD8\(^+\) T cells in mice receiving their priming immunization associated with anti-IFN-γ Ab treatment compared with 77.05% ± 5.78% (P < 0.05) of the same population generated by the priming immunization associated with control Ab treatment. By day 3 postboost, cell surface expression of IL-7Rα was reduced to approximately 80% of H-2Dd/p18\(^+\) CD8\(^+\) T cells in mice previously treated with the anti-IFN-γ Ab and to approximately 60% in mice previously treated with the control Ab. The vaccine-induced cells beginning expressing IL-7Rα almost immediately, with nearly 90% of H-2Dd/p18\(^+\) CD8\(^+\) T cells in both groups expressing IL-7Rα by 8 weeks post-boost immunization. At the same time, the expression of IL-7Rα on total CD8\(^+\) T cell populations was not different in the 2 groups of mice, with expression on <10% of the H-2Dd/p18\(^+\) CD8\(^+\) T cells for the duration of the study (Fig. 5B, lower left).

Further, the same p18-specific CD8\(^+\) T lymphocyte populations in mice treated with the anti-IFN-γ Ab following the experimental treatment C regimen and boosted with 3.3 \times 10^5 PFU rVac-gp160 at 46 weeks postinoculation had a significantly higher proportion of memory cell precursor IL-7Rα\(^+\) cells (36) than did p18-specific CD8\(^+\) T lymphocyte populations in mice treated with the control Ab. Between weeks 1 and 3 postboost, IL-7Rα\(^+\) CD62L\(^-\) p18-specific CTL populations constituted a significantly larger proportion of the total p18-specific CD8\(^+\) T lymphocyte population in mice treated with the anti-IFN-γ Ab than in mice treated with the control Ab: 53.59% ± 1.37% versus 44.58% ± 1.88% at 1 week, 51.55% ± 2.15% versus 47.95% ± 0.93% at 2 weeks, and 47.55% ± 1.82% versus 44.62% ± 0.43% at 3 weeks (P < 0.05 for all these time points) (Fig. 5C). At the same times postboost, equivalent proportions of IL-7Rα\(^+\) CD62L\(^-\) and IL-7Rα\(^-\) H-2Dd/p18\(^+\) CD8\(^+\) T cell populations were observed in both anti-IFN-γ Ab- and control Ab-treated mice (data not shown).

In a similarly performed experiment, mice originally treated with the anti-IFN-γ Ab following the experimental treatment C regimen but boosted with 3.3 \times 10^5 PFU rVac-gp160 at 27 rather than 46 weeks postinfection also exhibited significantly elevated levels of IL-7Rα\(^+\) CD62L\(^-\) p18-specific CD8\(^+\) T lymphocytes (data not shown).

**Vector specificity of anti-IFN-γ Ab effects.** Finally, we investigated whether the increases in surviving H-2Dd/p18\(^+\) CD8\(^+\) T cell populations that we observed in mice treated with the anti-IFN-γ Ab following immunization with rAd would also have been seen in mice treated with the anti-IFN-γ Ab following immunization with a different vaccine vector. To this end, we treated wild-type mice with the anti-IFN-γ Ab on days 4, 6, 8, 10, 12, and 14 following immunization with a recombinant vaccinia virus (2 \times 10^7 PFU rVac-gp160, administered i.p.). This schedule was designed to take into consideration the specific kinetics of the development of p18-specific CD8\(^+\) T lymphocytes in mice immunized with rVac-gp160, with peak p18-specific CD8\(^+\) T lymphocyte levels expected at day 7 and contraction expected by day 14 postinfection (35). Mice treated with anti-IFN-γ exhibited higher levels of H-2Dd/p18\(^+\) CD8\(^+\) T cells on day 5 postinfection than mice treated with the control Ab (6.60% ± 0.33% versus 4.93% ± 0.34% of CD8\(^+\) T cells; P < 0.01). However, these elevations in p18-specific CD8\(^+\) T lymphocyte populations were relatively small (approximately 35% greater than the populations in the controls) compared to the magnitude of the expansion of p18-specific CD8\(^+\) T lymphocytes observed in mice receiving a similar anti-IFN-γ treatment regimen after rAd-gp140 immunization (populations approximately 90% greater than those in controls [Fig. 3C]). Moreover, these differences were no longer observed by day 7 postinfection (data not shown). Therefore, the durable effect of anti-IFN-γ treatment on the tetramer-binding CD8\(^+\) T lymphocyte response was observed in mice immunized with a rAd but not a recombinant vaccinia virus vector.

**DISCUSSION**

We initiated these experiments to investigate the role that IFN-γ plays in the development of vaccine-elicited CTL. Previous studies have suggested that IFN-γ shapes both the expansion and contraction phases of the CTL response (3, 5, 10, 26, 51) and regulates the phenotype and function of Ag-specific T cells (25). We observed both effects of IFN-γ on vaccine-elicited CTL throughout primary and secondary immune responses. Furthermore, we observed that the effects of IFN-γ are influenced by the properties of the immunogen, since rAd-gp140-induced CTL responses were more affected by the presence of IFN-γ than were recombinant vaccinia virus-induced CTL responses.

It has been shown previously that IFN-γ inhibits transgene expression from rAd vectors by a transcription-related mechanism (46, 52), and the present results are consistent with these findings. We observed that IFN-γ decreased reporter gene expression from a rAd vector in a dose-dependent manner in vitro. Further, this effect on reporter gene expression could be counteracted by an anti-IFN-γ Ab. Moreover, the populations of H-2Dd/p18\(^+\) CD8\(^+\) T cells elicited by in vivo rAd-gp140 administration in GKO mice were elevated compared to those in wild-type mice. This finding suggests that the absence of IFN-γ in vivo may result in elevated levels of gp140 expression from the rAd vector. The absence of IFN-γ in vivo may also extend the duration of transgene expression (44), which could enhance the levels of CTL specific for the transgene product.

We sought to determine whether these observations with GKO mice were a consequence of increased p18 antigen expression or whether IFN-γ was playing another role during CTL development. We evaluated the effects of IFN-γ on rAd-transgene antigen levels in vivo in muscles at the site of rAd vector inoculation but did not detect differences in expression between wild-type and GKO mice. It is possible that low baseline levels of IFN-γR mRNA expression in the skeletal muscles of wild-type mice precluded the ability to alter transgene expression by manipulating IFN-γ expression. It is also possible that the times chosen for sampling were not optimal for the detection of differences in rAd-transgene expression in muscle. However, these days were chosen for analysis based on previous studies of CTL elicitation by rAd vectors and the kinetics of antigen expression in muscle following DNA-gp120 immunization (data not shown). Nevertheless, the present results do not rule out the possibility that differences in rAd-transgene expression in muscles that are important for immune stimulation cannot be detected in the experimental systems that were employed. Alternatively, the effects of IFN-γ on rAd-transgene expression that led to altered immune responses in GKO mice
may have occurred at sites outside the muscle. The absence of IFN-γR expression in muscle tissue in fact supports the notion that IFN-γ effects do not take place at the site of rAd vector inoculation in the muscle.

Experiments in which we administered the anti-IFN-γ Ab to wild-type mice following rAd-gp140 immunization suggested that blocking of IFN-γ effects during certain circumscribed periods could increase the expansion of rAd-induced CTL populations. The effects of delivering a blocking antibody according to the schedule shown for experimental treatment A (Fig. 3A) suggest an early effect of IFN-γ on CTL development. Such an effect of IFN-γ may be modified by specific components of the immune system, such as NK cells (10, 19, 31, 56). Alternatively, these results may be a consequence of the elimination of IFN-γ-mediated suppression of rAd-transgene expression (44, 46, 52), with a concomitant increase in p18 antigen expression levels, which drives CTL expansion. The demonstration of late elevations of the proportions of H-2D\(^{\alpha}\)/p18\(^+\) CD8\(^+\) T cells, illustrated for experimental treatment C (Fig. 3C), suggests a second phase of CTL development during which IFN-γ can influence the magnitude of CTL populations. These results may also reflect the elimination of IFN-γ-mediated suppression of rAd-transgene expression, with an associated increase in p18 antigen levels driving CTL expansion. Alternatively, they may reflect a decrease in the rate of CTL death during the contraction phase of the immune response, consistent with a proapoptotic role for IFN-γ during CTL contraction. Our results are at odds with the predictions of others (25) that IFN-γ exerts its regulatory mechanism(s) directly on T cells very early during the immune response (4, 24, 25). This finding may be attributable to differences between responses to live challenges and to replication-incompetent vectors.

Blocking IFN-γ activity in vivo both early (pre-peak CTL expansion) (Fig. 3A, treatment A) and late (post-peak CTL contraction and memory phase) (Fig. 3C, treatment C) following immunization led to increased levels of H-2DR\(^{d}\)/p18\(^+\) CD8\(^+\) T cells. Importantly, we observed no changes in the surface expression of CD62L in anti-IFN-γ Ab-treated mice that might suggest a difference in the functional properties of these memory CTL (Fig. 4). rVac-gp160 boosting of the mice subjected to experimental treatment C (Ab given between days 6 and 26 p.i.) did, however, uncover evidence of functional changes in the secondary H-2D\(^{d}\)/p18\(^+\) CD8\(^+\) T effector and memory cell populations. Mice that received the anti-IFN-γ Ab in association with the rAd-gp140 priming immunization exhibited no decline in H-2DR\(^{d}\)/p18\(^+\) CD8\(^+\) T memory cell populations through 7 weeks postboost, while the same cell populations in mice that had received the control Ab in association with the rAd-gp140 priming did exhibit such a decline postboost (Fig. 5A). Meanwhile, the kinetics of reexpression of CD62L following the boost immunization were not altered by anti-IFN-γ Ab treatment (Fig. 5B, top). The differences seen between these kinetics and those observed following the priming immunization (Fig. 4) are likely to reflect the usual differences between naïve and primed CD8\(^+\) T cell populations.

The expression of IL-7Ra\(^+\), a molecule whose expression is associated with long-lived memory T cells (2, 34, 36), on the surfaces of p18-specific effector and memory T cells was also elevated through 6 weeks postboost in mice that received the anti-IFN-γ Ab but not the control Ab following priming (Fig. 5B, bottom right). These results are of interest because they suggest that the addition of an anti-IFN-γ Ab to the vaccination regimen enhances the formation of memory CD8\(^+\) T cells, which are likely IL-7 responsive. Such IL-7Ra\(^+\) cells are important in that they should give rise to secondary effector CTL programmed for survival in the secondary memory pool. In fact, Bell et al. (7) determined that CD4\(^+\) T cells expressing high levels of IL-7R generate rapid and robust memory responses, and Cellerai et al. (12) demonstrated that higher IL-7R expression is associated with a high proliferation capacity.

The mechanism underlying the increase in cell surface IL-7R expression seen in our studies may be explained, at least in part, by the studies of Hidalgo et al. (33) demonstrating that IFN-γ reduces CTL generation largely by limiting the proliferation of IL-2-producing CD4\(^+\) T cells. This creates a feedback loop in which effectors produce IFN-γ, which limits the production of IL-2, leading, in turn, to the limitation of CTL generation. The CD127\(^+\) memory precursor CD8\(^+\) T cells produce IL-2 (34, 36). Wu et al. (61) demonstrated increased IL-2 production, T cell priming, and in vitro chemotaxis and recruitment to draining lymph nodes in bone marrow-derived DC from GKO mice. Moreover, Haring and Harty (25) found that the absence of IFN-γ signaling changed the phenotypes of both CD8\(^+\) and CD4\(^+\) T cells generated after infection. These cells reacquired CD127 expression more quickly than wild-type cells and maintained this expression longer, and a greater proportion were capable of producing both IFN-γ and IL-2 following Ag stimulation. Furthermore, Tewari et al. (53) demonstrated direct IFN-γ suppression of IL-7R expression on CTL.

Our data are consistent with recent studies showing that rAd5 vectors primarily induce T cell responses that are skewed toward producing IFN-γ, while rAd vectors of alternative serotypes (specifically rAd26 and rAd48) induce higher proportions of more balanced, polyfunctional IL-2\(^+\) T cell responses (39). IFN-γ regulates the contraction, phenotype, and function of T cells, and the generation of polyfunctional T cell responses by vaccines appears to confer better protection against viral pathogens than the generation of T cells producing IFN-γ alone.

Between weeks 1 and 3 postboost, IL-7Ra\(^+\) CD62L\(^-\) p18-specific CTL populations made up a significantly larger proportion of the total p18-specific CTL population in mice treated with the anti-IFN-γ Ab than in mice treated with the control Ab in association with the rAd-gp140 priming immunization (Fig. 5C). The fact that these populations were not different on the day of boosting provides further evidence that treatment with the anti-IFN-γ Ab during a priming immunization changed the resulting memory cell population, leading to significant changes in the effector and memory CTL populations that arose after the boosting immunization. While the effects of IFN-γ on the development of CTL observed in the present studies may be specific to responses elicited by the rAd5 vector, they suggest that anti-IFN-γ Ab treatment appears to enhance the efficacy of rAd5 vaccine vectors.
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