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Targeted Delivery of Murine Interferon- γ using a Recombinant Fowlpox Virus: NK Cell Recruitment to Regional Lymph Nodes and Priming of Tumor-Specific Host Immunity

Hasan Zeytin, Eva Reali, David A. Zaharoff, Connie J. Rogers, Jeffrey Schlom, and John W. Greiner

Abstract

IFN- γ is a pro-inflammatory cytokine which also acts as a potent immunomodulatory agent. In this study, a replication-deficient recombinant avian (fowlpox) virus was engineered to express the murine IFN- γ gene (rF-IFN- γ) with the rationale of delivering concentrated levels of the cytokine to a local tissue microenvironment. Subcutaneous (s.c.) rF-IFN- γ administration resulted in IFN- γ production that (1) was restricted to the tissue microenvironment of the injection site and (2) was biologically active as evidenced by a significant increase of class I MHC expression levels in s.c. growing tumors following rF-IFN- γ administration. Infection of a highly tumorigenic murine cell line, MC38, with rF-IFN- γ functioned as an effective tumor cell-based vaccine by protecting mice from the formation of primary tumors and from subsequent tumor challenge. The cell-based vaccine was completely ineffective if mice were vaccinated with MC38 cells either pretreated with recombinant IFN- γ or infected with the wild-type fowlpox virus. Analysis of the regional lymph nodes draining the site of injection of the rF-IFN- γ based tumor cell vaccine revealed the presence of tumor specific cell lysis (CTL) as well as significant amount of lysis directed at NK-sensitive YAC-1 cells. Flow cytometric analyses coupled with functional assays confirmed the sustained presence of NK1.1⁺ cells within those draining lymph nodes for up to 5 days following rF-IFN- γ injection. Mice treated with NK cell-depleting antibodies prior to the injection of the rF-IFN- γ -infected MC38 tumor cells were not protected from primary tumor growth; analysis of the lymph nodes draining the injection site in NK-depleted mice, revealed an accompanying loss of the tumor specific CTL activity. The findings provide evidence that NK cells, known for their contributions to host innate immunity, also provide immunoregulatory signals required for the development of an adaptive immune response which, in turn, protected vaccinated mice against tumor growth.

INTRODUCTION

Recombinant interferon-gamma protein (rIFN- γ) is a potent pro-inflammatory cytokine that exerts a variety of anti-proliferative and immunomodulatory actions which have implicated it as a potential anti-cancer agent. Among its immunomodulatory effects are (a) the upregulation of major histocompatibility complex (MHC), Fas molecules and tumor associated antigens on tumor cells (1,2) potentially making tumors better targets for the immune system, (b) the inhibition of the production of immunosuppressive factors such as TGF- β (3) and PGE₂ (4) and (c) potent anti-angiogenic effects which inhibit tumor metastasis (5,6). Despite those multiple actions that could negatively impact tumor growth, the effectiveness of rIFN- γ as a single anticancer agent has been disappointing. Clinical studies of patients diagnosed with late-stage

cancers have revealed some antitumor responses (7,8), but only at doses of rIFN- γ that are associated with severe and treatment-limiting systemic toxicities.

Unlike the type I interferons, IFN- γ is produced by a restricted group of cells that include T cells activated during adaptive immunity as well as NK and NK T cells that provide IFN- γ for the innate phase of the host immune response. Those observations indicate that IFN- γ actions are primarily local and, indeed, IFN- γ has been shown to enhance T_H1 polarization by: (1) facilitating IL-12 production by DCs primed by microbial products (9) and (2) synergizing with T cell receptor (TCR) signals to maximally induce T-bet which controls IL-12 receptor expression in naive T cells and acts as a master regulator of T_H1 differentiation (10). Several investigators have proposed that a more rational approach might be to deliver high concentrations of IFN- γ to the local tissue environment to exploit its antigen upregulating, angiostatic and immunomodulatory actions while avoiding the toxicities associated with its systemic administration. Indeed, research groups have engineered various recombinant viruses (11-15) and DNA-based technology (16) as delivery platforms to target IFN- γ to predetermined tissues. Those approaches have been shown to be safe, well-tolerated and capable of inducing anti-tumor immunity in experimental animals (14,16). For example, the intratumoral administration of recombinant retro- or adenoviruses expressing the IFN- γ gene revealed an excellent safety profile coupled with an enhanced tumor immunogenicity which correlated with disease stabilization in patients diagnosed with advanced melanoma (17,18). Other studies have shown that genetically modifying transformed cells to produce a type I interferon, particularly IFN- α , can also reduce their tumorigenicity (19,20).

Fowlpox virus, a member of the *Avipoxvirus* genus, with a double-stranded DNA genome of about 300 kb, has been developed as a live virus vector capable of delivering vaccines and immune adjuvants safely in mammalian hosts (11,13,21,22). Although the replication of fowlpox-based recombinant viruses is blocked in mammalian cells, inserted genes under transcriptional control of early promoters are expressed with high fidelity. Recent preclinical and clinical studies have shown the ability to administer recombinant fowlpox viruses multiple times without inducing neutralizing host immunity (21,23). A recombinant fowlpox virus expressing murine IFN- γ (rF-IFN- γ) is described in this study. Local IFN- γ production following a single rF-IFN- γ injection selectively expanded the lymphoid cell populations, particularly the NK1.1+ cells, in the regional draining lymph nodes. Those NK1.1+ cells exhibited unique YAC-1 lytic characteristics and provided crucial regulatory signals for priming of an antigen-specific T cell response that protected vaccinated mice from the establishment of a primary tumor. Finally, in vivo antibody depletion studies provided additional argument that the presence of NK cells provided critical immunoregulatory signals which supported the generation of an antitumor adaptive host immune response.

MATERIALS AND METHODS

Animals, cell lines and reagents

Six- to eight-week-old, female C57BL/6 (B6) mice (H-2^b) were obtained from NCI-Frederick, Frederick, MD. Mice were housed and maintained in microisolator cages under pathogen-free conditions.

The murine MC38 and B16/F10 (H-2^b), colon adenocarcinoma and melanoma cell lines, respectively were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose and 10% heat-inactivated fetal bovine serum (FBS). MC32A tumor cells, a subline of the MC38 cells transfected to express human carcinoembryonic antigen (CEA) (24), were grown in the same supplemented media. EL4, a murine T-cell lymphoma cell line (H-2b) and YAC-1 (mouse, A/Sn, lymphoma) tumor cell line which is sensitive to NK mediated lysis,

were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS and complete T cell media (21), respectively.

Lyophilized rIFN- γ was obtained from PBL Laboratories, Inc. (Piscataway, NJ) (carrier-free, specific activity = 1.02×10^7 units/mg protein) and stored at -80°C until use. Prior to injection, rIFN- γ was reconstituted in saline containing 1% mouse serum. Reconstituted murine IFN- γ was also stored at -80°C, and its biological activity was checked every 3-6 months for its ability to upregulate MHC antigen expression on a variety of murine tumor cell lines.

Construction of rF-IFN- γ

The recombinant fowlpox virus expressing the murine *IFN- γ* gene was constructed by inserting the foreign gene sequences into the *BamHI* *J* region of the genome of the POXVAC-TC (Schering Corporation, Kenilworth, NJ, USA) strain of fowlpox virus as described (25). Expression of murine IFN- γ in the recombinant fowlpox construct is under the control of the p40 promoter (25). IFN- γ production was confirmed by the in vitro infection of MC38 tumor cells as previously described (21). MC38 cells were harvested and washed twice with Opti-MEM media (Life Technologies Inc., Gaithersburg, MD). Two- 4×10^6 cells were added to 15 ml polypropylene conical tubes and pelleted by centrifugation. Cell pellets were suspended in 300 μ l of Opti-MEM media to which the indicated MOI (in 10 μ l) of rF-IFN- γ was added. Cells were incubated for 1 hr at 37°C, washed with complete medium twice and suspended in 10 ml of complete medium in T-25 flasks. At different time points, media supernatant was removed, stored at -20°C and IFN- γ levels were measured using a murine IFN- γ ELISA (Endogen, Inc., Cambridge, MA). Both uninfected MC38 cells and MC38 cells infected with wild-type fowlpox (FP-WT) served as controls. Serum IFN- γ levels were measured using the same ELISA.

Flow Cytometry

Flow cytometry was used to determine the level of expression of the MHC molecules on the tumor cell surface. Briefly, cells that had been either infected with rF-IFN- γ or FP-WT or incubated with rIFN- γ (1-10 ng/ml, 48 hr) were harvested and washed twice with cold Ca^{2+} - Mg^{2+} -free DPBS. Cells were resuspended in Ca^{2+} - Mg^{2+} -free DPBS at a concentration of 0.5 – 1.0×10^6 cells/ml. In order to block the Fc receptors, 1 μ g of the unlabeled CD16/CD32 Fc γ III/II receptor antibody (clone 2.4G2, rat IgG2b) was added to each sample. Cells were then incubated with 2 μ g of FITC-labeled antibodies against H-2K^b (clone AF6-88.5, mouse IgG2a, κ), H-2D^b (clone 28-14-8, mouse IgG2b, κ) and I-Ab (clone AF6-120.1, rat (Lou) IgG2a, κ) molecules or appropriate control antibodies (clone R19-15, mouse IgG2a, κ and clone 25-35, mouse IgG2b, κ) (PharMingen, Inc., San Diego, CA) for 1 h at 4°C. Cells (10,000-20,000) were analyzed using a Becton Dickinson FACScan or an LSR II. To determine changes of MHC expression of tumors treated with rF-IFN- γ in vivo, mice received an s.c. injection on their lower flank consisting of 3×10^5 of either MC38 or B16/F10 cells. When the tumors had achieved a volume of 75-100 mm³, an intratumoral injection of 1×10^7 pfu/50 μ l rF-IFN- γ or FP-WT or vehicle (HBSS) was administered. Twenty-four hours later, tumors were excised, cut into smaller pieces and incubated in 5 ml of complete medium containing 15 mg of Collagenase D (Roche, Nutley, NJ) for 1 hour. Every 20 minutes cells were mechanically separated by vigorous pipetting and at the end of the incubation period, cells were washed with cold Ca^{2+} - Mg^{2+} -free DPBS, centrifuged and counted. Flow cytometry was performed as described above.

Regional lymph nodes, consisting of the subiliac, para-aortic, and sacral nodes, were surgically isolated from groups of B6 mice following a single s.c. injection of rF-IFN- γ , FP-WT, rIFN- γ or vehicle. Lymph nodes from each treatment group were combined, dissected into small pieces and incubated in 3.0 ml of complete medium containing collagenase D, as previously

described. Cells were pelleted by centrifugation and washed twice with cold Ca^{2+} - Mg^{2+} -free DPBS. Cell aliquots from each group were resuspended and analyzed using the following antibodies: APC-Cy7-anti-mouse CD3e (clone 145-2C11), PE-Cy7-anti-mouse CD19 (clone 1D3), PE-anti-mouse NKG2D (clone CX5, rat IgG1, κ) (www.ebioscience.com), APC-anti-mouse NK1.1 (NKR-P1B and NKR-P1C) (PK136) (mouse IgG2a). Control antibodies included APC-Cy7 hamster IgG1 (anti-TNP), PE-Cy7 mouse IgG2a, PE-rat IgG1 (clone R3-34) and APC-mouse IgG2a (clone X13). In order to block the Fc receptors, 1 μg of the unlabeled CD16/CD32 Fc γ III/II receptor antibody (clone 2.4G2, rat IgG2b) was added to each sample. Cells (200,000-500,000) were analyzed using Becton-Dickinson LSR II.

NK-mediated lytic assay

Lysis of the NK sensitive YAC-1 cells was measured using either the 4-h Cr-51 or overnight In-111 release assays. YAC-1 cells ($2-4 \times 10^6$) were labeled with 250 μCi of $\text{Na}_2[^{51}\text{Cr}]\text{O}_4$ (PerkinElmer, Shelton, CT) or with 3.7 MBq of sodium [^{111}In] (Amersham, Arlington Heights, IL, USA) in serum-free RPMI 1640 for 30 min at 37°C. After each labeling, the cells were washed and plated in 96-well round bottom plates (Falcon) at a concentration of 5×10^3 cells/well in RPMI complete medium with 10% FBS. Effector cells, consisting of unfractionated or NK-enriched fractions from LNs or spleens, were added at varying effector to target (E:T) ratios. Depending on whether the YAC-1 cells were labeled with Cr-51 or In-111, the plates were incubated for 4-hr or overnight at 37°C after which the plates were centrifuged ($200 \times g$) for 1 minute, supernatants harvested and the radioactivity counted using a Beckman 5500 gamma counter (Beckman Scientific Instruments, Irvine, CA). Specific cellular cytotoxicity was determined by the following formula: percentage of specific lysis = (experimental cpm - spontaneous cpm) / (maximum cpm - spontaneous cpm) $\times$ 100. Maximum cpm was obtained by adding Triton X-100 (0.25%) (Sigma, St. Louis, MO) to the target cells in the absence of effector cells. Spontaneous cpm was the radioactivity from wells with target cells alone. EL-4 cells were used as negative control target cells. All assays were done in triplicate.

In selected experiments, NK cells were purified from either lymph nodes or spleens using an NK cell negative selection isolation kit (DynaL Biotech ASA, Oslo, Norway) that included rat IgG depleting antibodies to CD4, CD5, CD8 α , CD19, Ly-6G and Ter-119. Flow cytometric analyses revealed that >85% of the cells purified from either lymph nodes or spleens expressed NK1.1. In other experiments, different agents were analyzed for their ability to block LN-cell mediated YAC-1 lysis during the overnight lytic assay. Lymph node effector cells were preincubated for 1-2 hr in the presence of 100 nM concanamycin A (CMA) (Sigma, St. Louis, MO), 50 μg of the following antibodies: hamster anti-mouse CD178 (Fas Ligand, CD95 Ligand) (clone MFL3, hamster IgG1, κ) (PharMingen, Inc.), hamster anti-mouse CD120b (TNF receptor Type II, p75)(clone TR75-32, hamster IgG1, λ 1), hamster anti-mouse CD120a (TNF receptor Type I/p55)(clone MABTNFR1-B1, mouse IgG2a, κ). The CMA-treated effectors were washed prior to, whereas, the neutralizing antibodies were present during the assay.

In vitro T cell stimulation and CTL assay

The MC38 tumor-specific CTL line was generated by isolating the regional lymph nodes from mice administered rF-IFN- γ -infected MC38 cells. Lymph node cells were counted and added to T-25 flasks in 20 ml complete media at a ratio of 50:1 lymphocytes to irradiated (20,000 rads) MC38 tumor cells. Seven days later the cells were counted using trypan blue and 2.5×10^5 cells were restimulated with 1×10^5 irradiated MC38 cells and 5×10^5 fresh, irradiated splenocytes used as feeder cells in 2.0 ml/well of a 24-well plate. After an additional seven days of in vitro stimulation, T cells were harvested, MC38 and EL4 cells were labeled with Cr-51 and 4-hr cytolytic assays were carried out at predetermined E:T ratios.

Tumor Studies

Tumor studies included the s.c. administration of MC38, B16/F10 or MC32A tumor cells harvested from in vitro propagated cell lines. In other experiments, MC38 tumor cells were infected ex vivo at the indicated MOI with either rF-IFN- γ or FP-WT as previously outlined. Following infection, the cells were washed with complete medium twice, resuspended in 10 ml of complete medium and placed in T-25 flasks and grown in vitro for 2-3 days. Another group of mice received MC38 cells that were grown in vitro for 48 hrs in complete medium supplemented with 10 ng/ml of rIFN- γ . In all cases, mice received a single injection of $3-5 \times 10^5$ cells in 0.1 ml administered s.c. into the lower flank and checked 2x/week for tumor growth.

In Vivo Depletion of Immune Cells

The following were used for the *in vivo* immune cell depletion studies: GK1.5, an anti-mouse L3T4 (rat IgG_{2b}) (CD4); 2.43, an anti-mouse Lyt 2.2 (rat IgG_{2b}) (CD8) MAb; anti-mouse NK1.1 (PK136, mouse IgG2a), rabbit anti-mouse/rat asialo GM1 polyclonal antibody (Cedarlane Laboratories, Lmt. Hornby, Ontario, Canada); and Ly49D&H (gift from Drs. John Ortaldo and Robin Winkler-Pickett). GK1.5, 2.43 and PK136 were administered i.p. alone or in combination at a dose of 100 μ g/day for 5 consecutive days. The anti-asialo GM1 polyclonal antibody was reconstituted according to the manufacturer's instructions and mice received a single i.v. injection of 50 μ l of the reconstituted antibody. Ly49D&H was diluted to 1.0 mg/ml in PBS and 0.2 ml (200 μ g) was given as a single i.p. injection. Flow cytometric analyses of splenocytes revealed depletion of >98% of the CD4 and CD8 T cells following injection of the appropriate depleting antibodies. Short-term (4-hr) Cr-51 release cytotoxicity assay using YAC-1 cells as targets was used to verify NK cell depletion following the injection of PK136, anti-asialo-GM1 and Ly49D&H. In all cases, the percentage of YAC-1 killing by splenocytes (200:1 E:T) was <2.0% in those mice treated with the NK depleting antibodies. NK-mediated YAC-1 lysis of splenocytes isolated from normal mice ranged between 8-12% (200:1 E:T ratio). T and NK cell depletion was maintained with weekly injections.

Statistical Methods

Survival estimates among treatment groups were generated using Kaplan-Meier survival analyses. Differences in survivor functions between treatment groups were assessed using the log-rank test. Statistical significance of YAC-1 lysis and the number of NK1.1⁺, NK1.1⁺/NKG2D⁺ cells was based on Student's two-tailed *t* test. All *p* values are two-sided and not adjusted for the multiplicity of evaluation performed on the data. Analyses were conducted using STATA software (version 1.0, Stata Corp., College Station, TX) and statistical significance was set at a *P* value of <0.05.

RESULTS

Production of biologically active IFN- γ by rF-IFN- γ -infected cells

Murine colon carcinoma (MC38) cells infected with rF-IFN- γ (1.0 MOI) produced approximately 150 ng IFN- γ /10⁶ cells/day. No detectable IFN- γ levels were found in the supernatants from either uninfected or FP-WT-infected MC38 cells. Upregulation of MHC class I expression on the surface of tumor cells was used as readout to establish that biologically active IFN- γ was produced following rF-IFN- γ infection (Table 1). Infection of either MC38 or B16 melanoma cells significantly increased MHC Class I molecule (H-2K^b and H-2D^b) expression levels. A slight increase was observed following FP-WT infection. Supernatants from rF-IFN- γ -infected MC38 and B16/F10 cells also upregulated H-2^b antigens on both tumor cell lines, providing additional evidence of the production of biologically active IFN- γ (Table 1). Moreover, the extent of those increases were comparable to those measured following the addition of 10 ng/ml of rIFN- γ protein (Table 1).

To test whether rF-IFN- γ can upregulate MHC class I expression in vivo, mice bearing s.c. MC38 tumors (volume of 75-100 mm³; 8-12 day post-injection) received a single intratumoral injection of rF-IFN- γ or FP-WT or HBSS. Class I MHC expression levels were increased approximately 2-fold, as evidenced by MFI increases, on only those tumors isolated from mice given a single intratumoral injection of rF-IFN- γ (data not shown). The B16/F10 s.c. tumors in B6 mice provided a more convincing assessment of the ability of rF-IFN- γ to enhance MHC expression. Constitutive H-2^b expression was low on the B16/F10 tumors cells with <10% of the tumor cells expressing H-2K^b and -D^b (Fig. 1, panels A and B, respectively). Intratumoral inoculation with either HBSS or FP-WT did not change H-2^b expression levels, whereas at 24 hrs post-rF-IFN- γ injection, the percentage of H-2K^b as well as H-2D^b expressing B16/F10 tumor cells were increased to >90% with accompanying increases in MFI providing in vivo evidence for the production of biologically active IFN- γ .

Serum IFN- γ and changes in the regional lymph nodes after injection of FP-WT, rF-IFN- γ or rIFN- γ

Recombinant fowlpox viruses are, for the most part, non-lytic and replication incompetent with their infectivity range limited to the needle tract (26). This restricts the production of any inserted gene product, in this case, IFN- γ , to the local tissue microenvironment. To confirm this, mice received a single s.c. injection of 10⁸ pfu rF-IFN- γ or 1, 5 or 10 μ g rIFN- γ . Serum IFN- γ levels were measured at 1-48 hr post-injection. No detectable serum IFN- γ levels were found in mice that received rF-IFN- γ . In contrast, mice that received a single s.c. injection of 1, 5 or 10 μ g rIFN- γ had peak serum IFN- γ levels of 7, 83 and 121 ng/ml, respectively, at 1-h post-injection.

Because IFN- γ production from rF-IFN- γ was restricted to the injection site, any immune-related actions resulting would be examined in the regional lymph nodes (LNs) draining that site. LNs isolated from untreated B6 mice contain 2-3 million cells/node. A single s.c. injection of either rIFN- γ or rF-IFN- γ significantly increased lymph node cellularity (Fig. 2A). In the case of rIFN- γ treatment, the increase in the total number of cells/node was transient, peaking at 24 hrs and approaching baseline levels by 72 hr. Total number of cells/node draining the site of rF-IFN- γ administration was increased 2.5-fold at 72 hrs and that enrichment was sustained for 5-6 days (Fig. 2A). Changes in the number of immune cell subsets, i.e., T, B and NK cells, within the LNs of mice administered FP-WP, rF-IFN- γ and rIFN- γ mirrored the changes in total cells/node. For example, at 24 hr post-injection of FP-WT, rF-IFN- γ or rIFN- γ , the regional LNs contained significantly higher numbers of CD3⁺ (Fig. 2B), CD19⁺ (Fig. 2C) and/or NK1.1⁺ (Fig. 2D) cells, but at 72 hrs, only the LN from mice treated with rF-IFN- γ had sustained elevated numbers of each of the immune cell subsets. Interestingly, the number of NK1.1⁺ cells remaining in the regional LN 72 hr (Fig. 2D) after rF-IFN- γ was approximately 10-fold higher than any other treatment.

Flow cytometry identified NK1.1⁺ and NK1.1⁺/NKG2D⁺ cells in LNs 72 hrs post-injection of FP-WT, rF-IFN- γ or rIFN- γ - treated mice (Fig. 3). Within the LNs of untreated mice the percentage of cells expressing NK1.1 was <1% (Fig. 3B) with 14.7% of the NK1.1⁺ cells co-expressing NKG2D (Fig. 3F), an activation/cytotoxicity receptor on murine NK cells (27). Administration of FP-WT or rIFN- γ induced no significant changes in either the total number of NK1.1⁺ or NK1.1⁺/NKG2D⁺ cells. In contrast, rF-IFN- γ injection induced a significant ($P < 0.05$ vs untreated mice) enrichment of NK1.1⁺ and NK1.1⁺/NKG2D⁺ cells in the regional LNs. The percent NK1.1⁺ LN cells increased from 0.8% in untreated mice to 3.3% (Fig. 3C) in mice administered rF-IFN- γ . Furthermore, the percentage of those NK1.1⁺ cells which also expressed NKG2D rose from 14.7% in untreated mice to 42.6% in mice injected with rF-IFN- γ (Fig. 3G).

Identification of NK-mediated YAC-1 lysis in regional LNs draining the rF-IFN- γ injection site

While NK cells are usually thought to be restricted to the spleen and circulation during steady-state conditions, the flow cytometric data suggest that local IFN- γ production, particularly following rF-IFN- γ administration, results in a significant enrichment of activated NK1.1 cells within the regional LNs. LN were isolated from mice 72 hr post-injection of FP-WT, rF-IFN- γ or rIFN- γ and analyzed for their ability to lyse an NK sensitive target, YAC-1 cells, in both short-term (4 h) and overnight assays (Fig. 4). No appreciable YAC-1 lysis was detected in the LNs of mice injected with either FP-WT or rIFN- γ in either the short-term (Fig. 4A) or overnight (Fig. 4B) assays. In the short-term 4 h cytolytic assay, there was a measurable level of YAC-1 killing by the LN from rF-IFN- γ treated mice (Fig. 4A). However, the most dramatic amount of YAC-1 killing (>30% @ 200:1 E:T) was seen in an overnight assay using unfractionated LN cells from mice administered rF-IFN- γ (Fig. 4B).

Subsequent studies determined (1) the temporal aspects of YAC-1 lysis, (2) whether YAC-1 lysis was, indeed, localized to the regional LN draining the rF-IFN- γ injection site, (3) possible molecular events which may be responsible for YAC-1 lysis and (4) the effects of NK cell depletion on YAC-1 killing in the overnight assay. Mice received a single s.c. injection of rF-IFN- γ , FP-WT, rIFN- γ or HBSS and LN-mediated YAC-1 lysis was measured over 7 days (Fig. 5A). At 24 hrs post-injection of either rF-IFN- γ or rIFN- γ protein, the regional LNs of those mice contained enriched levels of YAC-1 lysis measured in the overnight assay (Fig. 5A). The difference was that the enrichment present in the regional LNs draining the site of mice injected with rIFN- γ was transient, peaking at 24 hrs post-injection and returning to baseline levels by 72 hrs. Delivery of IFN- γ as rF-IFN- γ resulted in sustained levels of YAC-1 killing in the draining LNs over 5-6 days with peak lysis occurring 72 hrs post-injection. The temporal aspect of YAC-1 lysis in the LN from mice administered rF-IFN- γ was very reminiscent of the changes seen in total cells and the number of NK1.1+ cells/node in the same treatment group (Fig. 2A and D). Enhanced YAC-1 lysis was present only in the regional LNs that drained the rF-IFN- γ injection site. No YAC-1 lysis was observed in LNs distal (contralateral) to the rF-IFN- γ injection site (Fig. 5B). LN cells from mice injected with rF-IFN- γ were isolated and co-incubated in the presence of an anti-FasL antibody, CMA or anti-TNF- α antibodies with significant inhibition of YAC-1 lysis ($P < 0.05$ vs. no treatment) associated with the addition of the anti-TNF- α antibodies (Fig. 5C). Mice were administered three different NK depleting antibodies – anti-asialo-GM1, anti-NK1.1 and Ly49D&H – prior to rF-IFN- γ administration. Since both anti-asialo-GM1 (28) and anti-NK1.1 (29) also deplete rare T cell populations, the Ly49D&H which selectively depletes activated NK cells (27,30) was added. The ability of the LN cells to lyse YAC-1 targets was completely lost in those mice receiving any of the three NK-depleting antibodies (Fig. 5D). Likewise, splenocytes isolated from those same mice also lost their ability to recognize and lyse YAC-1 cells in a conventional 4hr-Cr-51-release assay (data not shown). Therefore, both phenotypic and functional analyses provide a strong argument that the YAC-1 lysis identified in the regional LNs draining the rF-IFN- γ injection site was, indeed, NK cell-mediated.

IFN- γ produced by rF-IFN- γ -infected tumor cells prevents in vivo tumor development

Next, studies were designed to examine whether IFN- γ administration in the presence of antigen might influence host adaptive immunity. MC38 tumor cells were treated ex vivo with 6.25 MOI of either FP-WT or rF-IFN- γ or 10 ng/ml rIFN- γ prior to their s.c. injection into syngeneic B6 mice. Both rF-IFN- γ and rIFN- γ enhanced class I MHC expression levels on the MC38 tumor cells (Table 1). Despite higher H-2^b expression levels, only those mice receiving rF-IFN- γ infected MC38 tumor cells were protected from primary tumor growth (Fig. 6A, $P < 0.05$). Six of 10 mice within that treatment group remained tumor-free 8 weeks after tumor cell inoculation. Moreover, when those tumor-free mice were challenged with uninfected MC38, approximately 30% successfully rejected tumor growth with no such protection against

B16/F10 tumor growth (Fig. 6B). The findings argue that a single vaccination, consisting of rF-IFN- γ -infected MC38 tumor cells, generated sufficient antitumor immunity to protect those mice from subsequent tumor challenge. Both the protection from tumor growth as well as the generation of tumor-specific host immunity could not be explained simply by the upregulation of MHC molecules (31) on the tumor cell surface following rIFN- γ treatment. Sustained in vivo IFN- γ production in the microenvironment encompassing the injection site of the rF-IFN- γ -infected MC38 tumor cells seemed to be essential in preventing tumor growth.

Immune cell depletion studies attempted to identify those cell population(s) which conferred protection against primary tumor growth in mice administered rF-IFN- γ -infected MC38 tumor cells. Mice were administered immune cell depleting antibodies prior to their inoculation with rF-IFN- γ -infected MC38 cells (Fig. 6C). Protection from primary tumor growth was lost with CD4 or CD8 or their combined depletion. Interestingly, protection from the growth of rF-IFN- γ -infected MC38 tumors was also partially lost in mice depleted of their NK1.1⁺ cells with the PK136 antibody. Those observations suggested that protection from the growth of primary MC38 tumors infected with rF-IFN- γ required the presence of both innate and adaptive immune cells.

Role(s) of the different immune cell subsets in the protection of vaccinated mice were subsequently addressed. Mice received two vaccinations consisting of rF-IFN- γ -infected MC32A tumor cells that had been engineered to express the human CEA antigen (80-95% CEA⁺). CEA-expressing tumor cells were chosen in order to provide a stronger rejection antigen absent in the parental MC38 tumor cells. Indeed, 90% of the vaccinated mice remained tumor-free following challenge with parental MC32A tumor cells (Fig. 6D). In mice depleted of CD4, CD8 or both T cell subsets following the second vaccination, protection from subsequent tumor rechallenge with the MC32A tumor cells was lost. In contrast, NK cell depletion in vaccinated mice was not accompanied with a loss of protection against MC32A tumor challenge (Fig. 6D), indicating that resident effector T cell memory responses capable of protecting the host from tumor rechallenge does not require the presence of NK cells.

Tumor-specific CTL activity in the regional LNs: relationship with NK cells

Local delivery of IFN- γ via a recombinant fowlpox virus, rF-IFN- γ , enriches the regional LNs with immune cells (Fig. 2 and 3), particularly, NK cells and when administered with antigen, i.e., rF-IFN- γ -infected MC38 tumor cells, can induce a protective T cell response (Fig. 6A-C). It was hypothesized that the LNs draining the site of injection of MC38 cells infected with either FP-WT or rF-IFN- γ or treated in vitro with rIFN- γ might contain cells capable of antigen-specific tumor cell lysis.

Mice received a single s.c. injection of MC38 cells that were previously infected with FP-WT or rF-IFN- γ or treated ex vivo with rIFN- γ . Seventy-two hours after injection, regional lymph nodes were isolated from mice of each treatment group and analyzed for lysis MC38 and EL4 cells in short-term and overnight assays (Fig. 7). Within the LNs draining the site of injection of the rF-IFN- γ -infected MC38 cells was found MC38-specific cell-mediated lysis using either a 4-hr (panel A) or overnight assays (panel C). In both cases, MC38-specific lysis was weak (8-12%), but statistically significant ($P < 0.05$ vs. lysis from mice injected with FP-WT or rIFN- γ -treated MC38 cells), and could be selectively enhanced by in vitro stimulation with irradiated MC38 cells (Fig. 7A, insert). No measurable lysis was observed with EL4 cells as targets (Fig. 7B and D).

The presence of a large number of NK cells together with tumor specific CTLs within the regional LNs draining the site of injection of rF-IFN- γ -treated MC38 tumor cells prompted the examination as to whether any functional relationships existed between those cells. To address this question, mice were administered anti-asialo-GM, anti-NK1.1 or Ly49D&H (Fig. 8, panels

B-D) prior to the injection of rF-IFN- γ -infected MC38 cells. The low, but measurable tumor-specific lysis in the regional LNs of mice injected with rF-IFN- γ -infected MC38 tumors can be specifically enriched by in vitro stimulation with re-exposure to tumor antigen (i.e., irradiated MC38 tumor cells)(Fig. 8A). Depletion of NK cells by the administration of any of the three antibodies completely abrogated the ability of those mice develop a primary tumor-specific T cell response (Fig. 8, panels B-D). Those findings implicate NK cells as a crucial component in the development of a primary T cell response to MC38 tumors, in the draining lymph nodes of mice receiving rF-IFN- γ -infected MC38 tumors.

DISCUSSION

The present study underscores the advantages of targeted delivery of IFN- γ to a prescribed tissue microenvironment. Administration of IFN- γ as either a recombinant protein, rIFN- γ , or as a recombinant avipox virus, rF-IFN- γ , induces profound expansions of immune cells within the local, regional LNs. Several distinct advantages are gained, however, by delivering IFN- γ as rF-IFN- γ rather than injecting the rIFN- γ protein. First, the expansion of immune cells, particularly NK1.1+ cells, within the regional LNs was sustained for 5-6 days in comparison to a transient (approx. 24 hrs) expansion after rIFN- γ injection. Second, the enrichment within the regional nodes was achieved without any measurable circulating IFN- γ levels, thus negating any side effects associated with high doses and/or multiple injections of the rIFN- γ protein. And, finally, the combined administration of rF-IFN- γ , not rIFN- γ , with antigen (i.e., highly tumorigenic MC38 tumor cells) induced sufficient tumor-specific adaptive T cell immunity to protect mice against primary tumor growth and subsequent tumor challenge.

In the past, evaluations of rIFN- γ as a single antitumor agent have been predicated on the ability to deliver sufficient amounts of the cytokine to the tumor site. Upregulation of MHC, Fas and tumor associated antigens on tumor cells, inhibition of immunosuppressive factors and potent anti-angiogenic effects have all been document with systemic IFN- γ administration and have provided the argument for the immunomodulatory and, perhaps, antitumor actions of this cytokine. However, clinical studies revealed severe treatment-limiting toxicities that have blunted the use of rIFN- γ in cancer treatment. Yet, IFN- γ remains a potent proinflammatory cytokine as do questions on how best to exploit its actions. Our rationale was to target IFN- γ production to a tissue microenvironment via rF-IFN- γ injection, thus localizing its proinflammatory actions that would induce a controlled migration of host immune cells to the injection site. Under those conditions, with the addition of antigen, in the form of rF-IFN- γ -infected MC38 tumor cells, tumor specific CTL activity was generated in the antigen-stimulated LNs. Furthermore, T cell depletion studies established their requirement to protect mice from the growth of rF-IFN- γ -infected MC38 tumor cells. Thus, the findings of this study might provide the rationale to reexamine IFN- γ as a classic immune adjuvant when delivered with antigen to a selected tissue microenvironment.

The ability of rF-IFN- γ to act as an immune adjuvant was dependent on: (1) the sustainability of IFN- γ production at the injected site, consistent with using a fowlpox-based vector as a delivery vehicle and (2) the cellular enrichment (Fig.2), particularly NK1.1+ cells (Fig. 5A) within the regional lymph nodes of mice receiving rF-IFN- γ . Several intriguing findings focused our attention on the NK1.1+ cell population in the regional lymph nodes of mice injected with rF-IFN- γ . In vivo NK1.1 depletion studies suggested that they play an important role(s) in (1) the protection against primary tumor growth (Fig. 6C) and (2) the generation of a tumor-specific host immune response (Fig. 8). In contrast, in a tumor challenge experiment of vaccinated [rF-IFN- γ -infected MC32A (CEA-expressing) cells] mice, the ability to reject tumor was not lost with NK1.1+ cell depletion (Fig. 6D). Those findings indicate a role(s) for NK1.1+ cells in T cell priming but not for the recall of memory T cells.

The results argue that injection of rF-IFN- γ -infected MC38 initiates signals for the extravasation of NK1.1+ cells from the circulation to the site of inflammation. Traditionally, NK cells function as peripheral effector cells that recognize and kill stressed, transformed and virally infected cells, their principle role in innate immunity (32,33). Whether NK1.1+ cells recognize and kill rF-IFN- γ -infected MC38 tumor cells, contributing to the protection of mice against primary tumor growth is arguable. Infection of MC38 tumor cells with rF-IFN- γ enhances MHC class I expression (Table 1, Fig. 1) and might be expected to inhibit NK cell cytotoxicity through engagement of NK cell receptors containing a tyrosine-based inhibitory motif (34). Yet, when mice were depleted of NK cells, protection from the growth of rF-IFN- γ -infected MC38 tumor cells was lost (Fig. 6C). One explanation is that the NK1.1+ cells within the antigen-stimulated LNs are not tumoricidal, but their presence supports the development of tumor-specific adaptive immunity. Indeed, priming of naïve T cells to tumor-specific antigen occurs within secondary lymph sites and requires the presence of mature DCs. Within sites of chronic inflammation (i.e., allergen-induced atopic eczema/dermatitis syndrome), NK cells are in close contact with resident DCs (35) which results in DC maturation and local IL-12 production (36) driving a T_H1 cellular response. In the present study, flow cytometric analyses revealed that not only were the antigen-stimulated lymph nodes draining the rF-IFN- γ injection site enriched for NK1.1+ cells, but a significant percentage co-expressed the NKG2D-activating receptor, the primary cytotoxicity receptor for murine NK cells (Fig. 3). Usually, following NKG2D receptor-ligand interaction cytolysis by the NK, NKT and T cells is perforin-mediated (37,38), however, no perforin-mediated lysis was detected. Unfractionated LN cells from mice injected with rF-IFN- γ lysed YAC-1 cells in a TNF- α -dependent manner (Fig. 5C). A previous study (39) reported NK-dependent DC maturation/migration through a TNF-dependent mechanism. Absence of that pathway in NK cell-depleted mice would be expected to interrupt DC maturation and severely impair the cross-talk between innate and adaptive immune responses. Whether those findings signal a difference between “helper” (40) and “effector” NK cells or indicate a particular NK cell subset will be addressed in future studies.

More recent data have shown that NK cells also provide other “helper” function(s) to the adaptive immune system. Their presence in secondary lymphoid sites, particularly, antigen-stimulated LNs, provides a local IFN- γ source that assists in T-cell priming (41-44). One could speculate that the local IFN- γ production, by virtue of rF-IFN- γ injection, might substitute some needed “helper” functions in the NK cell-depleted mice, such as IL-12 or IL-18 induction in the microenvironment, and support some measurable T cell priming. However, depletion of NK cells prior to the administration of the cell-based vaccine (i.e., rF-IFN- γ -infected-MC38 tumor cells) led to a complete loss in the ability to generate a primary antigen-specific T cell response (Fig. 8). Those findings suggest that other NK-mediated events are required for T-cell priming which is consistent with recent evidence of an IL-18-dependent soluble factor released by activated NK which promotes DC maturation (45). Thus, NK cells provide crucial signals which can (1) impact DC editing and (2) provide a local source of IFN- γ and the absence of those signals due to NK cell depletion can severely impact T-cell priming (46,47). Indeed, patients with deficits in NK cell function have impaired induction of virus-specific immune response and a heightened susceptibility to recurrent viral infection (48). Therefore, it seems that NK cells are important early innate cells involved at multiple steps leading to the priming of adaptive T cell responses. An understanding of the cellular signals delivered to the innate immune system and how they shape the events upstream of the DC-T helper cell and/or NK-cytotoxic T cell interactions could contribute to purposefully driving a particular adaptive immune response and facilitate the design of novel immunization schema.

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Abbreviations

rIFN-γ	recombinant interferon-gamma
MHC	major histocompatibility complex
rF-IFN-γ	recombinant fowlpox virus expressing murine IFN- γ
FP-WT	

	fowlpox wild-type
DMEM	Dulbecco's Modified Eagle Medium
LN s	lymph nodes
s.c.	subcutaneous
MFI	mean fluorescence intensity
MOI	multiplicity of infection
DPBS	Dulbecco's phosphate buffered saline
HBSS	Hanks' Balanced Salt Solution
pfu	plaque-forming units

FIG. 1.

Enhanced H-2^b expression levels on B16/F10 tumors following intratumoral rF-IFN- γ injection. Mice received 3×10^5 B16/F10 cells which resulted in 75-100 mm³ s.c. tumors 8-12 days later at which time they were divided into three treatment groups (3/group). Each mouse received a single intratumoral injection (50 μ l) of HBSS (dotted lines) or 10^8 pfu of either rF-IFN- γ (solid lines) or FP-WT (dashed lines). Tumors were excised 24 hrs later and (A) H-2K^b and (B) H-2D^b expression levels analyzed by flow cytometry (see Materials and Methods). Data are from a single experiment that was repeated with similar results.

FIG. 2.

Increased LN cellularity and changes in CD3⁺, CD19⁺ and NK1.1⁺ cells following injection of rF-IFN- γ , FP-WT or rIFN- γ . (A) Mice (3-4/group) received single s.c. injections of either 5 μ g rIFN- γ (circles) or 10⁸ pfu FP-WT (triangles) or rF-IFN- γ (squares). Regional LNs were isolated 1-7 days later and the total number of cells/node determined using a hemocytometer. (B-D) Total number of CD3⁺ T cells (B), CD19⁺ B cells (C) and NK1.1⁺ cells (D) within the regional LN at 24 (open bars) and 72 hr (filled bars) of mice given a single s.c. injection of the indicated immune adjuvant. Data are the mean $\pm$ SEM from a representative experiment which was repeated with similar results. * P<0.05 (vs. untreated mice).

FIG. 3.

Changes in NK1.1+ and NK1.1+/NKG2D+ cells in the regional LN following a single injection of FP-WT, rF-IFN- γ or rIFN- γ . LNs were harvested from B6 mice (3-6/group) three (3) days after a single injection of HBSS, FP-WT rF-IFN- γ or rIFN- γ as described above. Panel A establishes the staining pattern for the isotype control and the region of interest for further analyses. Panels B-E illustrates the percentage of total LN cells that expressed NK1.1+ and panels F-I represent the percentage of the NK1.1+ cells co-expressing NKG2D. Data are the mean $\pm$ SEM from a representative experiment which was repeated with similar results.

FIG. 4.

YAC-1 cytotoxicity within the regional LNs of mice following a single injection of 10^8 pfu FP-WT (triangles), 10^8 pfu rF-IFN- γ (circles) or $5 \mu\text{g}$ rIFN- γ (squares). Regional LNs were isolated 72 hrs post-injection and tested for YAC-1 lysis in a 4-hr (A) and overnight (B) assays. Data are presented as the mean $\pm$ SEM from triplicate wells at each E:T ratio and are from a representative experiment performed 4-5 times with similar results.

FIG. 5.

Description of the overnight YAC-1 targeted lytic activity in the regional LNs. (A) Mice (3-4/group) received single s.c. injections of either 5 μ g rIFN- γ (squares) or 10^8 pfu FP-WT (triangles) or rF-IFN- γ (closed circles). Untreated mice (n=6) received a single s.c. injection of 100 μ l HBSS (open circles). Regional LNs were isolated after 24-168 hrs and YAC-1 lysis (E:T, 200:1) measured. (B) Mice (3-4/group) received a single injection (s.c.) of rF-IFN- γ (10^8 pfu) and control mice (n=6, open circles) received HBSS. LNs draining (circles) or distal (contralateral, triangles) to the s.c. injection site were isolated 72 hrs post-injection and YAC-1 lysis (E:T, 200:1) measured. (C) Mice (n=12) that received a single s.c. injection of 10^8 pfu rF-IFN- γ were sacrificed 72 h later, LNs removed, combined and an overnight YAC-1 lytic assay (E:T 200:1) was performed. Treatment with the different potential inhibitors was described in

the Materials and Methods. (D) Mice (3-5/group) received the NK cell-depleting antibodies, anti-asialo-GM (triangles), anti-NK1.1 (squares) or Ly49D&H (open circles) prior to receiving a single s.c. injection of 10^8 pfu rF-IFN- γ . LN cells were isolated, combined and YAC-1 lysis measured in an overnight assay. Untreated mice are represented by the closed circles. All data are presented as the mean $\pm$ SEM from triplicate wells from a representative experiment that was repeated with similar results.

FIG. 6.

rF-IFN- γ infection protects mice from tumor development: effects of immune cell depletion in naïve and vaccinated mice. (A) MC38 tumor cells were infected with 6.25 MOI of either FP-WT (squares) or rF-IFN- γ (triangles) or treated in vitro with 10 ng/ml rIFN- γ (circles) (see Materials and Methods). Control mice were injected with uninfected MC38 cells (dashed line). All mice ($n=20$ /group) received a tumor burden of 3×10^5 cells and were monitored daily for tumor appearance (minimum volume approx. 20-30 mm³). Results are presented as the percentage of tumor-free mice. (B) Tumor-free mice from the mice vaccinated with rF-IFN- γ -infected MC38 cells were challenged s.c. on the opposite side with 3×10^5 of either MC38 (diamonds) or B16/F10 (squares) cells and tumor appearance checked daily. (C) Prior to the injection of rF-IFN- γ infected MC38 tumor cells, mice (10/group) were administered the following immune cell-depleting antibodies: GK1.5 (anti-CD4, triangles), 2.43 (anti-CD8, squares), anti-asialo-GM1 (anti-NK, open circles), GK1.5/2.43 (open triangles) (see Materials and Methods). Control mice (solid circles) received a single injection of rF-IFN- γ -infected MC38 tumor cells alone. The percentage of tumor-free mice was determined as explained in panel A. (D) Mice (10/group) received two s.c. vaccinations of rF-IFN- γ (10 MOI)-infected MC32A (CEA-expressing) tumor cells (5×10^5 /injection). Prior to challenge with 3×10^5 parental MC32A tumor cells, mice were administered GK1.5 (open circles), 2.43 (anti-CD8, squares), anti-asialo-GM1 (closed triangles), GK1.5/2.43 (open triangles) or an isotype control

antibody (closed circles). Mice were monitored daily for tumor appearance (minimum volume approx. 20-30 mm³) and the results are presented as described in A. Data in all panels are the combined results from two separate experiments.

FIG. 7.

Tumor cell specific cytolytic activity in the regional LNs of mice following injection of rF-IFN- γ -infected MC38 cells. MC38 cells were either infected with 10 MOI of FP-WT (triangles) or rF-IFN- γ (circles) or treated in vitro with 10ng rIFN- γ /ml (squares) prior to injection s.c. into mice (3×10^5 cells/injection). Seventy-two hrs after injection, regional LNs were isolated and tested for lysis against MC38 (A and C) and EL4 (panels B and D) in 4-hr (A and B) and overnight (panels C and D) assays. Panel A, insert: LN cells from mice injected with rF-IFN- γ -infected MC38 cells were stimulated in vitro in the presence of irradiated MC38 tumor cells (see Materials and Methods) prior to 4-hr cytolytic assays with either MC38 (circles) or EL4 (triangles) cells as targets. Data are presented as the mean $\pm$ SEM from triplicate wells at each E:T ratio and are from a representative experiment performed 2-3 times with similar results.

FIG. 8.

In vivo NK cell depletion inhibits the development of an antigen-specific CTL response. Mice (3-5/group) received the NK cell-depleting antibodies, anti-asialo-GM1 (panel B), anti-NK1.1 (panel C) or Ly49D&H (panel D) as described in the Materials and Methods. Untreated mice which received an isotype control antibody are shown in panel A. All mice then received a single s.c. injection of rF-IFN- γ -infected (10 MOI) MC38 tumor cells. Three (3) days later the draining LNs were removed, combined and stimulated in vitro in the presence of irradiated MC38 cells (see Materials and Methods). Cells were harvested and 4-h lytic assays were carried out with MC38 (circles) and EL4 (triangles) cells as targets. All data are presented as the mean $\pm$ SEM from triplicate wells from a representative experiment that was repeated with similar results.

Table 1
MHC upregulation on MC38 and B16/F10 tumor cells following rF-IFN-γ infection.¹

MHC Cell Surface Expression			
		H-2K ^b	I-A ^b
MC38	none		
	FP-WT	N/A	
	rF-IFN-γ	61.8 [68.9] ² 70.1 [69.3] ³ 93.1 [177.6] ³ 95.5 [165.6] ³ 98.5 [89.8] ³	30.2 [54.4] 41.5 [45.8] 77.5 [45.8] ³ 92.2 [69.2] ³ 96.2 [47.6] ³
	rIFN-γ		neg neg neg neg neg
B16/F10	None		
	FP-WT	N/A	
	rF-IFN-γ	9.1 [14.1] 13.4 [18.8] 98.8 [92.2] ³ 87.8 [67.6] ³ 98.5 [89.8] ³	3.4 [12.2] 9.7 [17.7] 97.5 [52.6] ³ 81.5 [44.0] ³ 96.2 [47.6] ³
	rIFN-γ		neg neg neg neg neg

¹ MC38 and B16/F10 tumor cells were either infected with rF-IFN-γ or FP-WT at the indicated MOI or treated in vitro with supernatants from rF-IFN-γ-infected MC38 or by the exogenous addition of rIFN-γ as outlined in the Materials and Methods.

² Values represent the percentage of cells expressing the indicated MHC class I antigen measured by FACS. Data in parentheses are the mean channel fluorescence intensity. Data are from a single experiment that was repeated with similar results. neg. negative.

³ *P*<0.05 (vs. untreated cells).