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Kathleen C F Sheehan
Washington University School of Medicine in St. Louis

Koon Siew Lai
Washington University School of Medicine in St. Louis

Gavin P. Dunn
Washington University School of Medicine in St. Louis

Allen T. Bruce
Washington University School of Medicine in St. Louis

Mark S. Diamond
Washington University School of Medicine in St. Louis

See next page for additional authors

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Authors

Kathleen C F Sheehan, Koon Siew Lai, Gavin P. Dunn, Allen T. Bruce, Mark S. Diamond, Jennifer D. Heutel, Corazon Dungo-Arthur, Javier A. Carrero, J. Michael White, Paul J. Herzog, and Robert D. Schreiber

Blocking Monoclonal Antibodies Specific for Mouse IFN-α/β Receptor Subunit 1 (IFNAR-1) from Mice Immunized by *In Vivo* Hydrodynamic Transfection

KATHLEEN C.F. SHEEHAN,[1] KOON SIEW LAI,[1] GAVIN P. DUNN,[1] ALLEN T. BRUCE,[1]
MARK S. DIAMOND,[1] JENNIFER D. HEUTEL,[1] CORAZON DUNGO-ARTHUR,[1] JAVIER A. CARRERO,[1]
J. MICHAEL WHITE,[1] PAUL J. HERTZOG,[2] and ROBERT D. SCHREIBER[1]

ABSTRACT

Herein we report the generation of mouse monoclonal antibodies (mAbs) specific for the IFNAR-1 subunit of the mouse interferon-α/β (IFN-α/β) receptor (MAR1 mAbs) that block type I IFN receptor signaling and biologic response induction *in vitro* and *in vivo*. These mAbs were generated from *Ifnar1*$^{-/-}$ mice immunized by *in vivo* hydrodynamic transfection with a plasmid encoding the extracellular domain (ECD) of murine IFNAR-1. All MAR1 mAbs bound native receptor expressed on cell surfaces and immunoprecipitated IFNAR-1 from solubilized cells, and two mAbs also detected IFNAR-1 by Western blot analysis. *In vitro*, the mAbs prevented ligand-induced intracellular signaling and induction of a variety of type I IFN-induced biologic responses but had no effect on IFN-γ-induced responses. The most effective *in vitro* blocker, MAR1-5A3, also blocked type I IFN-induced antiviral, antimicrobial, and antitumor responses *in vivo*. We also explored whether murine IFNAR-1 surface expression required the presence of Tyk2. In contrast to *Tyk2*-deficient human cell lines, comparable IFNAR-1 expression was found on primary cells derived either from wild-type or *Tyk2*$^{-/-}$ mice. These mAbs represent much needed tools to more clearly elucidate the biochemistry, cell biology, and physiologic function of the type I IFNs and their receptor in mediating host-protective immunity and immunopathology.

INTRODUCTION

MOUSE TYPE I INTERFERONS (IFN-α/β) are a family of cytokines consisting of 14 functional IFN-α proteins and one IFN-β protein that play important roles in promoting and linking innate and adaptive immune responses.[1–3] All type I IFNs bind to the same heterodimeric receptor consisting of two subunits (IFNAR-1 and IFNAR-2)[4,5] that are coexpressed on nearly all cells. Ligand binding to the IFN-α/β receptor complex leads to the tyrosine phosphorylation and activation of IFNAR-1-associated Tyk2 and IFNAR-2-associated Jak1, which, in turn, phosphorylate cytosolic Stat1 and Stat2, giving rise to two types of activated transcription factors: a Stat1 homodimer and IFN-stimulated gene factor 3 (ISGF3), a tripartite complex of phosphorlyated Stat1, Stat2, and the constitutive DNA binding protein IFN regulatory factor-9 (IRF-9). The activated transcription factors enter the nucleus, bind to specific enhancer elements (IFN-γ activation sequence [GAS] and IFN-stimulated response element [ISRE] sequences, respectively) on certain genes, and by regulating the expression of these genes, induce the wide range of IFN-dependent effects on cellular function (reviewed in refs. 6–9).

Comparative functional analyses of several members of the type I IFN family revealed that they induce a significantly overlapping array of biologic responses, although quantitative differences have been noted in the specific activities of each particular form.[10,11] A small number of genes have been identified that are differentially regulated by IFN-α vs. IFN-β.[12,13] Based on IFN's wide-ranging activity, a reliable approach to studying the physiologic functions of the type I IFN family has been to use gene-targeted mice lacking a functional IFN-α/β receptor. For example, mice lacking the IFNAR-1 receptor subunit are

[1]Department of Pathology and Immunology, Center for Immunology, Washington University School of Medicine, St. Louis, MO 63110.
[2]Center for Functional Genomics and Human Disease, Monash Institute of Medical Research, Monash University, Clayton, Victoria 3168, Australia.

completely unresponsive to both endogenously produced and exogenously administered type I IFNs. Using these mice, it has been possible to validate the physiologically relevant roles of the type I IFNs in promoting antiviral, antimicrobial, antitumor, and autoimmune responses *in vivo*.[14–21]

Nevertheless, the use of *Ifnar1* gene-targeted mice is often complicated by the frequent need to breed the receptor deficiency into distinct genetic backgrounds and because of the high susceptibility that *Ifnar1*$^{-/-}$ mice display to certain viral infections. A proven alternative approach that circumvents these problems is the use of blocking, receptor-specific monoclonal antibodies (mAb) that can be administered to wild-type mice, rendering them as unresponsive to a particular cytokine as the corresponding receptor-deficient, gene-targeted mouse.[20,22–24] Thus, the availability of IFNAR-1-specific mAbs not only would help in analysis of the biochemistry and cell biology of the IFN-α/β receptor but also would facilitate the elucidation of IFN-α/β's physiologic *in vivo* actions. Although blocking mAbs specific for the human IFN-α/β receptor subunits have indeed been generated,[25–27] similar antibodies specific for the subunits of the murine IFN-α/β receptor are not available.

Herein we report the generation of blocking mAbs specific for murine IFNAR-1 derived from *Ifnar1*$^{-/-}$ mice immunized by *in vivo* hydrodynamic transfection using plasmid DNA encoding the extracellular domain (ECD) of murine IFNAR-1. We also characterize these mAbs using *in vitro* approaches and document their functional utility in assessing type I IFN's physiologic functions *in vivo*, using models of viral and bacterial infection and tumor development. Finally, we use the mAb to address the unresolved issue of IFN-α/β receptor expression in *Tyk2*$^{-/-}$ mice.

MATERIALS AND METHODS

Mice

Mice lacking the IFNAR-1 subunit of the IFN-α/β receptor (*Ifnar1*$^{-/-}$ mice) were obtained from M. Aguet (ISREC, Laussanne, Switzerland)[14] and were maintained on the same 129/Sv-Pas genetic background as the embryonic stem cell from which they were derived.[20] Wild-type 129/SvPas mice were obtained from Charles River (Wilmington, MA), and 129/SvEv (129 S6) or C57BL/6 mice were obtained from Taconic Farms (Germantown, NY). Mice were bred and maintained in our specific pathogen-free animal facility (Washington University School of Medicine) in accordance with American Association of Laboratory Animal Science guidelines. All animal protocols used in this study were approved by our Institutional Animal Review Board.

Cytokines and reagents

Purified recombinant cytokines were obtained from the following sources: MuIFN-γ from Genentech (South San Francisco, CA), HuIFN-$\alpha_{A/D}$ from Hoffmann-LaRoche (Nutley, NJ), MuIFN-αA and MuIFN-β from PBL Biomedical Laboratories (Piscataway, NJ), and MuIFN-α_5 from D. Fremont, Washington University School of Medicine (St. Louis, MO). Goat antimouse Ig secondary reagents were obtained from Cal-

tag Laboratories (Burlingame, CA). GIR-208 is a mouse IgG1 mAb specific for human IFNGR-1 that shows no reactivity with the mouse proteins[22] and was, therefore, used as an isotype-matched irrelevant control mAb in this study.

IFNAR-1 Plasmid DNA

A modified *Ifnar1* cDNA was prepared that encoded the following sequences in order: (1) the 26-amino acid IFNAR-1 signal sequence (nucleotides [nt] 1–78 of the full-length *Ifnar1* coding sequence), (2) a 10-amino acid N-terminal *myc* peptide-tag (EQKLISEEDL),[28] (3) the mature 404-amino acid IFNAR-1 ECD (nt 79–1287 of the full-length *Ifnar1* coding sequence), and (4) a C-terminal His$_6$ peptide tag. The modified *Ifnar1* cDNA was subcloned into the pRK5 mammalian expression vector using ClaI and XbaI to generate the pRK5.IFNAR1.ecd plasmid. Coding sequences of all constructs were verified by DNA sequencing (Big Dye method, Applied Biosystems, Foster City, CA). Plasmid DNA used for immunization was prepared using the Qiagen Endo-Free Mega Kit according to the manufacturer's instructions (Valencia, CA).

Generation of mouse mAbs against mouse IFNAR-1 ECD

Mouse mAbs specific for the ECD of mouse IFNAR-1 were developed from *Ifnar1*$^{-/-}$ mice immunized by hydrodynamic injection of plasmid DNA encoding the murine IFNAR-1 ECD. Briefly, *Ifnar1*$^{-/-}$ mice were injected intravenously (i.v.) over 7 sec with 100 μg of the pRK5.MuIFNAR1.ecd plasmid in a volume of Ringer's solution corresponding to 10% of the animal's body weight (i.e., 2.5 mL/25 g mouse).[29,30] Following an additional two rounds of injections on days 10 and 21, strong IFNAR-1-specific antibody responses were detected in the circulation of most of the injected mice. Seropositive mice with the highest anti-IFNAR-1 titers were boosted again 30 days later with 100 μg of the same plasmid DNA. To produce the IFNAR-1-specific mAbs, splenocytes were harvested 4 days after the final boost and were fused to the murine P3X63Ag8.653 myeloma cell line according to published procedures.[23] Supernatants from growth-positive wells were screened for the presence of IFNAR-1-specific mAbs by monitoring differential immunostaining of *Ifnar1*$^+$ vs. *Ifnar1*$^-$ murine sarcomas[20] as assessed by flow cytometry. MAR1 mAb were purified by protein A affinity chromatography.[22] Bulk quantities of purified endotoxin-free MARI-5A3 were generated by Leinco Technologies (St. Louis, MO).

Cell lines

Murine methylcholanthrene (MCA)-induced sarcoma cell lines derived from either IFNAR-1-expressing *Rag2*$^{-/-}$ mice (RAG2-R1, RAG2-R4), mice lacking the *Ifngr1* subunit of the IFN-γ receptor (RAD.gR.28), IFNAR-1-deficient *Ifnar1*$^{-/-}$ mice (IFNAR1-R1), or mice lacking both *Ifnar1* and *Ifngr1* (GAR4, GAR5) were isolated and propagated in R10 medium as described.[20,31,32] GAR4 and GAR5 tumor cells were transduced as described[33] with either empty retrovirus or a retrovirus encoding full-length murine *Ifnar1* to produce the IFN-α/β-responsive GAR4.AR1.18 and GAR5.AR1 cell lines.[20] 293 cells (6 $\times$ 10^6) were also transiently transfected with

pRK5.MuIFNAR1.ecd (25 μg) or empty vector by electroporation (0.25 kV) using a GenePulser and protein-containing supernatant harvested after 2 days. L929 cells were obtained from ATCC (Rockville, MD). Mouse embryo fibroblasts (MEFs) were generated according to standard procedures.[34] Bone marrow-derived macrophages were generated as previously described.[35] CosN cells (7×10^5) were transfected with 3 μg pRK5-MuIFNAR1.ecd using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to standard procedures, and supernatants were harvested after 24–48 h.

Flow cytometry

Samples (100 μL) of either hybridoma supernatants or purified mAb (2 μg) were incubated with IFNAR-1-deficient GAR4 cells or IFNAR-1-expressing GAR4.AR1.18 cells for 2 h at 4°C. Cells were washed and incubated for an additional hour at 4°C with biotin-conjugated goat antimouse IgG (Jackson Immuno-Research, West Grove, PA). After washing, the cells were resuspended in phosphate-buffered physiologic saline (PBS), incubated 40 min at 4°C with streptavidin-PE (Chromaprobe, St. Louis, MO), washed, resuspended in PBS containing 10% fetal bovine serum (FBS), and analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ). Immune serum, hybridoma supernatants, and purified MAR1 mAbs were also screened in a similar manner for their ability to detect natural IFNAR-1 on L929 cells, MCA sarcomas, primary splenocytes, and MEFs obtained from wild-type *Ifnar1$^{+/+}$* mice or gene-targeted *Ifnar1$^{-/-}$* mice. All staining was performed at 4°C.

Immunoprecipitation and Western blot analysis of IFNAR-1

Splenocytes (1×10^8) from 129/SvEv mice were lysed in 1 mL of 0.5% NP-40 lysis buffer (50 mM Tris, 150 mM NaCl, containing protease inhibitors [Sigma, St. Louis, MO], 1:500) and the lysates were precleared by two treatments with 50 μL of a 1:1 slurry of protein G sepharose (Pharmacia, Piscataway, NJ). Either 1 μL antisera (1:1000 dilution) or 20 μg purified antibody was added to the cleared lysates together with 50 μL of the 1:1 protein A/G sepharose slurry, and the mixture was incubated overnight at 4°C. Beads were washed three times with 0.5% NP-40 lysis buffer, followed by one wash with PBS, and then suspended in 25 μL Laemmli buffer (containing 1 μL 2-ME for reducing conditions) and heated for 8 min at 100°C. Samples were resolved on 4%–15% Criterion polyacrylamide gels (Bio-Rad, Hercules, CA), transferred to nitrocellulose, and analyzed by Western blotting as described elsewhere.[22] Specific proteins were detected by sequential treatments using biotin-conjugated antibodies (2 μg/mL) and steptavidin-Alexa-680 (1:10,000 dilution) (Molecular Probes, Eugene, OR) and visualized using a LICOR Odyssey imaging system (Lincoln, NE).

Phospho-Stat1 immunoblot

L929 cells (5×10^6) were preincubated with either IFNAR-1-specific or irrelevant GIR-208 mAb and stimulated for 15 min at 37°C with 300 U/mL MuIFN-α. Reactions were terminated by addition of cold medium. The cells were washed and lysed for 20 min at 4°C by the addition of two volumes of RIPA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% DOC, containing a protease inhibitor cocktail [Sigma, 1:500]) and 2 mM sodium orthovanadate. Lysate (200 μg/lane) was run on 10%–20% Criterion SDS-PAGE gels and transferred to nitrocellulose. Membranes were incubated with Odyssey blocking buffer (LICOR) for 1 h prior to the addition of polyclonal rabbit antibodies specific for murine phospho-Stat1 (Cell Signaling, Beverly, MA, 1:1000) and overnight incubation at 4°C. Nitrocellulose membranes were washed, incubated with goat antirabbit-IgG-Alexa Fluor-680 (Molecular Probes) for 45 min and, after washing, analyzed on a LICOR Odyssey imaging system. To visualize total Stat1 protein, the membrane was incubated with a mouse Stat1 N-terminal specific antibody (G16920, Transduction Laboratories, South San Francisco, CA) for 1 h, followed by incubation with goat antimouse-IgG-Alexa Fluor-800. Staining with mouse antiactin (Sigma, St. Louis, MO) followed by goat antimouse-IgG-Alexa Fluor-800 (1:10,000) was used to confirm equivalent loading of cellular protein into each lane. Staining was visualized as described.

In vitro assays for IFN function

IFN-induced antiviral effects were assessed using a cytopathic effect (CPE) assay that employed L929 cells and vesicular stomatitis virus (VSV Indiana strain, 5×10^4, TCID$_{50}$) as previously described.[36] Induction of nitric oxide (NO) was determined using L929 cells stimulated with different types of IFN and lipopolysaccharide (LPS) as previously described.[37,38] Enhancement of MHC class I expression was assessed by flow cytometry after treatment of the RAG-R4 MCA sarcoma cell line with different types of IFN for 72 h as described.[20]

Phamacokinetics

Groups of wild-type or *Ifnar1$^{-/-}$* mice were injected with either 0.2 mg or 2.0 mg MAR1-5A3 or GIR-208 control mAb intraperitoneally (i.p.) in a volume of 0.5 mL PBS. At each time point, blood was collected from 2–5 animals (200 μL each via retroorbital sinus). MAR1-5A3 antibody levels in the serum were determined using an indirect binding inhibition ELISA. Briefly, plates were coated with purified MAR1-4D12 mAb (200 ng/well), washed, and then 50 μL IFNAR-1.ecd-containing supernatant was added per well. After 1 h, plates were washed, incubated with dilutions of either serum or unconjugated, purified MAR1-5A3 antibody, and washed again. Biotin-conjugated MAR1-5A3 (0.1 μg) was added to each well, and plates were developed using streptavidin-peroxidase (Zymed, Carlsbad, CA) and ABTS substrate (Roche, Indianapolis, IN). Values were determined based on a standard curve of purified MAR1-5A3 diluted in normal mouse serum. Levels of GIR-208 were directly quantitated by ELISA using plates coated with murine IFNGR1.ecd protein,[39] with values determined based on a standard curve of purified GIR-208 diluted in normal mouse serum.

In vivo assays for IFN function

VSV infection. Groups of 5 wild-type or *Ifnar1$^{-/-}$* mice were pretreated with either PBS, 1.25 or 2.5 mg MAR1-5A3, GIR-208 mAb, or saline 1 day prior to infection with VSV Indiana strain (1×10^6, TCID$_{50}$, i.v., in 200 μL) as described.[40] Animals were monitored daily for survival.

Listeria *infection*. Groups of 5 wild-type or $Ifnar1^{-/-}$ mice were pretreated with PBS or 2.5 mg of either MAR1-5A3 or control GIR-208 mAb 1 day prior to infection with *Listeria monocytogenes* EGD strain as previously described.[18,41] Four days postinfection, the spleens were harvested, and the numbers of bacteria were counted.

Growth of transplanted tumors. Wild-type or $Rag2^{-/-}$ mice ($n = 3–5$) were treated for various times beginning on day -1 with MAR1-5A3 or control GIR-208 mAb (e.g., 500 μg on days -1, 0, $+1$, and $+2$ plus; 250 μg every other day thereafter) and challenged with 1×10^6 RAG2-R1 sarcoma cells subcutaneously (s.c.) on the flank as described.[20] Tumor growth was monitored and recorded as the mean tumor diameter of two perpendicular measurements.

Generation of Tyk2-*deficient mice*

Targeting vector. Genomic clones containing portions of the murine *Tyk2* gene were isolated from a Lambda Fix II 129/Sv genomic library (Stratagene, La Jolla, CA). A 16-kb genomic *Tyk2* clone encompassing the exons for the 5′-untranslated region (5′-UTR) and the first ATG start codon was used to generate the *Tyk2* targeting vector. A 1.2-kb AvaI-SacI fragment containing the 5′-UTR, a 1.5-kb SacI-EcoRI fragment containing the exon coding for the first ATG codon, and a 4.7-kb EcoRI fragment containing the three exons were cloned, respectively, into the NotI, AscI, and PmeI sites of the TNLOX1-3 targeting vector.[42]

Transfection of ES cells and generation of Tyk2-*deficient mice*. The *Tyk2* targeting vector (20 μg) was linearized by SalI digestion and electroporated into the MC-50 embryonic stem cell line generated in our laboratory. Following double selection, single colonies were analyzed for homologous recombination by PCR using the following set of primers: 5′Sac412F primer ATGCATCATACACACACATGTGAG and Neo1547F primer GAAGACAATAGCAGGCATGCT. The frequency of homologous recombination events was 1:236. The complete integration of the 3′ region, including the downstream loxP3 site, was verified by PCR using 3P1F primer GATCCT-CACAAGTTCCAGTC and 3P4R primer CTGGGA-GAGCAGCCACTGCTC. The targeted ES cell clone, M83, was then transiently transfected with an expression vector pMC-cre, which encodes the Cre-recombinase.[42] Colonies were screened by PCR using M180F primer CGGATGCACATAG-CACTGTGTAGC and 3P4R primer as described. The ES cell clone, GK-76, in which the PGK-neo cassette plus the 1.5-kb gene segment were deleted, was used to microinject into blastocysts to generate *Tyk2*-deficient mice.

Genotyping by Southern blot, PCR, and Western blot. Southern blot analysis was performed on 25 μg BamHI-digested genomic DNA as described.[40] The filter was probed with a ^{32}P-labeled external probe (3EP). To genotype mice by PCR genomic DNA samples were amplified with M180F and 3P4R primers. The samples were amplified in the following manner: 1 cycle at 94^{0}C for 5 min; 35 cycles at the following conditions: 94°C for 30 sec, 65°C for 30 sec, and 72°C for 90 sec; and finally 1 cycle at 72°C for 5 min. A PCR product of 240-bp fragment derived from the targeted allele and a 1.5-kb fragment derived from the wild-type allele were resolved on a 1.5% TBE agarose gel. Western blot analysis was performed on

lysates of bone marrow macrophages as described[40] using Tyk2 antisera obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

In vivo *genetic hydrodynamic immunization*

In vivo hydrodynamic transfection of naive wild-type mice or $Ifnar1^{-/-}$ mice with a plasmid encoding the ECD of murine IFNAR-1 engineered to express an N-terminal *myc* tag and a C-terminal His$_6$ tag resulted in the accumulation of soluble tagged forms of IFNAR-1.ecd protein in the blood in both types of mice. As evidenced by immunoprecipitation with anti-*myc* and Western blot analysis with labeled anti-*myc*, the tagged IFNAR1.ecd was detected as early as 10 h postinjection and persisted for >1 week in both types of mice (data not shown). However, when the mice were subjected to a second round of *in vivo* hydrodynamic transfection, soluble IFNAR-1 was detected only in the blood of wild-type mice but not $Ifnar1^{-/-}$ mice. The sera obtained from the latter but not the former contained IFNAR-1-specific antibodies as evidenced by flow cytometry using $Ifnar1^{+/+}$ MCA-induced murine sarcomas generated in $Rag2^{-/-}$ mice (RAG2-R4) (Fig. 1A and 1B, respectively). Serum titers in some of these twice-transfected $Ifnar1^{-/-}$ animals exceeded 1:10,000, as detected by FACS analysis (data not shown). Thus, the subsequent absence of detectable IFNAR-1.ecd in the circulation of hydrodynamically transfected $Ifnar1^{-/-}$ mice was explained by the development of an antibody response against the foreign soluble IFNAR-1 protein.

To further assess the specificity of the antibody response generated in hydrodynamically transfected $Ifnar1^{-/-}$ mice, sera from these mice were tested by flow cytometry using a variety of IFNAR-1-expressing and IFNAR-1-deficient cell lines. Whereas sera from transfected $Ifnar1^{-/-}$ mice failed to stain the *Ifnar1*-deficient GAR4 MCA sarcoma line derived from a mouse lacking both IFNAR-1 and IFNGR1[20] (Fig. 1D), homogeneous positive staining was observed on GAR4 cells that had been stably transduced with a retrovirus encoding full-length, wild-type IFNAR-1 (GAR4.AR1.18 cells)[20] (Fig. 1C). The same sera also stained an MCA sarcoma from a mouse that naturally expressed wild-type IFNAR-1 (RAG2-R1) (Fig. 1E) but not a sarcoma derived from a gene-targeted mouse that lacked the protein IFNAR1-R2 (Fig 1F).[20] Furthermore, the serum detected IFNAR-1 on type I IFN-responsive murine L929 fibroblasts, and staining was blocked if the cells were preincubated at 4°C with a combination of 10,000 U each of MuIFN-αA and MuIFN-β (Fig. 1G,H). Thus, *in vivo* hydrodynamic transfection of $Ifnar1^{-/-}$ mice with plasmid DNA encoding the ECD of IFNAR-1 led to induction of antibodies reactive to the native receptor expressed on cell surfaces.

Generation of IFNAR-1-specific mAbs

Following multiple rounds of *in vivo* hydrodynamic transfection of $Ifnar1^{-/-}$ mice with the IFNAR-1.ecd plasmid, the spleen of 1 immunized mouse was harvested, and splenocytes were fused to murine myeloma cells to generate mAb.[22] As assessed by differential staining of GAR4 and GAR4.AR1.18 cells, one third of the growth-positive wells (138 of 415) con-

tained antibody that specifically reacted with the ectopically expressed receptor. Culture supernatants from 23 of 65 strongly positive cell lines also reacted with IFNAR-1-expressing RAG2-R1 cells. All but 2 of these antibodies were of the IgG1 isotype. Five separate hybridomas (MAR1-1H5, MAR1-3A7, MAR1-4D12, MAR1-4F12 and MAR1-5A3) were selected, subcloned by limiting dilution, and expanded, and the specific IgG1 that was purified from spent culture supernatants was used in the subsequent analyses.
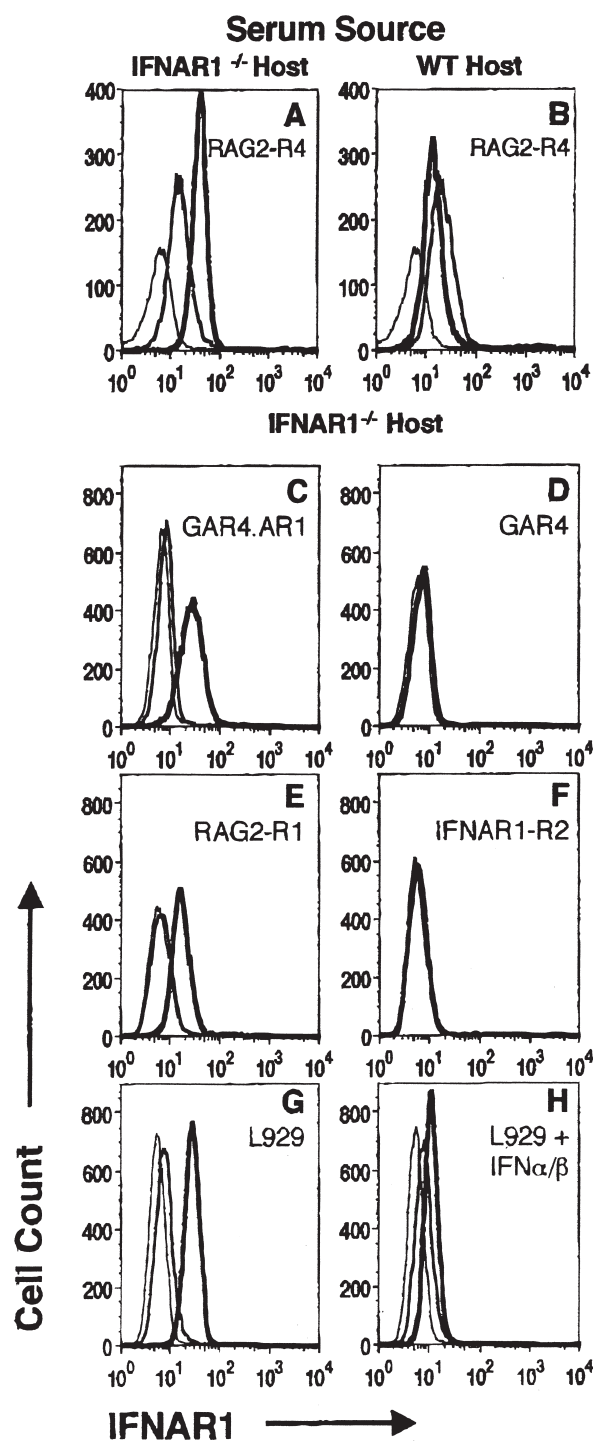

FIG. 1. Detection and specificity of plasmid-induced humoral response. Indicated cell lines were stained using preimmune or immune sera at a 1:1000 dilution, followed by PE-conjugated goat antimouse Ig. Fluorescence intensity was analyzed by flow cytometry. Gray line indicates secondary antibody alone, solid line signifies control normal mouse sera, and bold line symbolizes immune sera. (**A** and **B**) RAG2-R4 sarcoma stained with sera obtained from $Ifnar1^{-/-}$ or wild-type mice, respectively, following hydrodynamic genetic immunization. Serum obtained preimmunization or postimmunization of $Ifnar1^{-/-}$ mice using plasmid encoded IFNAR-1.ecd was used in **C, D, E, F, G,** and **H**. (**C** and **D**) The murine fibrosarcoma GAR4 (derived from an MCA-treated $Ifngr1^{-/-} \times Ifnar1^{-/-}$ mouse) and GAR4.AR1.18 (transfected with full-length murine $Ifnar1$). (**E** and **F**) MCA-induced sarcoma lines obtained from either wild-type 129/SvEv (RAG2-R2) or $Ifnar1^{-/-}$ mice (IFNAR1-R2). (**G** and **H**) L929 cells preincubated in buffer or cytokines (10,000 U each of IFN-α and IFN-β).

MAR1 mAbs recognize native IFNAR-1

The specificity of each purified MAR1 mAb was assessed using two different approaches. First, when analyzed by flow cytometry, each mAb stained IFNAR-1-expressing RAG2-R1 sarcoma cells (Fig. 2A, top) but did not stain IFNAR1-R2 sarcoma cells derived from an $Ifnar1^{-/-}$ mouse (Fig. 2A, bottom). Second, each MAR1 mAb was examined for the ability to immunoprecipitate natural IFNAR-1-derived from cell lysates of 129/Sv mouse splenocytes. When these immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting using biotin-conjugated MAR1-5A3 (Fig. 2B), each MAR1 mAb precipitated a single 100-kDa component that displayed an M_r consistent with that reported for natural murine IFNAR-1. This band was not present in precipitates generated with the GIR-208 control mAb. In contrast, when used as detection reagents in Western blot analyses, only MAR1-5A3 and MAR1-1H5 reacted with IFNAR-1 separated by SDS-PAGE under either reducing (data shown) or nonreducing (data not shown) conditions (Fig. 2C).

MAR1 antibodies block type I IFN-induced Stat1 phosphorylation

To assess the effects of the MAR1 antibodies on IFN-α/β-induced cellular responses, we first asked whether the mAbs could block IFN-α/β-dependent phosphorylation/activation of the transcription factor Stat1. Pretreatment of murine L929 cells with each of the MAR1 mAbs led to a 60%–88% inhibition of MuIFN-α5-dependent Stat1 phosphorylation (Fig. 3). Inhibition was not observed in cells treated with control GIR-208 mAb. Similar results were obtained in other experiments in which IFN-α/β receptor signaling was assessed by monitoring nuclear translocation of activated Stat1 as detected using confocal microscopy.[43] Here again, pretreatment of either L929 cells or RAG-R2 sarcomas with individual MAR1 mAb inhibited Stat1 nuclear accumulation following stimulation for 15–30 min with different forms of type I IFNs (100 U/mL HuIFN-$\alpha_{A/D}$, MuIFN-αA, or MuIFN-β). Thus, all five MAR1 mAbs blocked IFN-α-induced receptor signaling, and four of five (all except MAR1-4D12) prevented IFN-β-induced Stat1 activation (data not shown).
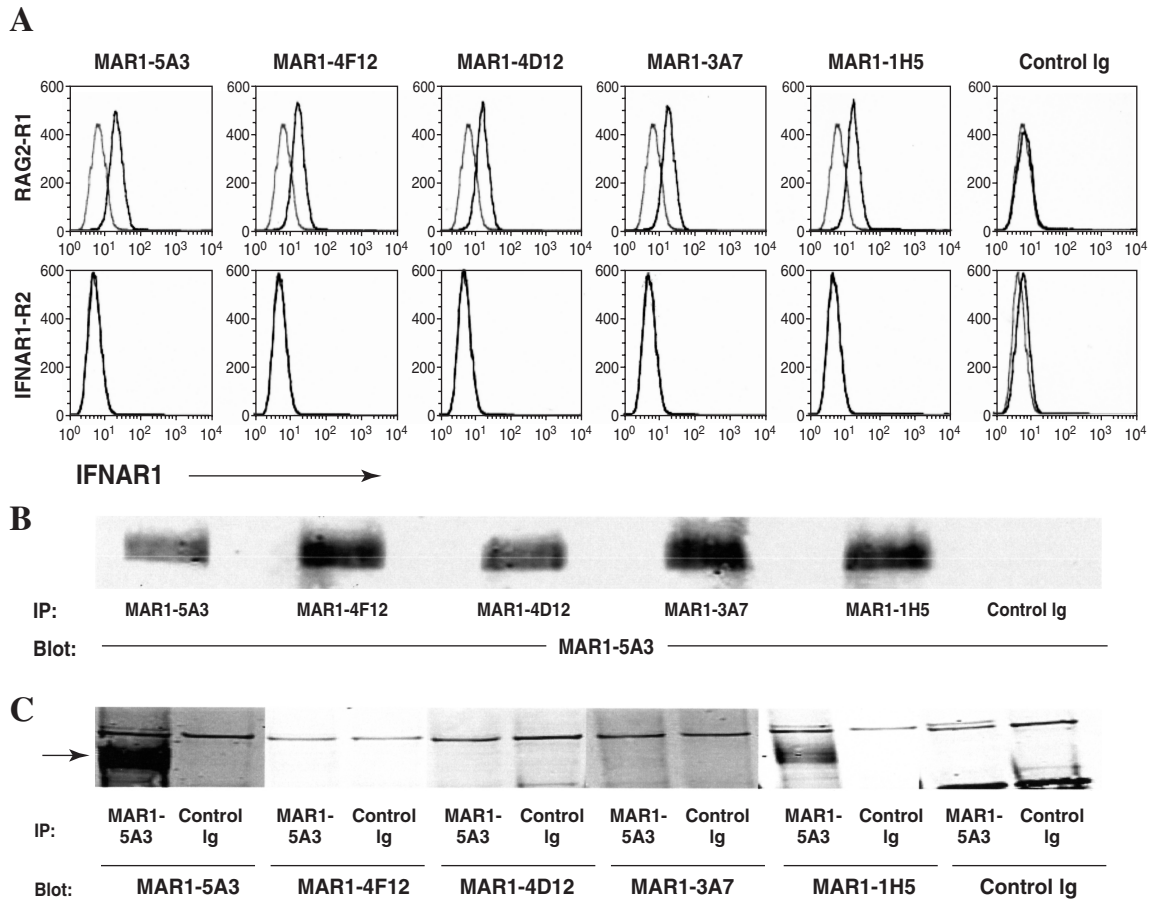
FIG. 2. Detection of native IFNAR-1. (**A**) The IFNAR-1-expressing wild-type tumor RAG2-R1 and the *Ifnar1*-deficient tumor cell line IFNAR1-R2 (10^6 cells/well) were stained with 2 μg of each purified MAR1 mAb (thick line) or control Ig (thin line) and detected with secondary antibody, PE-conjugated goat antimouse Ig. (**B**) Immunoprecipitation of 129/SvEv splenic lysates (1×10^8 cells/lane) using 5 μg of individual MAR1 mAb or control Ig plus protein G sepahrose. Protein was visualized with biotin-conjugated MAR1-5A3. (**C**) Western blot analysis of 129/SvEv splenic lysates immunoprecipitated with either MAR1 mAb or control Ig and detected with individual biotin-conjugated MAR1 mAb or control Ig. Native IFNAR-1 appears as a 100-kDa band.

MAR1 antibodies block classic type I IFN-induced cellular responses in vitro

Because the signature functional activity of the type I IFNs is to protect cells against viral infection, we tested the capacity of the MAR1 mAbs to block IFN-induced antiviral activity using a CPE assay that employed L929 cells and VSV. Each of the five MAR1 mAbs displayed a dose-dependent ability to block antiviral activity induced by 10 U of either HuIFN-$\alpha_{A/D}$ (Fig. 4A) or MuIFN-αA (Fig. 4B). Whereas four of the MAR1 mAbs also blocked IFN-β-induced antiviral activity, MAR1-4D12 did not (Fig. 4C). MAR1-5A3 consistently displayed the most potent blocking activity of any of the receptor-specific mAbs tested regardless of the specific type I IFN used. None of the mAbs inhibited IFN-γ-induced antiviral activity in the same assay system (Fig. 4D; data not shown).

Stimulation of a variety of cells with type I IFNs plus LPS causes the expression of the inducible form of nitric oxide synthase (iNOS), leading to production of NO, which has potent

antimicrobial effects.[44–46] Therefore, we also tested the capacity of the MAR1 mAbs to block iNOS induction in L929 cells treated with a combination of LPS and either HuIFN-$\alpha_{A/D}$, MuIFN-αA, or MuIFN-β. Again, all the mAbs specifically inhibited HuIFN-$\alpha_{A/D}$ (Fig. 4E) and MuIFN-αA (Fig. 4F) induced iNOS induction (as evidenced by monitoring accumulation of the stable NO product, nitrite, in the medium) in a dose-dependent fashion. Four mAbs also inhibited IFN-β-induced iNOS expression (Fig. 4G). As was the case for antiviral activity, MAR1-5A3 displayed the most potent and most generalized inhibitory capacity of all the mAbs, and MAR1-4D12 inhibited iNOS induction by MuIFN-α and HuIFN-α but not by MuIFN-β (Fig. 4E, F, G). None of the mAbs inhibited NO induction by MuIFN-γ (Fig. 4H; data not shown).

Type I or type II IFNs also upregulate expression of many of the components that participate in the MHC class I antigen processing and presentation pathway. For example, H-2K^b expression on the surface of RAG2-R4 sarcoma cells increases significantly (50–90 channel shift) when cells are cultured for
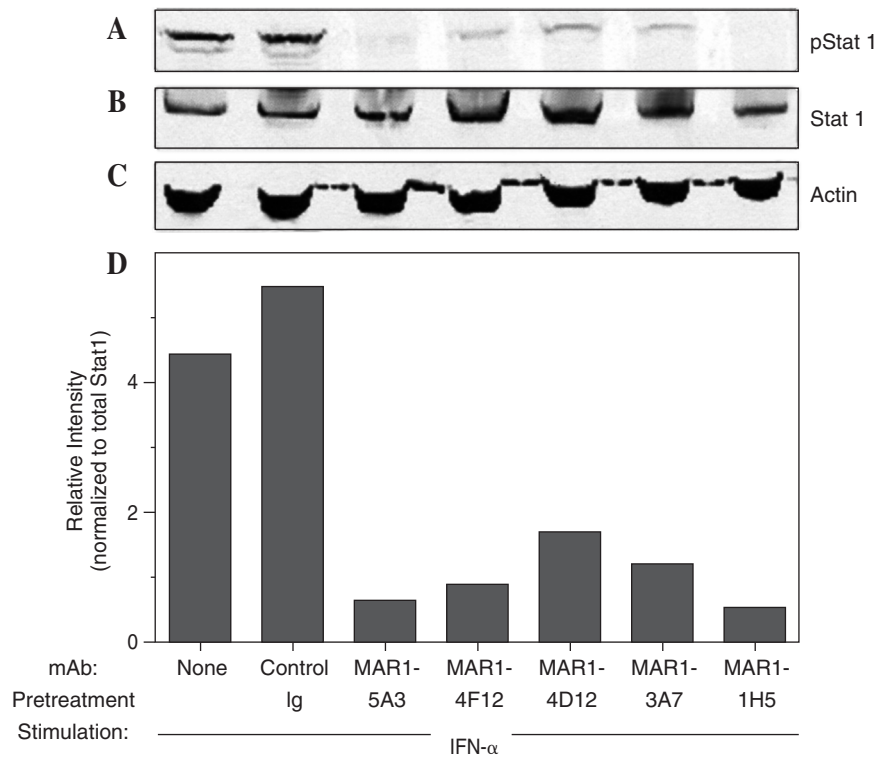
FIG. 3. Inhibition of Stat1 phosphorylation. L929 cells (5×10^6) were first treated with MAR1 or control Ig (10 μg/mL) and then stimulated with 300 U/mL MuIFN-α for 15 min. Cellular lysates were separated on SDS-PAGE and transferred to nitrocellulose. Blots were stained for (**A**) phospho-Stat1, (**B**) total Stat1 protein, and (**C**) actin, and binding was visualized using a LICOR Odyssey imaging system. (**D**) Values represent the relative staining intensity for phospho-Stat1 normalized to total Stat1 staining.

72 h with 300 U/mL of either HuIFN-$\alpha_{A/D}$, MuIFN-αA, or MuIFN-β (Fig. 4 I, J, K). In contrast, MHC class I upregulation was attenuated in a dose-dependent manner when the cells were pretreated with each of the MAR1 mAbs. Although MAR1-5A3 completely suppressed IFN-α/β-dependent enhancement of H-2K^b expression, its specific activity was not significantly greater than that of any of the other MAR1 mAbs. MAR1-5A3 did not inhibit IFN-γ-induced MHC I expression (Fig. 4L). Similar results were obtained when either L929 murine fibroblasts or a variety of MCA-induced sarcoma lines were used as indicator cells (data not shown).

MAR1-5A3 pharmacokinetics

Before assessing the *in vivo* efficacy of the IFNAR-1-specific mAbs, we first determined their pharmacokinetics. When naive 129/Sv mice were injected i.p. with a single 0.2-mg dose of MAR1-5A3, we were surprised to find that the mAb displayed a circulatory half-life ($t_{1/2}$) of only 1.8 days compared with 10 days for the irrelevant, isotype-matched GIR-208 control mAb (Fig. 5A). This unusually rapid clearance rate was not immune mediated, as no anti-MAR1-5A3 reactivity could be detected by ELISA in the sera of either naive mice or mice that received multiple injections of the antibody (data not shown). Moreover, the $t_{1/2}$ of 0.2 mg of MAR1-5A3 was the same in both naive animals and mice that had been treated weeks before with similar doses of MAR1-5A3 (Fig. 5A). We subsequently found that two other IFNAR-1-specific mAbs (MAR1-

1H5 and MAR1-4D12) displayed similar rapid clearance kinetics (data not shown). Therefore, we considered the possibility that the rapid disappearance of the MAR1 antibodies from the circulation reflected their absorption by an unanticipated large cellular pool of IFNAR-1 expressed on cells *in vivo*. To test this hypothesis, we repeated the mAb clearance study in *Ifnar1*$^{-/-}$ mice and found that the serum half-life of low-dose MAR1-5A3 increased to 7.5 days and was now similar to that of control IgG (Fig. 5B). Based on this finding, we repeated the pharmacokinetics analysis in wild-type mice but injected 2 mg of MAR1-5A3. Under these conditions, MAR1-5A3 displays a $t_{1/2}$ of 5.2 days, which was comparable to that of the control GIR-208 mAb ($t_{1/2}$ = 7 days) (Fig. 5C). Taken together, these data suggest that a large pool of IFNAR-1 may be present in normal mice that would require a substantial bolus of mAb to saturate this receptor pool. No significant differences were observed in leukocyte populations derived from spleen and blood obtained from mice treated with one or more injections of the MAR1-5A3 mAb, indicating that this mAb did not cause cell depletion (Table 1). Because MAR1-5A3 was the IFNAR-1 mAb that displayed the most potent *in vitro* receptor blocking activity, all subsequent *in vivo* experiments were conducted using purified, pyrogen-free, and aggregate-free MAR1-5A3.

Enhanced mortality to VSV in MAR1-5A3-treated mice

Mice unable to respond to IFN-α/β (e.g., *Ifnar1*$^{-/-}$ mice and *Stat1*$^{-/-}$ mice) are more susceptible to viral infection
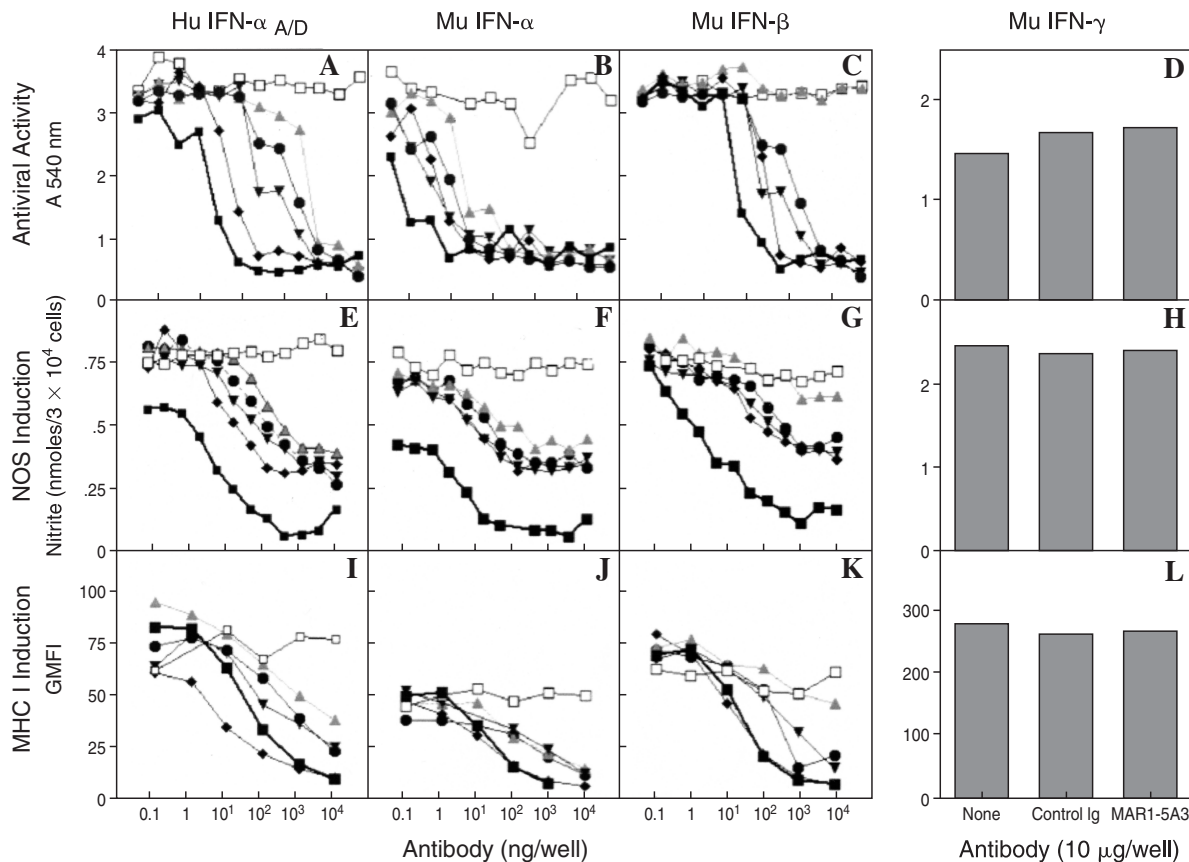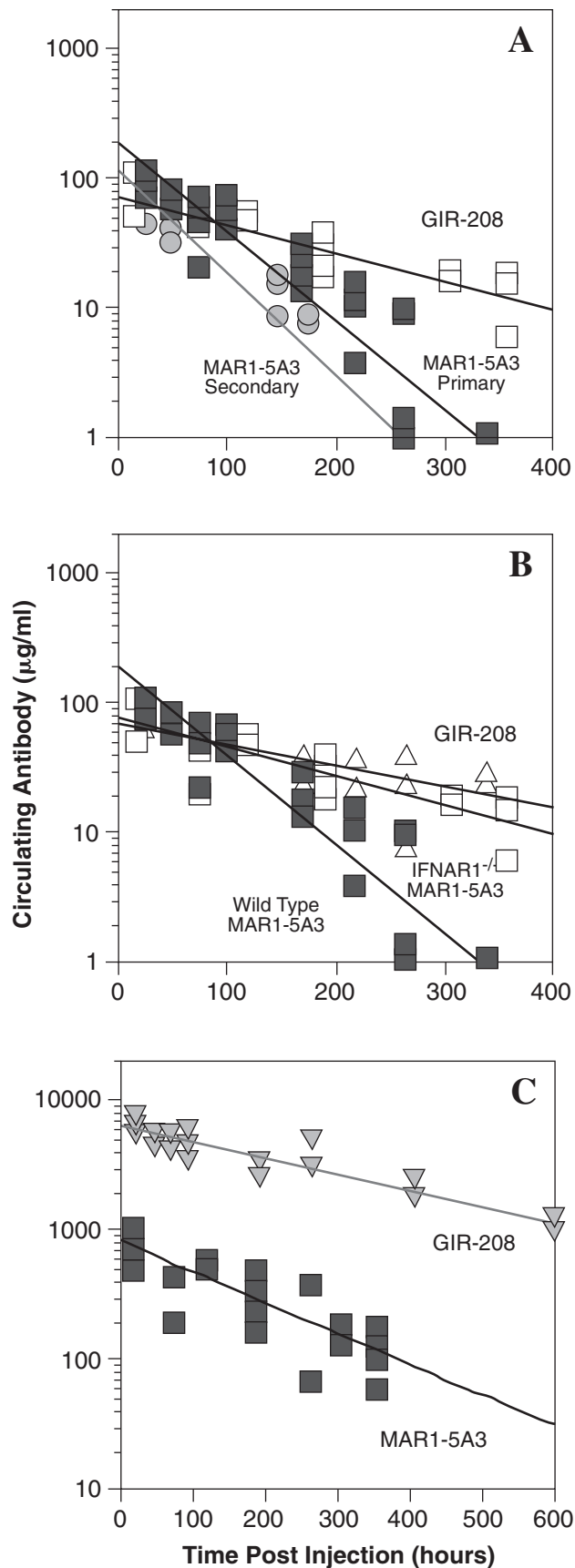
FIG. 4. MAR1 mAbs neutralize type I IFN activity *in vitro*. (**A, B, C,** and **D**) Blockade of type I IFN induced antiviral activity. L929 cells (3×10^4/well) were preincubated with titrations of MAR1 (MAR1-5A3, black squares; MAR1-4F12, inverted black triangles; MAR1-4D12, gray triangles; MAR1-3A7, black circles; or MAR1-1H5, black diamonds) or control Ig (open squares) for 1 h at 37°C, and the cultures were stimulated with type I IFN (10 U/well) overnight. Monolayers were washed to remove free antibody and cytokine, infected with VSV Indiana, and cultured an additional 48 h. Cell viability was assessed visually and by vital dye staining with crystal violet. (**D**) L929 cells were preincubated with buffer, MAR1-5A3, or control Ig (10 μg/mL) prior to stimulation with MuIFN-γ (10 U/well). (**E, F, G,** and **H**) Blockade of IFN-α-induced NOS. L929 cells (6×10^4/well) were preincubated 1 h at 37°C with titrations of MAR1 mAbs (as described) and exposed to HuIFN-$\alpha_{A/D}$, MuIFN-α, or MuIFN-β (100 U/well) plus LPS (5 ng/well). After 48 h of culture, supernatants were harvested and assayed for production of nitrites. (**H**) L929 cells were preincubated with buffer, MAR1-5A3, or control Ig (10 μg/mL) prior to stimulation with MuIFN-γ (100 U/mL) plus LPS. (**I, J, K,** and **L**) Inhibition of type I IFN-induced MHC I. RAG2-R4 sarcoma cells were pretreated for 1 h with the indicated concentrations of MAR1 mAb or control Ig and stimulated with type I IFN (1000 U/mL) for an additional 72 h of culture. Cells were harvested and stained for H2-K^b or irrelevant H2-K^k and analyzed by flow cytometry. Data are representative of four similar experiments using L929, murine sarcoma lines, or MEFs. (**L**) RAG2-R4 cell culture with buffer, MAR1-5A3, or control Ig (10 μg/mL) prior to stimulation with MuIFN-γ (300 U/mL).

than are wild-type mice. As shown in Figure 6, *Ifnar1*$^{-/-}$ mice succumbed rapidly to infection with VSV, whereas wild-type mice pretreated with either PBS or 2.5 mg control GIR-208 mAb displayed no morbidity. In contrast, wild-type 129/Sv mice treated with a single injection of two different doses of MAR1-5A3 showed enhanced susceptibility to VSV infection similar to that observed in the *Ifnar1*$^{-/-}$ mice.[40,47] Whereas all the *Ifnar1*$^{-/-}$ mice died within 4 days of infection, the time to death of wild-type mice treated with 2.5 mg MAR1-5A3 was approximately 10 days. Wild-type mice treated with 1.25 mg of IFNAR-1 mAb died at the same rate as mice receiving the higher mAb dose, but only 80% of this group succumbed to infection. MAR1-5A3 is thus able to block the protective effects of endogenously produced type I IFN during viral infection *in vivo* as well as *in vitro*. Similar

effects were seen following administration of MAR1-1H5 (data not shown).

Control of Listeria infection

Type I IFNs have been shown to control the *in vivo* response to *L. monocytogenes* in mice but, surprisingly, inhibit development of host-protective anti-*Listeria* responses. Specifically, *Ifnar1*$^{-/-}$ mice are more resistant to *Listeria* infection, clear the bacteria more efficiently, and show considerably less lymphocyte apoptosis in their spleens than their wild-type counterparts.[17,18,48] We, therefore, examined the effect of MAR1-5A3 treatment on the course of *Listeria* infection in normal mice. Wild-type 129/Sv mice treated with either PBS or control GIR-208 mAb were highly susceptible to *Listeria* infection, as in-

dicated by the presence of high numbers of the organisms in the spleens of infected mice. In contrast, *Ifnar1*$^{-/-}$ mice were resistant to infection and displayed 2 logs fewer bacteria in the spleen. Wild-type mice pretreated with a single 2.5-mg dose of MAR1-5A3 were also more efficient in controlling *Listeria* infection than control wild-type mice, and the spleens of the MAR1-5A3-treated mice contained significantly less *Listeria* than did controls (Fig. 7). Thus, these results demonstrate that antibody blockade of the IFN-α/β receptor mimics the genetic receptor deficiency in this model of bacterial infection challenge.

Temporal blockade of tumor rejection

We previously showed that endogenously produced type I IFNs are critical to the development of the host antitumor responses against primary, carcinogen-induced, and transplantable tumors.[20] Continuous treatment of wild-type mice with MAR1-5A3 inhibited rejection of several immunogenic MCA sarcomas that were efficiently eliminated in either untreated, naive, syngeneic wild-type hosts, or wild-type mice treated with GIR-208 mAb. To establish the temporal requirements for IFN-α/β during successful antitumor immune responses, we asked whether antibody blockade needed to be maintained throughout the entire rejection process. Groups of wild-type 129/Sv mice were, therefore, treated for varying time periods with MAR1-5A3 or control GIR-208 mAb and challenged with highly immunogenic RAG2-R1 sarcoma cells. As reported previously, RAG2-R1 sarcomas grew progressively when transplanted into immunodeficient *Rag2*$^{-/-}$ mice (Fig. 8 A, B, C), but were rejected when transplanted into wild-type mice treated with control GIR-208 mAb (Fig. 8 D, E, F). Also, consistent with our earlier report, RAG2-R1 tumors grew progressively in 4 of 5 wild-type mice treated continuously with MAR1-5A3 (i.e., treatment on days −1, 0, +1, +2, and every other day until day +21) (Fig. 8G). Interestingly, RAG2-R1 tumors also grew progressively in wild-type mice treated with MAR1-5A3 even when mAb treatment was discontinued after day 2 (treated days −1, 0, +1, and +2) (Fig. 8H). More importantly, progressive tumor growth was still observed in the majority of mice (3 of 5) treated with MAR1-5A3 only during the time frame of days +6 to +9 (Fig. 8I). These data indicate that type I IFNs act not only early, at the initiation of the immune response to an immunogenic tumor, but also late during the effector phase of tumor rejection.

TABLE 1.   MAR1-5A3 IS NONDEPLETING *IN VIVO* CELL[a]

Marker	Saline treatment	MAR1-5A3 treatment (2.5 mg)	Control Ig treatment (2.5 mg)
CD3	16.7×10^6 (32.2)[b]	14.3×10^6 (32.5)	12.4×10^6 (28.1)
CD4	10.7×10^6 (20.6)	9.3×10^6 (21.2)	8.2×10^6 (18.7)
CD8	5.7×10^6 (10.9)	4.9×10^6 (11.1)	4.2×10^6 (9.5)
B220	33.4×10^6 (64.2)	27.8×10^6 (63.1)	30.3×10^6 (68.9)
F4/80	4.6×10^6 (8.8)	4.5×10^6 (10.2)	4.9×10^6 (11.1)

[a]Mice (129/Sv) were treated with saline, MAR1-5A3 (2.5 mg), or control Ig (2.5 mg) i.p., and 4 days later, splenocytes were harvested and analyzed for surface marker expression by flow cytometry. Numbers represent cells expressing the indicated marker.
[b]% of total cells.

Detection of IFNAR-1 on *Tyk2*-deficient cells

Having demonstrated the specificity and blocking activity of the MAR1 mAbs, we used them to address the unanswered question of whether murine IFNAR-1 expression at the cell surface required an interaction with Tyk2. Previous studies using human cell lines have shown that membrane expression of human IFNAR-1 is stabilized by the association of Tyk2 with the intracellular domain of IFNAR-1.[49–51] These studies further showed that a human cell line lacking Tyk2 was unresponsive to HuIFN-α. These data suggest that in human cells, Tyk2 may be a key factor regulating IFNAR-1 surface expression, recycling, and function. In contrast, genetic disruption of the *Tyk2* gene in mice leads to only partial cellular insensitivity to many of the cytokines that engage Tyk2-associated receptor subunits

(e.g., IFN-α/β, interleukin-10 [IL-10], and IL-12). Because of the absence of antibodies that could detect murine IFNAR-1 on cell surfaces, it has not been possible to assess whether Tyk2 plays as important a role in IFNAR-1 expression in mice as it does in humans. We, therefore, used the MAR1-5A3 mAb to examine murine IFNAR-1 expression levels on MEFs or splenocytes obtained from wild-type, *Tyk2*-deficient or *Ifnar1*$^{-/-}$ animals. Staining with biotin-conjugated MAR1-5A3 revealed similar levels of IFNAR-1 expression on MEF lines derived from genetically matched wild-type or *Tyk2*-deficient animals (Fig. 9A, B). *Tyk2* deficiency was confirmed by Southern blot analysis (data not shown) and Western blot analysis (Fig. 9D). No MAR1-5A3 staining was detected on primary MEFs obtained from *Ifnar1*$^{-/-}$ mice (Fig. 9C). In addition, no statistically significant differences in surface IFNAR-1 levels between wild-type and *Tyk2*$^{-/-}$ splenocyte subpopulations (CD3$^+$, CD4$^+$, CD8$^+$, B220$^+$, F4/80$^+$, or DC3$^-$/DX5$^+$) were detected (paired *t*-test, $p = 0.53$–0.182) (Fig. 9E). Identical results were also obtained using the MAR1-4D12 mAb, which binds an epitope distinct from that recognized by MAR1-5A3 (data not shown). Thus, these novel MAR1 reagents provide the first opportunity to study murine IFNAR-1 expression on cell surfaces and should facilitate the analysis of cytokine receptor trafficking, particularly with respect to the effects of Tyk2 on receptor localization.
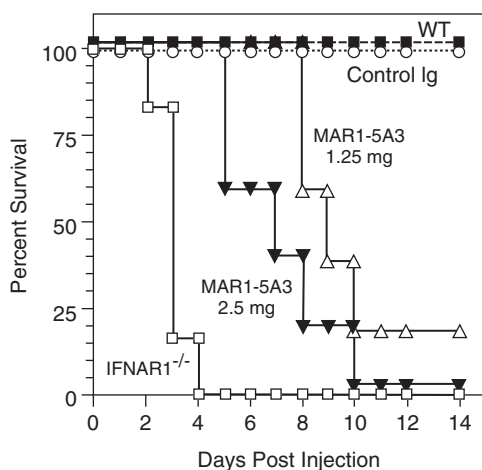
DISCUSSION

This paper describes a strategy to generate murine mAbs specific for soluble murine proteins, using a combination of *in vivo* genetic immunization and *in vitro* somatic cell fusion techniques. It also presents a novel use for these mAbs to neutralize IFN-α/β at different times during an ongoing *in vivo* response, an advantage that the use of gene-targeted mice does not allow. In this study, we took advantage of the availability of gene-targeted *Ifnar1*-deficient mice capable of mounting a humoral response against the soluble IFNAR-1 ectodomain whose expression in adult mice was transiently induced by genetic hydrodynamic transfection. Genetic immunization provided an efficient alternative to work-intensive, conventional procedures for producing and purifying soluble antigens. The
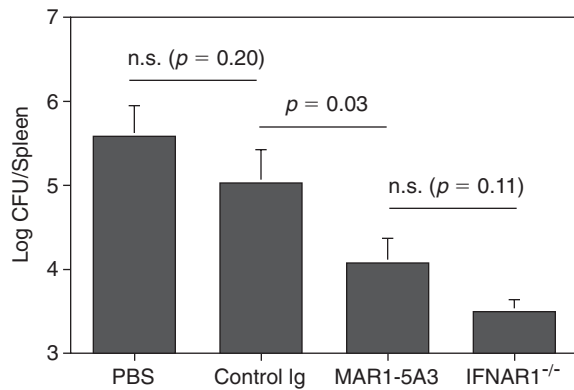

FIG. 6.   Protection from viral infection/neutralization of endogenous type I IFN. IFN-$\alpha\beta$R$^{-/-}$ (open squares) or wild-type 129/Sv mice ($n = 5$–6 per group) were left untreated (open circles), pretreated with either saline (black circles), control IgG1 (black squares), MAR1-5A3 (2.5 mg/mouse) (inverted black triangles), or 1.25 mg/mouse (open triangles) i.p. on day -1. Mice were infected with VSV (Indiana strain) 4.5×10^4 TCID$_{50}$ i.v. and observed daily. Data are representative of three experiments.

FIG. 7. Control of *Listeria* infection. 129/SvEv or *Ifnar1*$^{-/-}$ mice were pretreated with PBS, control Ig, or MAR1-5A3 (2.5 mg/mouse) 1 day prior to infection with *Listeria*. Four days later, spleens were harvested and homogenized, and the number of bacterial colonies was enumerated. Colony counts for MAR1-5A3-treated animals were significantly different from those obtained from animals treated with control Ig (Mann Whitney test, $p = 0.0343$). Data represent two combined experiments.

use of gene-targeted mice provided a host that recognized the reintroduced murine protein as a foreign antigen.

The *in vivo* hydrodynamic transfection procedure used in this study was developed by others[29,30] and is based on hepatocyte uptake of cDNAs injected i.v. under high pressure, which in turn leads to high-level production of the encoded protein by the cells. In the current study, we employed a cDNA that encoded a secreted form of the mouse IFNAR-1 protein that constituted the entire ECD of the receptor subunit. At present, we do not know if this same procedure can be used to generate antibodies against intracellular proteins, such as kinases and transcription factors, and it may require reengineering an intracellular protein for secretion by addition of a hydrophobic signal sequence at the N-terminus. Moreover, the hydrodynamic transfection/splenocyte fusion technique used herein has only been optimized thus far for matched pairs of gene-targeted mice and the corresponding proteins they lack. Nevertheless, we assume this approach will be applicable to situations involving wild-type mice and cDNAs encoding proteins of other species. A recent report by Bates et al.[52] compares two methods of i.v. delivery of plasmid DNA for the development of antigen-specific polyvalent antisera in mice rats and rabbits. They, too, found that this method of plasmid-based immunization is effective in generating humoral responses to a range of protein antigens in mice and that these mice are a suitable source of immune B cells for hybridoma generation and the isolation of mAb.[52]

The immune sera from hydrodynamically transfected, gene-targeted mice were particularly well suited for use in immunostaining/flow cytometry and also immunoprecipitation/Western blot analyses (data not shown) and did not require preabsorption, as is often needed with conventionally produced antiprotein sera generated using adjuvants. Moreover, the fact that most of the hybridomas produced blocking IgG1 mAbs showed that *in vivo* hydrodynamic transfection/immunization not only induced class switching but also caused affinity maturation of B cells that recognized functionally relevant anti-

genic determinants on the IFNAR-1 protein such that the mAbs they produced inhibited the effects of IFN ligands that bind to the IFN-α/β receptor with pM affinities. Finally the protocol produced at least one mAb (MAR1-5A3) that blocked type I IFN effects *in vivo* without depleting IFNAR-1-bearing cells, demonstrating that the procedure was useful in developing mAb suitable to probe the physiologic functions of this cell surface receptor. Thus, the protocol outlined in this study yielded heretofore unavailable mAbs suitable for analyzing IFNAR-1 on a biochemical, cell biologic, and functional basis both *in vitro* and *in vivo*.

Each of the five MAR1 mAbs characterized in this report bound IFNAR-1 either when it was expressed on the surface of intact cells or when it was released from solubilized cell membranes. When analyzed for antigen-binding specificity the mAbs were found to cluster into two distinct groups. The mAbs MAR1-5A3, MAR1-1H5, MAR1-3A7, and MAR1-4F12 comprised one group and cross-competed with one another for binding to wild-type IFNAR-1, as assessed by flow cytometry and ELISA, but did not compete for IFNAR-1 binding with the second group comprising only MAR1-4D12. In fact, MAR1-4D12 and MAR1-5A3 can function as capture and detection reagents, respectively, in an ELISA that is specific for soluble IFNAR-1. Interestingly, the two specificity groups displayed distinct capacities to block cellular responses induced either by IFN-α or IFN-β. Whereas each of the four antibodies in the first group inhibited a variety of cellular responses induced by either IFN-α or IFN-β, MAR1-4D12 inhibited only IFN-α-induced responses. This result suggests that IFN-α and IFN-β may differ in the manner in which they interact with the IFN-α/β receptor complex, a result consistent with the currently held concept of human type I IFN binding to its receptor.[53,54]

Although more work is needed to better define the epitope specificities seen by each of the MAR1 antibodies, our findings are consistent with the work of others who have proposed distinctive receptor binding specificities of IFN-α vs. IFN-β.[53–57] Previous structure-function analyses using human cell lines revealed that although IFNAR-2 appeared to be the primary ligand-binding chain,[5] coexpression of IFNAR-1 and IFNAR-2 was required for high-affinity ligand binding and cellular activation (reviewed in ref. 9). Several groups have also presented evidence characterizing the binding of specific human IFN-α subtypes with distinct human IFNAR-1 domains and have linked the strength of ligand binding with the range of biologic activities induced.[12,47,58] In addition, studies with *Ifnar1* gene-targeted mice show a lack of functional activity despite the detection of limited ligand binding. Clearly, more definitive analyses will be required to understand the mechanism by which specific ligands interact with IFNAR-1 and IFNAR-2 to trigger biologic responses.

As we prepared to use the mAbs in *in vivo* experiments, we were struck by the highly unusual pharmacokinetics that they displayed in wild-type mice. Specifically, when injected into normal mice at relatively low doses (200 μg/mouse), the antibodies rapidly disappeared from the circulation, displaying a serum half-life of 1.8 days, which was more than 5 times faster than that of isotype-matched control IgG ($t_{1/2} = 10$ days). The accelerated clearance of the MAR1 mAbs is, thus, distinct from that of mAbs specific for other cytokine receptors (e.g., IFNGR-1, tumor necrosis factor receptor 1 [TNFR1], or TNFR2).[22,23]
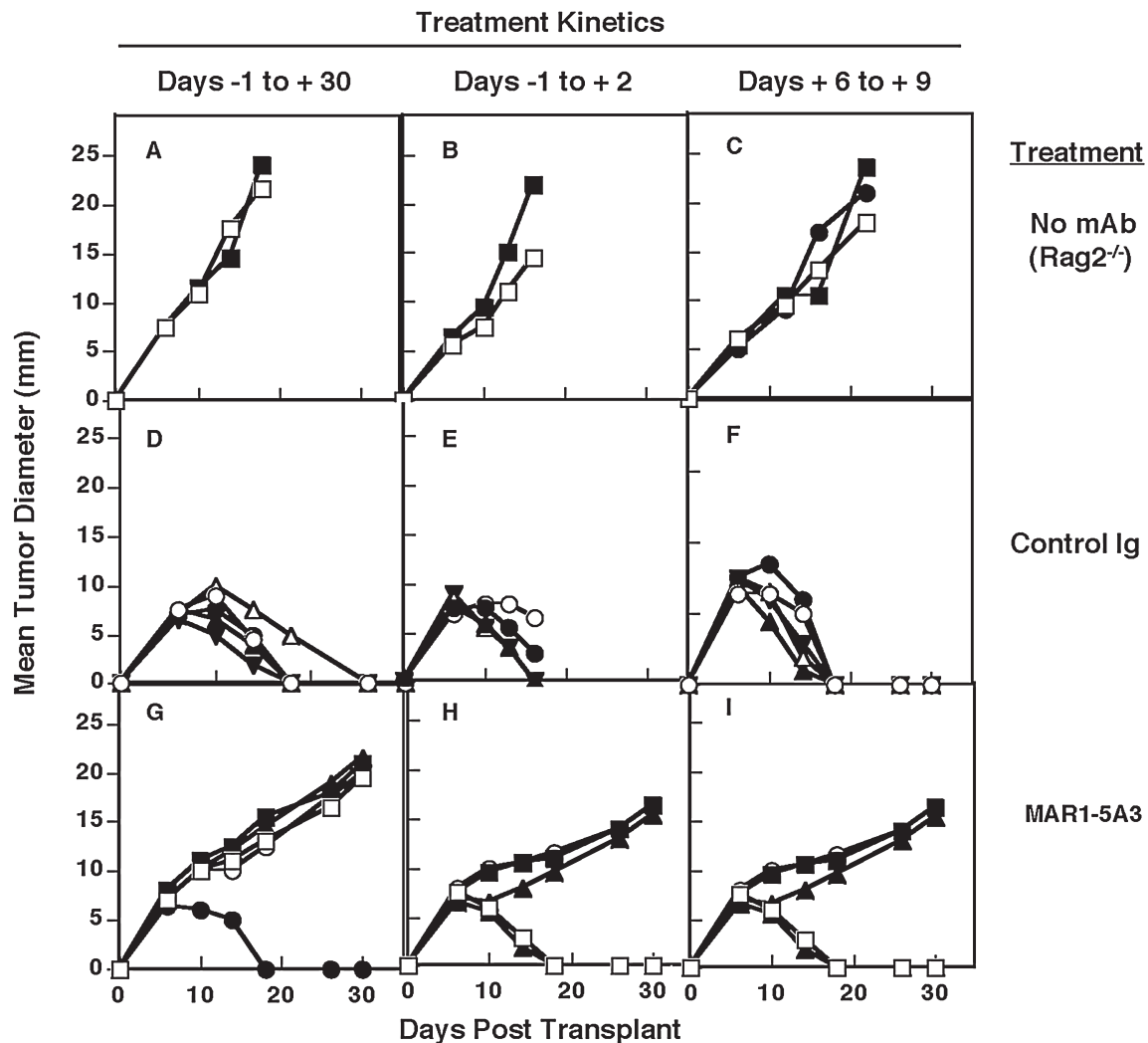
FIG. 8. Blockade of tumor rejection. 129/SvEv mice were treated with MAR1-5A3 (**G, H,** and **I**) or control Ig (**D, E,** and **F**) for various periods of time as indicated and inoculated with RAG2-R1 tumor cells (1×10^6 /mouse). Tumor growth was monitored over 30 days. $Rag2^{-/-}$ mice (**A, B,** and **C**) were similarly inoculated with tumor lines as a control for cell growth *in vivo*. Each line represents the tumor growth kinetics in individual mice. Data are representative of two experiments.

This anomalous behavior could not be explained by the presence of soluble forms of IFNAR-1, as immunoprecipitation/Western blot analysis of sera from unmanipulated mice did not reveal detectable levels of soluble IFNAR-1. This is consistent with the prediction for the gene structure of IFNAR-1, which indicates no alternative splicing. Importantly, this result is distinct from studies with IFNAR-2, which produces a soluble isoform by alternative splicing and which has been detected in serum.[59] Nor could it be explained by the presence of preexisting or induced antibodies against the IFNAR-1 mAbs as assessed by ELISA.

Two important clues helped resolve this dilemma. The first came from the observation that the serum half-life of the receptor mAbs normalized when the pharmacokinetics were performed in $Ifnar1^{-/-}$ null mice ($t_{1/2} = 7.5$ days). The second came from the finding that the half-life of MAR1-5A3 stabilized when we injected 10 times more antibody. These results

are consistent with the conclusion that wild-type mice possess a large depot of IFNAR-1 that needs to be saturated before excess antibody can appear in the circulation. Yet, as determined in preliminary Scatchard analyses, primary murine lymphocytes, macrophages, and fibroblasts express only approximately 1000 surface receptors per cell (unpublished observations). In addition, as assessed by flow cytometry, we have not yet found a primary cell population that expresses extremely high levels of cell surface IFNAR-1. Unfortunately, the MAR1 mAbs do not function in immunohistology using either frozen or fixed tissue sections, although we are currently pursuing imaging studies to compare IFNAR-1 tissue expression. Thus, we currently favor the hypothesis that a large pool of IFNAR-1 may be present within certain cells, and as intracellular IFNAR-1 cycles to the cell surface, it absorbs IFNAR-1 mAb out of the circulation. This hypothesis is consistent with the finding that in human cell lines, IFNAR-1 can cycle rapidly between the
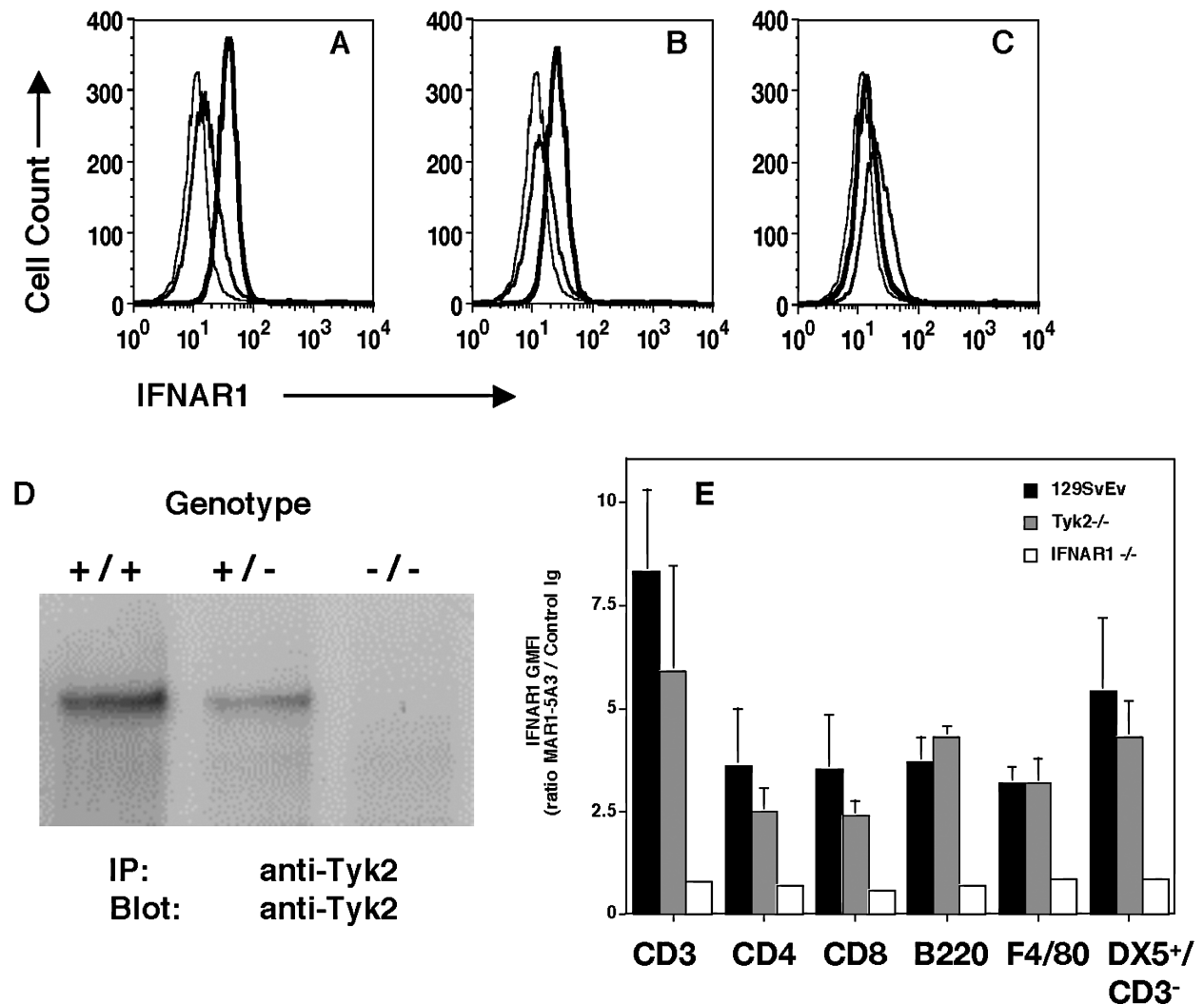
FIG. 9. IFNAR-1 expression on *Tyk2*-deficient cells. MEF derived from 129/SvEv (**A**) wild-type, (**B**) *Tyk2*-deficient, or (**C**) *Ifnar1*$^{-/-}$ mice were analyzed for expression of IFNAR-1 using biotin-conjugated MAR1-5A3 or control Ig, followed by streptavidin-PE. Gray lines represent unstained cells, thin lines represent control Ig staining, and bold lines indicate MAR1-5A3-stained cells. (**D**) *Tyk2* deficiency is confirmed in gene-targeted mice used to generate primary MEF lines by Western blot analysis. (**E**) Splenocytes from wild-type 129/SvEv, *Tyk2*$^{-/-}$, or *Ifnar1*$^{-/-}$ mice were analyzed for dual expression of FITC-conjugated or PE-conjugated cell surface markers and biotin-conjugated MAR1-5A3, followed by streptavidin-APC. The ratio of the geometric mean fluorescent intensities of MAR1-5A3/control Ig staining are shown. Data represent the average of three separate experiments expressed as the mean ± SD.

plasma membrane and intracellular stores.[49] More work is needed to quantitate the absolute amounts and relative distribution of IFNAR-1 within cells vs. on the cell surface and to explore whether the subcellular compartmentalization of IFNAR-1 differs between distinct cell populations.

The demonstration that MAR1-5A3 effectively inhibited all type I IFN responses *in vitro* and could be injected multiple times *in vivo* and not provoke an antibody response suggested that it might be useful to block type I IFN responses *in vivo*. Clearly, the availability of gene-targeted mice lacking *Ifnar1* has significantly enhanced our understanding of the role of IFN-α/β in host defense and autoimmunity. However, the use of gene-targeted mice is often complicated by genetic issues of mouse background (e.g., we have recently found that the

Ifnar1$^{-/-}$ mouse is actually on a 129/SvPAS genetic background, and not on a 129/SvEv background as reported by the distributor[20]) and often experimental *in vivo* models require that the mice be on particular genetic backgrounds. Thus, the ability to render a wild-type mouse insensitive to type I IFNs using a blocking IFN-α/β receptor mAb has distinct advantages, including the ability to examine the effect of type I IFN blockade at different stages of immune induction or disease progression.

To validate the use of MAR1-5A3 *in vivo*, we chose three *in vivo* mouse models known to be dependent on type I IFN that covered a range of functional processes. First, antibody blockade of IFNAR-1 in wild-type mice produced the same increased sensitivity to viral infection as deletion of the *Ifnar1*

gene.[15,47] Second, mAb blockade of IFNAR-1 in normal mice led to the same protective effects against *Listeria* infection as was noted in *Ifnar1*$^{-/-}$ mice.[17,18,48] Third, MAR1-5A3 treatment of wild-type mice not only produced the same effects as *Ifnar1* gene deletion in ablating host-protective immune responses against tumors[20] but also provided the added benefit of establishing at least some of the temporal requirements for type I IFN action during the process. Thus, the mAb allowed us, for the first time, to assess the effects of type I IFN depletion at different stages of disease pathogenesis and thus obtain insights that could not have been achieved using gene-targeted mice. The fact that MAR1-5A3 does not deplete IFNAR-1-bearing host cells *in vivo* adds significant support to the conclusion that MAR1-5A3 blockade in mice recapitulates many of the deficits observed in gene-targeted animals.

Having thus produced and characterized the first function-blocking mAbs to murine IFNAR-1, we used them to address the still unanswered question of whether, in murine cells, cell surface expression of IFNAR-1 depends on the presence of the Tyk2 Janus kinase. It is well established that Tyk2 constitutively associates with the intracellular domains of human and murine IFNAR-1 and is required to maintain expression of human IFNAR-1 at the cell surface.[60,61] Mutated U1A human tumor cells that lack *Tyk2* are unresponsive to IFN-α because they fail to sustain cell surface expression of IFNAR-1.[50,51,62] Subsequent work revealed that the association of Tyk2 with the IFNAR-1 intracellular domain modifies the trafficking and rate of internalization of IFNAR-1,[49] yet cells from mice with a targeted disruption of the *Tyk2* gene are still able to manifest substantial amounts of type I IFN-dependent antiviral activity,[45,63,64] suggesting that significant differences exist between the roles of Tyk2 in mouse vs. human type I IFN receptors. Using the MAR1 mAbs produced in this study, we found comparable expression of IFNAR-1 on the surface of cells from mice that either express or lack Tyk2. Thus, this observation reveals that Tyk2's chaperone functions differ between humans and mice and, although critical for maintaining expression of human IFNAR-1 at the cell surface, are not needed to maintain expression of the mouse protein. Additional studies will be needed to dissect the mechanisms by which murine cells respond to type I IFNs in the absence of Tyk2.

Thus, in sum, we have documented a method involving *in vivo* genetic hydrodynamic transfection/immunization of *Ifnar1*-deficient mice to develop murine IFNAR-1-specific mAbs capable of interacting with the native murine receptor protein at the cell surface. These mAbs inhibit type I IFN responses *in vitro*, and one has been shown to effectively block type I IFN responses *in vivo*. The availability of these monoclonal murine IFNAR-1-specific reagents should facilitate investigations into the biosynthesis and trafficking of IFNAR-1, help to elucidate the interactions between different type I IFN subtypes with the IFN-α/β receptor, and assist in identifying the physiologic roles of type I IFN in normal and disease states *in vivo* to complement questions that cannot be answered ty the use of *Ifnar1*-deficient mice.

ACKNOWLEDGMENTS

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Address reprint requests or correspondence to:
Dr. Robert D. Schreiber
Department of Pathology and Immunology
Center for Immunology
Washington University School of Medicine
660 South Euclid
St. Louis, MO 63110

Tel: (314) 362-8747
Fax: (314) 362-8888
E-mail: Schreiber@immunology.wustl.edu