SUPPORTING TEXT FOR SMITH ET AL.

DETAILED METHODS

Cells and Cell Lines

The murine C1498, J774, 3T12, and IC-21 cell lines were acquired from American Type Culture Collection (ATCC, Manassas, VA) and grown in RPMI 1640 containing 10% FCS, 2mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 100 µM 2-mercaptoethanol (R10 medium). Chinese Hamster ovary (CHO) cells were obtained from Pamela Stanley (Albert Einstein School of Medicine, New York, NY) and grown in R10. The PLAT-E packaging cell line (1), was provided by Dr. Toshio Kitamura, (University of Tokyo, Japan), and cultured in DMEM containing 10% fetal calf serum (FCS)(Harlan Bioproducts, Indianapolis, IN), 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 100 µM 2-mercaptoethanol (D10 medium) with 10 µg/mL blasticidin (Invitrogen, Carlsbad, CA) and 1 µg/mL puromycin (Sigma Chemical, St. Louis, MO).

Preparation of primary BM macrophages was performed as described previously(2). Bone marrow derived macrophages were cultured in D10 media with 5% horse serum and 20% L-cell conditioned medium at 37° C in 5% CO_2. For functional assays, bone marrow macrophages were harvested with Versene-EDTA (Gibco) followed by gentle scraping with a cell scraper. Conditioned medium was produced as previously described(2) from L-cells provided by Dr R. Schreiber (Washington University, St. Louis).

Antibodies, MCMV, and viruses

The 3D10 {IgG1 anti-Ly49H (3)}, 4E4 {anti-Ly49D (4)}, 28-8-6S (anti-K^bD^b) , 28-8-14 (anti-D^b), 2.4G2 (anti-FcγRII/III), 9E10 (anti-c-myc IgG1 isotype control) and B8-24-3 (anti-K^b IgG1 isotype control) mAbs (ATCC) were purified from spent culture supernatants. Smith strain MCMV and γHV68 were provided by the laboratory of Skip Virgin (Washington University, St. Louis, MO). The SMsubm02-16 mutant MCMV clone lacking the entire m02 family(5) was obtained from Tom Shenk (Princeton University, Princeton, NJ). Herpes simplex type 1 virus (KOS strain) was provided by David Leib (Washington University, St. Louis). MCMV was cultured in monolayers of NIH 3T12 cells. Culture supernatants were harvested when essentially all of the cells displayed a rounded, enlarged shape consistent with infection. MCMV was isolated from the culture supernatants using standard methods(6). Viral suspensions were aliquoted, frozen at -70°C and titer was determined by standard plaque assay(6).

Generation of BWZ transfectants

Ly49H cDNA was ligated into the pMX-IRES vector under control of the 5' LTR promoter; DAP12 cDNA was inserted into the same construct 3' from the IRES, allowing separate ribosomal translation of the DAP12 sequence. The resulting Ly49H-DAP12 bicistronic plasmid expression vector was transfected into PLAT-E cells to generate culture supernatants expressing infectious retroviruses as previously described (1). BWZ cells were infected with Ly49H-DAP12 expressing retroviruses and the Ly49H-DAP12 transfected BWZ cell population (HD12 cells) was expanded in R10. BWZ cell lines stably expressing Ly49H and DAP12 were isolated by cell sorting at the

Washington University flow cytometry facility. Fab fragments of anti-Ly49H mAb 3D10(6) were labeled with Alexa-488 (Molecular Probes) and used to sort Ly49H expressing cells without cross-linking of cell surface Ly49H. Similarly, a Ly49D-DAP12 bicistronic vector was generated and used to produce DD12 cells.

To generate a signaling-deficient murine DAP12 mutant capable of associating with Ly49H, site-directed mutagenesis of tyrosine residues in both ITAM at positions 64 and 75 (Tyr64 and Tyr75, respectively) was performed using a PCR-based strategy. A forward murine DAP12 primer (5'-GAGCTTCAGGGT CAGAGACCAGAAGTATTCAGTGACCTCAAC-3') encoding a Tyr75 to Phe75 mutation and a reverse primer derived from the murine DAP12 3'-untranslated region and containing a new *Sal* I site (5'-CTTGTTGAGTCGACATCTGGA ATGAC-3') were used to amplify a 134 bp fragment from a murine DAP12 cDNA template. Similarly, a 321-bp 5' fragment was amplified from the same DAP12 cDNA template using a 5'-untranslated region forward primer that contains the start codon and an endogenous *Nco* I site (5'-GCATATCTGGCCACCATGGGGGCTCTG-3') in combination with a reverse primer encoding a Tyr64 to Phe64 mutation (5'-CTTCTGGTCTCTGACCCTGAAGCTCCTGAAAAGGCGACTC-3') and which contains an overlapping region of identity of 25 bp with the forward Tyr75 to Phe75 internal primer. The resulting PCR fragments were separated and purified from a ~1% agarose gel using a Gel Extraction kit (Qiagen, Valencia, CA), and then used as template for a second round of PCR employing the external 5'-*Nco* I-containing forward and 3'-*Sal* I-containing reverse primers to generate a full-length DAP12 cDNA cassette harboring a double ITAM Phe64, Phe75 mutation. This PCR product was digested with *Nco* I and

Sal I and the resulting 400-bp fragment was then subcloned into pMX-IRES-GFP in place of the GFP cassette.

Reporter assay with MCMV infected cells

The macrophage cell line IC-21 or bone marrow-derived macrophages were infected with MCMV at a multiplicity of infection (MOI) of 5 at 37°C and 5% CO_2 either in individual wells of a 6 well plate or in 10 cm tissue culture dishes. Cells were infected by overlaying with MCMV in minimal R10 medium to cover the culture surface and rocked or agitated every 10 minutes for 60 minutes duration. Infections were begun at specific time points in order to overlay the cells onto HD12 monolayers at the same time. γHV68 and HSV infections were performed similarly except in media containing 2% FCS and adsorption time was extended to 2 hrs. After co-culture, β-gal activity was quantitatively assayed with the substrate CPRG (Calbiochem, San Diego, CA) or qualitatively by fixation and staining with X-GAL as described(7). For antibody blocking experiments, antibodies were added into R10 culture medium at a final concentration of 20 to 50 μg/mL and mAb 2.4G2 (20 μg/ml) was added to prevent inadvertent Fc receptor mediated effects. Photomicrographs were acquired using a Nikon TMS-F microscope and a Nikon model 990 digital camera (Nikon USA, New York, NY).

For ultraviolet radiation-mediated inactivation of MCMV (UV-MCMV), tissue culture-propagated viral stocks (titer = 3.02 X 10^7 pfu/ml) were thawed on ice and split into 2 aliquots. One aliquot was transferred to a single well of a 12-well flat-bottomed dish (lid removed) and exposed to a 30-W germicidal UV lamp inside the biosafety hood at a distance of ~7 cm for 30 min. with agitation every 5-10 min. The unirradiated aliquot

was maintained on ice until needed. We determined the titer of the UV-inactivated virus to be $\leq 10^2$ pfu/ml (limit of detection) in a plaque assay with 3T12 fibroblasts.

Flow cytometric analysis and intracellular staining

After staining, cell lines were analyzed with a FACSCalibur instrument (BD Biosciences, San Jose, CA) as previously described(3). Secondary reagent streptavidin-PE was used at optimum final concentration as determined by titration experiments. Cells were gated according to forward and side scatter and fluorescence data from 10^4 to 10^5 gated events were collected.

Freshly isolated B6.RAG1-/- splenocytes (devoid of mature B and T cells) were co-cultured for 6-8 h with either non-infected or MCMV-infected IC21 cells at a ratio of 1:1 in a 96-well plate (150,000 cells each). In a different experiment, we co-cultured B6.RAG1$^{-/-}$ splenocytes with BaF3 cells (1:1 ratio) either untransfected, or transfected with m157, or GFP. Where indicated, the co-cultures were performed in the presence of F(ab')$_2$ fragments directed against either Ly49H or Ly49D in the final concentration of 50 μg/ml. In all experiments, brefeldin A was added for the last 5-7 h. Cells were harvested with Versene-EDTA and surface stained with anti-NK1.1-APC, anti-CD3-PerCP-Cy5.5 (PharMingen) and anti-Ly49H-biotin (clone 3D10) in the presence of mAb 2.4G2 to block mAb binding to Fc receptors on NK cells. This was followed by staining with streptavidin-PE. Cells were then fixed and permeabilized with the Cytofix/Cytoperm kit according to the manufacturer's instructions (Pharmingen). Intracellular IFN-γ was detected with XMG1.2-FITC (PharMingen) and intracellular ATAC/Lymphotactin was stained with mAb MTAC-2 (8). For data analysis the cells were gated on the

$NK1.1^{+}CD3^{-}$ population and the staining of Ly49H or Ly49D versus cytokine or chemokine was depicted.

Cloning of Ly49H ligand

A cDNA library was produced from MCMV-infected IC-21 cells. Approximately 10^8 IC-21 cells were infected with MCMV (MOI = 5) for 24 hours. mRNA was isolated using the FastTrack kit (Invitrogen), and cDNA was generated using the Superscript Choice kit (Invitrogen). BstXI adapters (Invitrogen) were ligated and the cDNA was size selected by agarose gel electrophoresis and excision of the gel portion containing cDNAs larger than 1 kb. cDNA was purified by digestion of the agarose with beta-agarase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions and ligated into the pMX vector. The completed library contained approximately 5×10^6 independent clones. ORFs encoded by the MCMV genome were inspected and ranked according to the presence of a transmembrane domain, signal peptide sequence and sites for N-linked glycosylation. Of particular interest were ORFs showing predicted structural homology to MHC-related molecules as determined by 3D-PSSM program (9). PCR primers were designed to amplify each ORF from 3 nucleotides (nt) before the predicted start codon to 3 nt after the predicted stop codon. Primers contained restriction enzyme sites for directional cloning into the pMX-puro vector. PCR amplification was carried out using the ThermalAce DNA polymerase (Invitrogen) or *Pfu* DNA polymerase (Stratagene) and readily generated amplicons were ligated into pMX-puro vector. To account for possible polymerase errors during PCR amplification, 5 independent clones of each amplicon were isolated. The 5 clones were

then pooled and used as a group to transfect PLAT-E cells. Transfectants were then used to stimulate BWZ transfectants or to generate retroviruses that were used to infect BaF3, C1498, or J774 cells. Puromycin resistant cell lines expressing each ORF were co-cultured with HD12 cells for 12-22 hours. Stimulation of the HD12 line was assayed by fixation and staining with X-GAL. The sequence of multiple m157 clones was verified.

IL-2 activated NK (LAK) and cytotoxicity assays

Generation of LAK cells, sorted Ly49H^{+} and Ly49H^{-} NK cells and standard 4hr ^{51}Cr-release assays were performed as previously described (3, 10). Alexa 488 labeled Fab fragments of mAb 3D10 were used for flow sorting of Ly49H subsets.

RESULTS

Expression of Ly49H and Ly49D constructs

There was comparable expression of Ly49H and Ly49D on the respective BWZ indicator lines as detected by mAbs specific for Ly49H and Ly49D respectively (Supplemental Fig 1).

MCMV specificity of Ly49H activation

During the course of macrophage infection, type I interferons are produced that could induce the ligand for Ly49H on infected cells. Despite the capacity of IFN-α to induce up-regulation of MHC class I and II expression (data not shown), IFN-α did not stimulate HD12 cells (Supplemental Fig 2A). Also, MCMV-infected cells fixed in paraformaldehyde could stimulate HD12 cells that were not activated by cell-free

supernatants taken from infected cells (data not shown). Since interferons and other soluble mediators do not induce expression of the Ly49H ligand, we suspected that the ligand is a cell surface associated molecule.

Inasmuch as the known ligands for other Ly49 family receptors are surface expressed proteins, namely MHC class I molecules (11), we examined MCMV-infected primary BM macrophages from H-2 $D^b/K^b/\beta$2m-triple deficient (3KO) mice (C57BL/6 background)(Supplemental Fig 2B) for capacity to specifically stimulate HD12 reporters. The capacity of infected 3KO cells to stimulate Ly49H reporter cells provided evidence against the possibility that host MHC class I molecules serve as ligands for Ly49H.

The Ly49H receptor has restricted specificity even among herpesviruses. We exposed HD12 cells to primary BM macrophages infected with MCMV or either of two other herpesviruses, γHV68 and Herpes simplex (HSV), all of which have related genomes (12) and can infect rodent cells. Despite efficient infection (data not shown), γHV68- or HSV-infected macrophages did not trigger HD12 (or DD12) reporter cells (Supplemental Fig 2C).

Importantly, exposure of macrophages to UV-inactivated MCMV failed to induce expression of the Ly49H ligand (Supplemental Fig 3A). Moreover, kinetic analyses (Supplemental Fig 3B) indicated that activation of HD12 cells by MCMV-infected stimulators could be detected as early as 8 hours after infection (infection + activation time). Taken together, these data strongly suggested that the putative ligand for Ly49H is an MCMV encoded gene product expressed in the early phase of infection.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. FACS analysis of the BWZ reporter lines. BWZ.36 cells transduced with Ly49H- or Ly49D-containing retroviral vectors were stained with mAbs 3D10, or 4E4, respectively. GFP expression is shown for the control DAP12-GFP line. For DAP12-GFP, HD12, HD12.Y2F, DD12, and DD12.Y2F, high-expressing sublines were established by high-speed cell sorting and expression levels of the post-sorted reporter lines (bold tracings) are compared to the parental lines (dashed tracings). There was no cross-reactivity of the antibodies with reciprocal cell lines (data not shown), as previously reported (3).

Supplemental Figure 2. Ly49H recognizes putative MCMV encoded ligand. (A) Cells treated with type I interferon do not stimulate Ly49H-expressing reporter cells. Left panel: CPRG analysis of parental BWZ.36 and derivative HD12 and DD12 lines following co-culture with IC-21 stimulators infected with mock (open bars) or mock + CHO cells for DD12 (stippled bars), MCMV (shaded bars), or treated with increasing amounts of recombinant murine interferon-α (10-10^4 U/ml; dotted bars). Conditions are as outlined above. Data are expressed as a percent of maximal β-gal induction by PMA + ionomycin for each reporter-stimulator combination. Right panel: X-Gal analysis of HD12 cells following 18-hours co-culture with IC-21 cells infected with MCMV (top) or treated with 1000 U/ml interferon-α (bottom panel) under conditions outlined above. Inset represents maximal β-gal induction following stimulation with PMA + ionomycin. These doses of IFN-α induced upregulation in MHC class I and II expression (data not shown). (**B)** Host MHC class I is not required for Ly49H-mediated activation by MCMV-

infected cells. CPRG analysis of parental BWZ.36, HD12 and DD12 reporter cells co-cultured with mock- (diagonal stippled bars) or MCMV-infected (open bars) primary bone marrow macrophages derived from wild-type C57BL/6 or $K^b/D^b/\beta_2$m-triple-deficient (3KO; shaded bars) mice. The effects of anti-Ly49H (mAb 3D10; dotted bars) or anti-MHC class I (mAbs 28-8-14 + 28-8-6S; horizontal stippled bars) antibodies are shown for the HD12 responses. Stimulators were infected at MOI=5 for 28 hours; co-culture time=20 hours; CPRG assay time=16 hours. Data are expressed as a percent of maximal β-gal induction by PMA + ionomycin for each reporter-stimulator combination. **(C)** Ly49H-expressing reporters are stimulated by MCMV-infected cells but not by cells infected with other herpesviruses. Parental BWZ.36 and derivative HD12 and DD12 reporter cells were co-cultured with primary bone marrow macrophages following infection with MCMV (open bars), γHV68 (shaded bars), HSV (dotted bars), or mock-infected (stippled bars; only mock-HSV infection is shown). Stimulators were infected at MOI=5 for 42 hours for each virus; co-culture time=24 hours; CPRG assay time=18 hours. Similar percentages of infected cells were noted as indicated by cytopathic effect. Data are expressed as a percent of maximal β-gal induction by PMA + ionomycin for each reporter-stimulator combination.

Supplemental Figure 3 (A) UV-inactivated MCMV fails to stimulate HD12 cells. UV-inactivated MCMV does not stimulate Ly49H-expressing reporter cells. Left panel: CPRG analysis of parental BWZ.36 and derivative HD12 and DD12 lines following co-culture with IC-21 stimulators infected with mock (open bars) or mock + CHO cells for DD12 (stippled bars), MCMV (shaded bars), or UV-inactivated MCMV (dotted bars).

Data are expressed as a percent of maximal β-gal induction by PMA + ionomycin for each reporter-stimulator combination. Right panel: X-Gal analysis of HD12 cells following 18-hours co-culture with IC-21 cells infected with MCMV (top) or UV-inactivated MCMV (bottom panel). Inset represents maximal β-gal induction following stimulation with PMA + ionomycin. **(B) Kinetic analysis of the induction of Ly49H-mediated activation in HD12 cells in response to MCMV infection.** IC-21 cells were infected with MCMV (MOI=5) for varying lengths of time (x axis), harvested and then co-cultured with Ly49H-expressing HD12 reporter cells for either 4.5 (circles) or 12 (boxes) hours. β-gal activity was measured colorimetrically using CPRG.

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