An *in vitro* reporter cell system for analysis of functional Ly49 receptor binding of cognate ligands in *cis* and *trans*

Benjamin D. Edmonds, Colleen Fullenkamp, Natalya V. Guseva, Jonathan W. Heusel

Department of Pathology, and MSTP SUMR Program,
Roy J. and Lucille A. Carver College of Medicine,
University of Iowa 200 Hawkins Dr., Iowa City, IA 52242.

Abstract

Activation of natural killer (NK) cells requires the integration of stimulatory and inhibitory signals mediated in part by members of the allelic Ly49 receptor family. Inhibitory Ly49 receptors bind cognate MHC class I ligands in *trans* (ITIM activation) and in *cis* (no ITIM activation). We have developed an *in vitro* reporter cell system for analysis of the functional interaction between the activating Ly49H receptor (C57BL/6 strain, B6) and its murine cytomegalovirus (MCMV)-encoded ligand, m157. Since m157 also binds inhibitory Ly49 receptors, including Ly49I from 129 mice, we exploited our reporter cell system to determine: 1) whether Ly49H^{B6} and/or Ly49I^{129} bind the GPI-linked m157 ligand in *cis*, and 2) whether the induction of beta-galactosidase (β-gal) reporter activity of Ly49H-expressing HD12 cells could be inhibited or attenuated by the co-expression of Ly49I^{129} (HD12-I129 cells) binding to the same m157 ligand. We found that Ly49H^{B6}, but not Ly49I^{129}, binds m157 in *cis*, as measured by flow cytometry and by activation of Ly49H reporter (HD12) cells. When Ly49I^{129} is co-expressed in cis with Ly49H and m157, partial m157 staining is restored, possibly reflecting trogocytosis of m157 by Ly49I^{129}. When Ly49H is co-expressed with Ly49I^{129} and m157, the mean fluorescence intensity of m157 is slightly reduced and correlates with a minor reduction in HD12 activation (in *trans*). We also show that co-expression of Ly49H^{B6} and Ly49I^{129} on HD12-I129 cells results in lower β-gal induction following co-incubation with m157-expressing stimulator cells compared with HD12 cells (expressing only Ly49H). These findings represent a novel demonstration of *cis* ligand binding for an activating Ly49 receptor, and also demonstrate the utility of this reporter cell system for analysis of other relevant inhibitory and activating Ly49 receptor interactions (e.g. Ly49G2 and H-2D^{k}).

Introduction

NK cells are innate lymphocytes capable of potent cytotoxicity as well as the secretion of cytokines, chemokines and growth factors that influence the subsequent phases of immune responses (Fig.1). NK cells are regulated by a diverse array of endogenous (self), inflammatory and exogenous (non-self) signals. Further, each NK cell may respond to multiple activating specificities, each tuned to a slightly different activation threshold, resulting in a complex and heterogeneous NK cell compartment capable of diverse biological responses. The lectin-like Ly49 receptor family includes both activating (e.g., Ly49H, Ly49D) and inhibitory (e.g., Ly49A, C, G, and I) members that are expressed combinatorially on NK cells. Inhibitory Ly49 receptors repress intracellular phosphorylation through their cytoplasmic domain immunoreceptor tyrosine inhibitory motifs (ITIM) when cognate MHC class I ligands are engaged in *trans*, providing a mechanism for self-tolerance. However, inhibitory Ly49 receptors also bind their MHC class I ligands in *cis* (on the same cell), a mechanism that regulates the participation of inhibitory Ly49 receptors in the NK cell immune synapse, and may lower the overall activation threshold (Fig. 1).

To reduce the complexity of multiple inhibitory and stimulatory receptor input mediated by Ly49 and other NK cell receptor systems (e.g., CD94/NKG2), we developed a system to analyze the functional interactions of discrete Ly49 receptors and their ligands by expressing them in BWZ.36 cells using highly efficient retroviral-mediated transduction. BWZ.36 cells contain an inducible β-gal reporter that is activated by ITAM-associated receptors, including Ly49H. In addition, BWZ cells expressing one Ly49 receptor may be sequentially transduced to express additional receptors and/or cognate ligands (e.g., MHC I and MCMV m157). We exploited this system to test whether Ly49H^{B6} and/or Ly49I^{129} could bind m157 in *cis*, and to determine whether ITIM-mediated inhibition of β-gal reporter activity could be used to further model NK cell activation (Fig. 2).

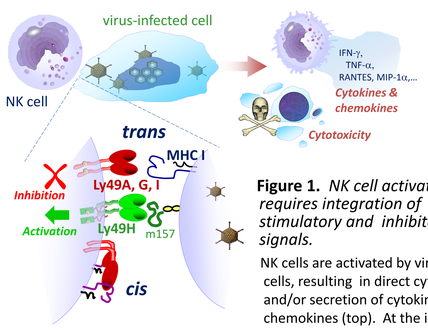

Figure 1. *NK cell activation requires integration of stimulatory and inhibitory signals.*

NK cells are activated by virus-infected cells, resulting in direct cytotoxicity and/or secretion of cytokines and chemokines (top). At the immune synapse (bottom), inhibitory Ly49 receptors (e.g., Ly49A, G, or I) bind MHC ligands in *trans*, whereas the activating Ly49H receptor binds the MHC I decoy ligand, m157, on MCMV-infected target cells. In other strains, MCMV m157 engages inhibitory Ly49 receptors (e.g., Ly49I^{129}). The binding of self-MHC I ligands on NK cells sequesters inhibitory Ly49 receptors away from the immune synapse and effectively lowers the activation threshold.

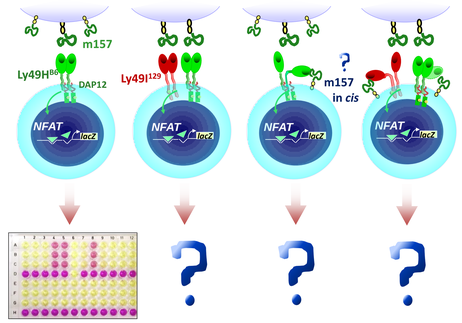

Figure 2. *Schematic of the BWZ.36 inducible β-gal reporter cell system used to assess functional cis and trans binding of Ly49H^{B6} and Ly49I^{129}.*

BWZ.36 ('BWZ') and retrovirally transduced derivative cells contain NFAT-inducible beta-galactosidase reporter activity , which is activated by immunoreceptor tyrosine activation motif (ITAM)-associated receptors such as Ly49H (coupled to the ITAM adaptor, DAP12). This system may be used to assess the effect of inhibitory Ly49 receptors co-expressed with Ly49H or the potential for *cis*-binding of Ly49 receptor ligands, such as MCMV m157.

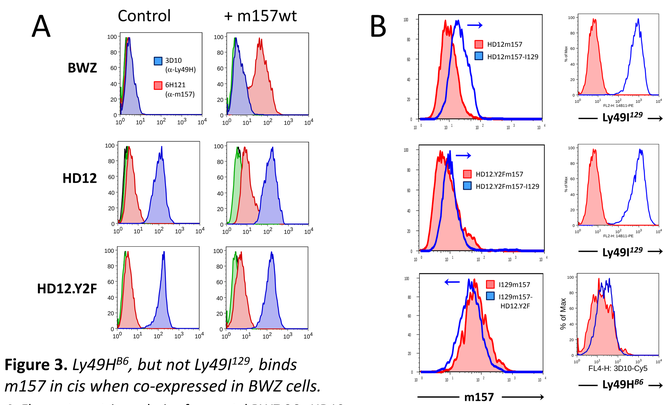

Figure 3. *Ly49H^{B6}, but not Ly49I^{129}, binds m157 in cis when co-expressed in BWZ cells.*

A. Flow cytometric analysis of parental BWZ.36 , HD12 or HD12.Y2F cells before (left panels) and following (right panels) transduction with the MCMV ligand, m157. Note the high-level m157 expression on BWZ, compared to the barely detectable m157 expression on Ly49H-expressing cells. ***B.*** Flow cytometric expression of m157 in HD12m157, HD12.Y2Fm157, and I129m157 cells following transduction of either Ly49I^{129} (left top 2 panels) or Ly49H (as non-signaling HD12.Y2F receptor; left bottom panel). Histograms on the right indicate that the level of expression for Ly49I^{129} is much higher on HD12m157 and HD12.Y2Fm157 cells than the expression of Ly49H on I129m157 cells.

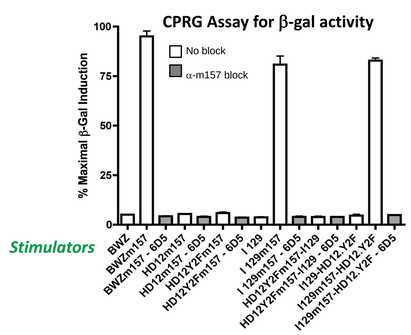

Figure 4. *Ly49H binding of m157 in cis prevents activation of HD12 reporter cells.*

Measurement of β–gal reporter activity in HD12 cells following co-incubation (16 hr) with the indicated stimulator cells using the colorimetric substrate, chlorphenol red β–D-galactopyranoside ('CPRG Assay'). Note that Ly49H-mediated activation by BWZ-m157 cells is comparable to that of I129-m157 cells, but that no activation is detected following stimulation by cells co-expressing m157 and Ly49H in *cis*. Specificity for m157-mediated activation is indicated by pre-incubation of stimulator cells with blocking anti-m157 MAb, 6D5. Data shown were measured 4 hr after addition of CPRG and expressed as a percentage of maximal induction by stimulation with PMA + ionomycin.

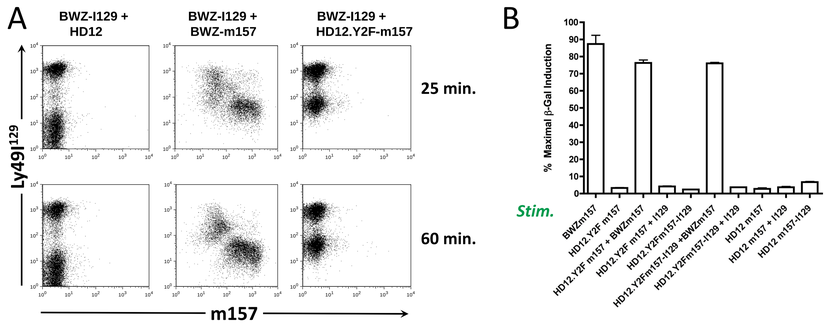

Figure 5. *Increased m157 expression on HD12m157-I129 (or HD12.Y2Fm157-I129) cells may results from trans-acquisition of m157 (trogocytosis), but is not available for Ly49H activation in trans.*

A. Flow cytometry showing that BWZ-I129 cells acquire m157 staining after co-incubation (25 and 60 min. shown) with BWZ-m157 , but not HD12-m157, cells. Note that the BWZ-m157 cells also acquire Ly49I^{129} staining. ***B.*** CPRG assay of HD12 reporter cells co-incubated with the indicated stimulator cells ("Stim."). Note that although HD12.Y2Fm157 cells have increased m157 expression in the presence of I129 (in *cis*), the HD12 reporters are not activated above background. Data are expressed as a percentage of maximal induction by PMA + ionomycin.

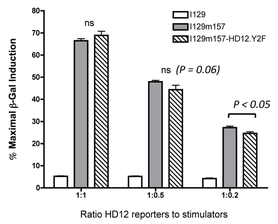

Figure 6. *Reduction of m157 expression on I129m157-HD12.Y2F cells results in lower Ly49H-mediated activation at sub-saturating stimulation.*

CPRG assay of HD12 reporters coincubated (16 hr.) with the indicated stimulator cells at cell ratios of 1:1, 1:0.5, and 1:0.2 (submaximal stimulation). Note that the BWZ-I129m157 cells transfected with Ly49H (I129m157-HD12.Y2F) have a slightly lower surface expression for m157 (Fig. 3B), which may explain the slightly lower activation of HD12 reporters at the submaximal stimulator cell ratios. Data are expressed as a percentage of maximal induction by PMA + ionomycin.

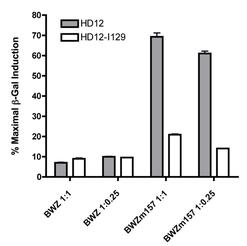

Figure 7. *Potent inhibition of Ly49H-mediated activation by Ly49I^{129} is cell-intrinsic.*

CPRG assay of HD12 and HD12-I129 reporters coincubated (16 hr.) with the indicated stimulator cells at 1:1 and submaximal 1:0.25 cell ratios. Note that Ly49I^{129}-mediated inhibition of HD12 reporter activity is significant (70-75%) when stimulated with m157-expressing cells at both stimulator cell inputs, suggesting a cell-intrinsic (ITIM-dependent) mechanism. Data are expressed as a percentage of maximal induction by PMA + ionomycin.

Conclusions & Future Directions

- Co-expression of Ly49H and m157 on the same cell results in loss of detectable cell surface m157 and no *trans* activation of Ly49H reporter cells; this is the first demonstration of *cis* ligand-binding for an activating Ly49 receptor (Ly49H^{B6}).
- Expression of Ly49I^{129} on HD12m157 (or HD12.Y2Fm157) partially rescues surface m157 expression, but does not measurably activate Ly49H reporters in trans. This most likely reflects trogocytosis of m157 by the higher-affinity Ly49I^{129} receptors (to be confirmed).
- Co-expression of inhibitory Ly49I^{129} on HD12 cells significantly attenuates reporter activation when stimulated in *trans* by m157-expressing stimulators; this demonstrates the utility of the BWZ reporter system for future studies modeling NK cell activation. Confirmation of a cell-intrinsic (ITIM-mediated) inhibition requires generation and co-expression on HD12 cells of an ITIM signaling-deficient Ly49I^{129} receptor (in progress).
- Future studies will address the Ly49G2-mediated regulation of NK cell activation mediated by Ly49H^{B6} and Ly49P$^{MA/My}$ in the context of MCMV infection.

Acknowledgements

Special thanks to M.G. Brown for helpful discussions and reagents. B.D.E. was supported by the University of Iowa Medical Scientist Training Program's Summer Undergraduate Medical Research (SUMR) internship, with assistance from Leslie Harrington and Elizabeth Snyder (MSTP administrators).