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J Immunol 2013; 190:6269-6276; Prepublished online 8 May 2013;
doi: 10.4049/jimmunol.1202533
http://www.jimmunol.org/content/190/12/6269

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/05/09/jimmunol.1202533.3.DC1

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Markers of Nonselective and Specific NK Cell Activation

Leslie A. Fogel,* Michel M. Sun,* Theresa L. Geurs,* Leonidas N. Carayannopoulos,†,1 and Anthony R. French*

NK cell activation is controlled by the integration of signals from cytokine receptors and germline-encoded activation and inhibitory receptors. NK cells undergo two distinct phases of activation during murine CMV (MCMV) infection: a nonselective phase mediated by proinflammatory cytokines and a specific phase driven by signaling through Ly49H, an NK cell activation receptor that recognizes infected cells. We sought to delineate cell surface markers that could distinguish NK cells that had been activated nonselectively from those that had been specifically activated through NK cell receptors. We demonstrated that stem cell Ag 1 (Sca-1) was highly upregulated during viral infections (to an even greater extent than CD69) and serves as a novel marker of early, nonselective NK cell activation. Indeed, a greater proportion of Sca-1+ NK cells produced IFN-γ compared with Sca-1− NK cells during MCMV infection. In contrast to the universal upregulation of Sca-1 (as well as KLRG1) on NK cells early during infections, such as MCMV, the nonredundant role of Ly49H in mediating resistance to MCMV was demonstrated that a combination of Sca-1, CD27, and KLRG1 can distinguish NK cells nonselectively activated by cytokines from those specifically stimulated through activation receptors. The Journal of Immunology, 2013, 190: 6269–6276.

Natural killer cells are innate immune lymphocytes that were initially characterized by their ability to kill transformed and infected cells without prior sensitization. Studies of rare humans with isolated NK cell deficiencies (1), as well as mice depleted of NK cells (2), demonstrated that NK cells are a critical component of host defense against viruses, particularly herpesviruses. Murine CMV (MCMV), a β herpesvirus, has been an invaluable tool for delineating the role of NK cell activation receptors in early host defense.

NK cell responses are controlled by cytokines and signals from activation and inhibitory receptors. Previous studies showed that mouse strains that express the Ly49H activation receptor, such as C57BL/6 (B6), are resistant to MCMV infection, whereas those that do not, such as BALB/c, are susceptible to infection (3–5). Furthermore, transgenic expression of Ly49H in susceptible mouse strains results in resistance to MCMV (6), and congenic B6 mouse strains that lack the Ly49H locus are susceptible to MCMV (3, 7).

Ly49H recognizes the MCMV-encoded protein m157, resulting in NK cell activation and the killing of infected cells (8, 9). Moreover, when infected with an m157-deficient virus, B6 mice become susceptible to MCMV infection (10, 11), demonstrating the nonredundant role of Ly49H in mediating resistance to MCMV.

NK cells undergo two distinct phases of activation during viral infections, such as MCMV. Early during MCMV infection, there is nonselective activation of both Ly49H+ and Ly49H− subsets of NK cells, which results in NK cell proliferation and IFN-γ production. This phase is driven by proinflammatory cytokines, such as IL-15 and IL-12 (12, 13). Subsequently, there is specific activation of Ly49H+ NK cells, which is dependent on Ly49H recognition of m157 and results in preferential expansion of the Ly49H+ subset of NK cells (14–16). Other activation receptor/ligand pairs have been identified in a variety of infection systems and/or mouse strains (17–20). Moreover, preferential expansion of specific NK cell subsets was also observed following a number of human infections (21–23), implicating a role for specific NK cell activation in human health and disease.

To our knowledge, there have been no reports of distinct cell surface proteins (“markers”) associated with the specific activation of NK cells. In contrast, several markers of early, nonspecific NK cell activation have been described, including the C-type lectin receptor CD69 (also called very early Ag) and the killer cell lectin-like receptor G1 (KLRG1) (24, 25). Although CD69 is not expressed on resting NK cells (26), ~30–40% of resting NK cells express KLRG1 (24, 27, 28). Despite this difference, both CD69 and KLRG1 are highly upregulated on NK cells following activation by a variety of stimuli, including viral infections (26, 28–30). However, CD69 is only transiently upregulated on NK cells following MCMV infection, whereas there is prolonged upregulation of KLRG1 expression for >1 wk (28, 31). Previous studies showed that KLRG1+ NK cells are less activated (28) and more prone to apoptosis following MCMV infection (31) than are KLRG1− NK cells. However, none of these studies examined the

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Received for publication September 10, 2012. Accepted for publication April 5, 2013.

This work was supported by National Institute of Allergy and Infectious Diseases Grants R01 AI078994 and AI073552 and Washington University Institutional Training Grant T32-AI007172.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ATCC, American Type Culture Collection; B6, C57BL/6; GCC, ganciclovir; HSV, HSV type 1; KLRG1, killer cell lectin-like receptor G1; MCMV, murine CMV; MCMV-Am157, m157-deficient murine CMV; MFI, mean fluorescence intensity; p.i., postinfection; Sca-1, stem cell Ag 1; VV, vaccinia virus; wt, wild-type.

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relationship between KLRG1 and Ly49H expression during the specific phase of NK cell activation during MCMV infection.

Although stem cell Ag1 (Sca-1; also called Ly-6A/E) has been best characterized as a marker of hematopoietic stem cells (reviewed in Ref. 32), it was initially identified on activated lymphocytes (33). Interestingly, there is conflicting evidence as to whether Sca-1 has an activating or inhibitory effect on lymphocytes. Initial studies demonstrated that Ab cross-linking of Sca-1 resulted in T and B cell activation in vitro (34–37). However, subsequent experiments demonstrated that effector T cells from Sca-1–deficient mice had increased proliferative responses (38) and that double-negative regulatory T cells from Sca-1–deficient mice are unable to suppress skin allograft rejection (39). However, the expression and function of Sca-1 on NK cells are unknown.

We hypothesized that the nonselective and specific phases of NK cell activation would result in characteristic expression patterns of cell surface proteins (or markers). Markers of early, nonselective activation would be highly expressed early following infection on most NK cells but have negligible expression on naive NK cells (similar to that of CD69). Conversely, markers of specific activation would be differentially modulated on cells expressing the appropriate activation receptor. For example, one would expect differential expression of the markers on Ly49H+ and Ly49H− subsets of NK cells during MCMV infection of B6 mice. The identification of such a panel of cell surface proteins will facilitate the study and identification of activation receptor/ligand pairs in other systems.

In this study, we identified and characterized cell surface markers of NK cell activation during MCMV infection of B6 mice. The identification of cell surface proteins (or markers). Markers of early, nonselective activation would be highly expressed early following infection on most NK cells but have negligible expression on naive NK cells (similar to that of CD69). Conversely, markers of specific activation would be differentially modulated on cells expressing the appropriate activation receptor. For example, one would expect differential expression of the markers on Ly49H+ and Ly49H− subsets of NK cells during MCMV infection of B6 mice. The identification of such a panel of cell surface proteins will facilitate the study and identification of activation receptor/ligand pairs in other systems.

In this study, we identified and characterized cell surface markers of both nonselective and specific NK cell activation.

Materials and Methods

Mice

B6 mice were obtained from the National Cancer Institute (Charles River, MA). 129 IFNαR-deficient mice (IFNαR−/−) were backcrossed onto a B6 background for 10 generations, as previously described (16). B6. Sca-1+/GFP mice (Sca-1+/−) (41), which have an enhanced GFP cassette knocked into the Sca-1 locus, were a generous gift from Timothy Graubert (Washington University). B6.DAP12 loss-of-function knock-in (DAP12−/−) mice (42) were a kind gift from Eric Vivier (Centre National de la Recherche Scientifique-INSERM-Universite de la Mediterranee, Marseille, France). Mice were maintained under specific pathogen–free conditions and used between 8 and 14 wk of age. All experiments were conducted in accordance with institutional guidelines for animal care and use.

Abs

Biotinylated anti-Ly49H Ab (3D10) was a kind gift from Wayne Yokoyama (Washington University). The following Abs and PE-streptavidin were purchased from eBioscience (San Diego, CA); unless indicated otherwise: biotinylated anti-KLRG1 (2F1); FITC-conjugated anti-KLRG1 (2F1), Ly49H (3D10), and -NKp46 (29A1.4; R&D Systems); PE-conjugated anti-CD27 (LG.7F9), -CD69 (H1.2F3), –IFN-γ (XMG1.2), and -KLRG1 (2F1); PerCP/Cy5.5-conjugated anti-CD3 (145-2C11) and -NK1.1 (PK136); PE/Cy7-conjugated anti-CD27 (LG.7F9) and -Sca-1 (D7); eFluor 450-conjugated anti-NKp46 (29A1.4); allophycocyanin-conjugated anti-Ly49H (3D10); allophycocyanin/fluor 780-conjugated anti-CD3 (17A2); allophycocyanin/Cy7-conjugated anti-CD3e (145-2C11); BD Pharmingen, San Diego, CA); V500-conjugated anti-Sca-1 (D7; BD Biosciences, San Jose, CA) and V450-conjugated anti-CD69 (H1.2F3; BD Biosciences).

Viruses and injection of mice

Salivary gland stock of Smith strain MCMV (American Type Culture Collection [ATCC], Manassas, VA) was prepared using young BALB/c mouse that were injected i.p. with 1 × 106 PFU tissue-culture propagated MCMV. The titer of the stock was determined using a standard plaque assay (3) in permissive NIH3T3 fibroblasts (ATCC, MCMV-AT1.5 (G881A mutation creating a premature stop codon) is an m157-deficient isolate of MCMV (MCMV-Δm157), which was described previously (31). Unless otherwise indicated, mice were injected i.p. with 5 × 104 PFU salivary gland stock MCMV.

Vaccinia virus (VV; ATCC) was propagated as previously described (43). All VV infections were done by i.p. injection with 5 × 105 PFU/mouse. HSV strain 17 was a kind gift from David Leib (Dartmouth Medical School, Lebanon, NH). HSV was propagated and titered as previously described (43). All HSV infections were done by i.p. injection of 1.5 × 106 PFU/mouse.

Splenocyte preparation, intracellular staining, and flow cytometry

Single-cell suspensions of splenocytes were prepared using standard techniques (14). To block nonspecific binding of Abs to FcRs, splenocytes were incubated in 2.4G2 anti-FcγRIIIb (hybridoma from ATCC) prior to staining with labeled Abs. Data acquisition was performed with a FACScalibur flow cytometer (BD Pharmingen) using CellQuest software (BD Biosciences) or with a FACSscan (BD Pharmingen) with DxF multicolor upgrade (Cytom Development, Fremont, CA) and FlowJo CE software (TreeStar, Ashland, OR). Data analysis was performed using FlowJo software. Intracellular IFN-γ staining was done as previously described (14). NK cells were identified as CD3−, NK1.1+ or as CD3−, NK1.1+, NKp46+. All histograms were scaled for the purpose of comparison.

Quantification of MCMV viral loads using real-time PCR

MCMV viral loads were determined by quantifying the copies of the IE1 gene using quantitative real-time PCR (TaqMan), as previously described (44), with the following modifications. DNA was isolated using the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA) from homogenized spleens diluted 1:10 in PBS. Five microliters of 100- and 1000-fold dilutions of each sample were run in triplicate. Samples were run in 96-well plates on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

In vitro IL-15–proliferation assay

Single-cell suspensions of splenocytes were prepared for various proteins on NK cells by negative selection with magnetic beads (Miltenyi Biotec, Auburn, CA). Splenocytes were cultured in 96-well plates for 72 h with various concentrations of murine IL-15 (PeproTech, Rocky Hill, NJ).

Statistical analysis

Data analysis was done with Microsoft Excel and GraphPad Prism (GraphPad Software, La Jolla, CA). Unless otherwise noted, unpaired, two-tailed t tests were used to determine statistically significant differences. Error bars in the figures represent SDs from the mean value.

Results

Sca-1 is a novel marker of early, nonselective NK cell activation

We used flow cytometry to analyze the expression of various proteins on NK cells from naive mice and from mice 2 d postinfection (p.i.) with MCMV. In these experiments, we observed that there was negligible expression of Sca-1 on NK cells from uninfected mice (6.7 ± 2.7%), whereas it was highly expressed on almost all NK cells as 2 d.p.i. with MCMV (99.1 ± 0.6%) (Fig. 1A, 1B). Table I). Sca-1 is also upregulated following other viral infections, including VV and HSV type I (HSV) infections (Fig. 1A, 1B).

Subsequently, we compared the expression of Sca-1 with the expression of previously identified activation markers, CD69 (26, 29, 30) and KLRG1 (28) (Fig. 1C, 1D). Like Sca-1, the frequency of KLRG1+ and CD69+ NK cells increased between naive and infected mice (Table I). Moreover, there was an increase in the abundance of each marker per cell, as measured by an increase in the mean fluorescence intensity (MFI) (Fig. 1E). Sca-1 abundance increased by an average of 125 ± 10-fold, KLRG1 abundance increased by an average of 4.9 ± 0.5-fold, and CD69 abundance increased by an average of 31.7 ± 11-fold. Similarly, there was increased expression of Sca-1, KLRG1, and CD69 on hepatic NK cells at 2 d.p.i with MCMV (Supplemental Fig. 1A, 1B). Together these results demonstrate that Sca-1 is more strongly induced on
NK cells following MCMV infection than other previously described markers of early NK cell activation, KLRG1 and CD69.

Prior studies showed that type I IFNs can induce the expression of both Sca-1 (46) and CD69 (29). To determine whether type I IFNs are required for the induction of Sca-1 and CD69 during the early, nonspecific response to MCMV infection, we analyzed Sca-1 and CD69 expression on NK cells from wt B6 or IFNαβR−/− mice 2 d p.i. with MCMV (Fig. 1F). Although the increased frequency of Sca-1+ NK cells from IFNαβR−/− mice 2 d p.i. with MCMV was only slightly reduced compared with that seen in wild-type (wt) B6 mice (71.0 ± 17.1% and 91.6 ± 9.0%, respectively), the frequency of CD69+ NK cells was markedly less in IFNαβR−/− mice (35.9 ± 15.6%) compared with wt B6 mice (91.8 ± 10.6%). These results suggest that type I IFNs are not necessary for the induction of either marker during MCMV infection. However, based on the significant decrease in the frequency of CD69+ NK cells in IFNαβR−/− mice compared with wt B6 mice, type I IFNs contribute substantially to the induction of CD69 expression during MCMV infection. In contrast, it appears that other factors can compensate for the absence of type I IFN signaling in inducing Sca-1 expression during MCMV infection, consistent with previous reports that IFN-γ, IL-6, and IL-9 can also induce expression of Sca-1 (47–49).

**Functional consequences of Sca-1 upregulation**

We investigated whether the upregulation of these activation markers had any functional implications. We looked at marker expression with respect to IFN-γ production in both splenic (Figs. 2A, 2B, Supplemental Fig. 1G) and hepatic (Supplemental Fig. 1C, 1D, 1F) NK cells 1.5 d p.i. with MCMV, the time point of maximal production of IFN-γ by splenic NK cells (14) following MCMV infection (Supplemental Fig. 1E). There was a statistically significant increase in the proportion of splenic Sca-1+ and CD69+ NK cells that made IFN-γ (51.3 ± 9.0% and 56.5 ± 8.0%, respectively)

### Table I. Frequency (%) of marker expression on NK cells from naive mice or mice 1.5 or 2 d.p.i. with MCMV

<table>
<thead>
<tr>
<th></th>
<th>Sca-1</th>
<th>KLRG1</th>
<th>CD69</th>
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<tbody>
<tr>
<td>Naive</td>
<td>6.7 ± 2.7</td>
<td>42.8 ± 7.8</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td>1.5 d.p.i.</td>
<td>44.7 ± 11.9</td>
<td>59.5 ± 4.0</td>
<td>65.9 ± 17.3</td>
</tr>
<tr>
<td>2 d.p.i.</td>
<td>99.1 ± 0.6</td>
<td>83.8 ± 5.7</td>
<td>92.8 ± 4.4</td>
</tr>
</tbody>
</table>

Data are mean ± SD from three or four independent experiments with a total of 8–13 mice/time point.
compared with Sca-1− and CD69− NK cells (39.2 ± 11.2% and 20.1 ± 5.4%, respectively). Contrary to a previous report by Robbins et al. (28), we did not observe a difference between the proportion of KLRG1+ and KLRG1− NK cells producing IFN-γ (45.3 ± 13% and 43.5 ± 11%, respectively, *p* = 0.22). Similar patterns were seen for hepatic NK cells (Supplemental Fig. 1C, 1D), although the overall magnitude was lower than that seen in spleen (Supplemental Fig. 1E). From these results, we concluded that Sca-1 and CD69 upregulation specifically identify activated NK cells early during MCMV infection.

To investigate the function of Sca-1 on NK cells, we examined NK cell responses in Sca-1−/− mice (41). We did not observe any difference in splenic viral titers or the frequency of proliferating NK cells during MCMV infection (data not shown). Furthermore, no differences were seen in NK cell–mediated killing by cytokine-stimulated LAK cells generated from Sca-1−/− or wt splenic NK cells (data not shown). However, 1.5 d p.i. with MCMV, there was a significant increase in the frequency of NK cells producing IFN-γ from Sca-1−/− mice (65.3 ± 7.3%) compared with those from wt B6 mice (44.1 ± 12.6%) (Fig. 2C). These differences in IFN-γ production occurred in the absence of any significant differences in serum concentrations of IL-12 from wt B6 or Sca-1−/− mice 1.5 d p.i. with MCMV (data not shown). These results suggest that Sca-1 has an inhibitory function on NK cells during MCMV infection.

**KLRG1, CD27, and Sca-1 are markers of specific NK cell activation**

In contrast to markers of early, nonselective activation during MCMV infection, markers of specific activation should be differentially expressed on Ly49H+ and Ly49H− NK cells. Although Sca-1 and KLRG1 are uniformly upregulated on NK cells early during MCMV infection, regardless of Ly49H expression (Fig. 3C), we observed differential expression of both Sca-1 and KLRG1 on Ly49H+ and Ly49H− NK cells late during MCMV infection (6–10 d p.i.) (Fig. 3A–C). In addition, Ly49H+ and Ly49H− NK cells differentially express CD27, a costimulatory molecule of the TNFR superfamily that has been described as a marker of NK cell maturation (reviewed in Ref. 50). KLRG1 expression is maintained on Ly49H+ NK cells (98.8 ± 0.4% at 8 d p.i.), whereas it is gradually lost on Ly49H− NK cells (66.6 ± 5.7% at 8 d p.i.). Conversely, the expressions of CD27 and Sca-1 decrease more rapidly on Ly49H+ NK cells (8.8 ± 6.3% and 17.8 ± 9.7%, respectively, at 8 d p.i.) than on Ly49H− NK cells (33.2 ± 8.3% and 42.8 ± 11.6%, respectively, at 8 d p.i.). The differential expression of KLRG1, CD27, and Sca-1 during MCMV infection suggests that these cell surface molecules function as markers of specific NK cell activation.

The evaluation of the coexpression patterns of KLRG1, CD27, and Sca-1 supported this conclusion. The coexpression of these markers was compared on NK cells that had been specifically activated (Ly49H+KLRG1+ NK cells) and NK cells that had not been specifically activated (Ly49H−KLRG1− NK cells) (Fig. 3D). Although there was no predominant CD27/Sca-1 subset among the Ly49H+ KLRG1− NK cells, most Ly49H+KLRG1+ NK cells became CD27 Sca-1− over time (88.1 ± 1.8% at 8 d p.i.). Moreover, when we gated NK cells based on the expression of these three markers, we observed that the KLRG1+CD27+ Sca-1− population was enriched for Ly49H+ cells (ratio of Ly49H+/Ly49H− NK cells).
NK cells of 11.8 ± 3.4 at 8 d p.i.), whereas very few of the KLRG1 CD27 Sca-1+ NK cells were Ly49H+ (ratio of Ly49H+/Ly49H− NK cells of 0.24 ± 0.1 at 8 d p.i.) (Fig. 3E). Therefore, it may be possible to use KLRG1, CD27, and Sca-1 expression on NK cells during viral infections to enrich for populations with high or low expression of receptors responsible for specific activation.

**Differential expression of KLRG1, CD27, and Sca-1 is dependent on Ly49H recognition of m157**

To determine whether the distinct expression patterns of KLRG1, CD27, and Sca-1 on Ly49H+ and Ly49H− NK cells depend on the binding of Ly49H to m157, we analyzed the expression of KLRG1, CD27, and Sca-1 on NK cells from mice infected with either wt MCMV or MCMV-Δm157 (16). In all previous experiments, mice were infected with 5 × 10^5 PFU wt MCMV; however, because of the increased virulence of MCMV-Δm157 in B6 mice (11, 16), we inoculated mice with 1 × 10^6 PFU of virus to provide sufficient stimulation but prevent excessive mortality. In contrast to the differential expression of KLRG1, CD27, and Sca-1 that we observed when mice were infected with wt MCMV (Figs. 3, 4A), infection of mice with MCMV-Δm157 resulted in similar proportions of Ly49H+ and Ly49H− NK cells expressing KLRG1, CD27, or Sca-1 (Fig. 4B). Furthermore, studies with DAP12^−/− mice, which lack the ability to signal through Ly49H, revealed similar findings with no differential expression of Sca-1, KLRG1, or CD27 following infection with wt MCMV (Fig. 4C). Together, these studies demonstrated that the modulation of expression levels of KLRG1, CD27, and Sca-1 on Ly49H+ NK cells was dependent on the recognition of m157 by Ly49H and subsequent DAP12-mediated signaling.

Given that there was no stimulation through Ly49H in the mice infected with MCMV-Δm157, we expected that the expression levels of KLRG1, CD27, and Sca-1 on NK cells from mice infected with MCMV-Δm157 would resemble those on Ly49H− NK cells from mice infected with wt MCMV. However, the observed expression levels of KLRG1 and CD27 on NK cells from mice infected with MCMV-Δm157 were intermediate between the levels on Ly49H+ and Ly49H− NK cells from mice infected with wt MCMV, whereas the expression levels of Sca-1 were substantially higher in mice at day 6 p.i. with MCMV-Δm157 compared with either Ly49H+ or Ly49H− NK cells from mice infected with wt MCMV (Fig. 4B). These observations were consistent with the higher viral loads (100-fold at days 4 and 6 p.i.) found in mice infected with MCMV-Δm157 compared with mice infected with wt MCMV (Fig. 4D). The differences in viral loads following infection with wt MCMV and MCMV-Δm157 were abrogated by days 8 and 10 p.i. (Fig. 4D). We hypothesized that the prolonged elevation of viral loads in mice infected with MCMV-Δm157 resulted in persistent nonspecific NK cell activation compared with infection with wt MCMV, reflected in higher than expected expression levels of KLRG1, CD27, and Sca-1. We verified this hypothesis in wt B6 mice infected with MCMV-Δm157 treated daily with either ganciclovir (GCV) or a control solution. The viral loads in mice infected with MCMV-Δm157 and treated with GCV were reduced to levels comparable to those seen in untreated mice infected with the wt MCMV (Supplemental Fig. 2A, 2C). The higher than expected levels of Sca-1 observed in both Ly49H+ and Ly49H− NK cells on day 6 p.i. with MCMV-Δm157 (Fig. 4B; and in mice treated with control solution, Supplemental Fig. 2B) were abrogated in GCV-treated mice (Supplemental Fig. 2B).

Importantly, no significant differences were observed in the proportions of Ly49H+ and Ly49H− NK cells that expressed KLRG1, CD27, or Sca-1 when GCV treatment decreased the viral loads in mice infected with MCMV-Δm157 to levels comparable to those seen in mice infected with wt MCMV (Supplemental Fig. 2B).

**Differential expression of KLRG1, CD27, and Sca-1 is not the result of preferential proliferation**

During the specific activation phase of MCMV infection, Ly49H+ NK cells proliferate substantially more than do Ly49H− NK cells (12, 14, 16, 51), raising the possibility that the differential regulation of KLRG1, CD27, and Sca-1 expression on Ly49H+ and Ly49H− NK cells reflects a by-product of cellular proliferation rather than NK cell receptor–mediated activation. Moreover, previous studies showed that KLRG1 and CD27 are upregulated following adoptive transfer of NK cells into lymphopenic hosts (RAG/common γ-chain–deficient mice) and subsequent homeostatic proliferation (52, 53). To determine whether proliferation.

![Figure 4: Differential expression of KLRG1, CD27, and Sca-1](http://www.jimmunol.org/DownloadedFrom/.../Figure_4.png)
alone was sufficient to change the expression of these markers, we analyzed their expression on CFSE-labeled NK cells cultured in vitro with various concentrations of IL-15 for 72 h (Fig. 5). Based on CFSE dilution, we identified subsets of NK cells that had divided (diluted CFSE) and those that had not (no CFSE dilution) (Fig. 5A). Although no differences (Fig. 5B, 5C) were seen in CD27 expression on subsets of NK cells that had divided versus those that had not (with 50 ng/ml IL-15, 97.4 ± 1.5% and 92.6 ± 4.2%, respectively), there was a decreased frequency of KLRG1+ NK cells among those that had divided compared with those that had not (with 50 ng/ml IL-15, 17.2 ± 4.6% and 37.3 ± 2.0%, respectively). In contrast, there was a slightly greater frequency of Sca-1+ NK cells that had divided compared with those that had not (with 50 ng/ml IL-15, 23.8 ± 1.3% and 9.88 ± 3.7%, respectively) (Fig. 5B, 5C). These minor perturbations of KLRG1 and Sca-1 expression contrast with the modulation of these markers during MCMV infection. Furthermore, the patterns of change are the opposite of what we saw for Ly49H+ (analogous to the proliferative subset) and Ly49H− NK cells in vivo during MCMV infection. Taken together, these results demonstrate that the differential expression of KLRG1, CD27, and Sca-1 during MCMV infection is not a mere consequence of preferential proliferation.

**Discussion**

Although it is well-established that distinct mechanisms mediate the nonselective and specific activation of NK cells, we demonstrated for the first time, to our knowledge, the presence of cell surface markers that distinguish NK cells that have been nonselectively activated by cytokines from those that have been specifically stimulated through activation receptors. Although markers of nonselective activation are universally upregulated on all NK cells early during viral infection, markers of specifically activated NK cells become differentially expressed at later times during infection on NK cells that express or lack expression of an activating receptor able to recognize infected cells. We showed that Sca-1 is highly upregulated early during viral infections, and at later times it can be used to distinguish cells that have been specifically activated through an activation receptor. Additionally, KLRG1 and CD27 expression levels are differentially modulated on specifically activated cells. Together, the differential expression patterns of KLRG1, CD27, and Sca-1 can identify a population enriched for specifically activated NK cells.

We identified the upregulation of Sca-1 as a novel marker of nonselectively activated NK cells. Similar to CD69 and KLRG1, which were previously shown to be upregulated following NK cell activation (28, 54), there is a dramatic increase in the frequency of Sca-1+ NK cells following MCMV infection, as well as VV and HSV infections (Fig. 1A, 1B). However, the increase in the abundance of Sca-1 per cell is substantially greater than that of either KLRG1 or CD69 (Fig. 1F). Our observations at the protein level are corroborated by similar findings at the transcript level in recent data from the Immunological Genome Project (55), which reveal that Sca-1 is the most highly upregulated transcript on NK cells (>65-fold) at 1 d p.i. with MCMV. In contrast, CD69 transcript levels increase by only 6-fold.

Both Sca-1 and CD69 identify functionally active NK cells, as demonstrated by the increased proportion of Sca-1+ and CD69+ NK cells that make IFN-γ compared with NK cells that do not

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**FIGURE 5.** Proliferation alone does not result in the differential expression of KLRG1, CD27, or Sca-1. (A) Representative histograms showing CFSE dilution and peak identification of NK cells that had (solid, thick line) or had not (dashed line) divided. (B) Representative graphs showing relative expression of KLRG1, CD27, and Sca-1 on splenic NK cells that had (solid line) or had not (dashed line) divided. (C) Proportion of NK cells that had (▪, solid line) or had not (○, dashed line) divided that expressed KLRG1 (left panel), CD27 (middle panel), or Sca-1 (right panel) following 72 h of culture in 3, 20, or 50 ng/ml of IL-15. Data are a composite of two or three independent experiments with pooled triplicates.
express these markers (Fig. 2A, 2B). Although a greater proportion of Sca-1+ NK cells made IFN-γ compared with Sca-1− NK cells in wt mice, NK cells from Sca-1−deficient mice produced more IFN-γ compared with those from wt B6 mice during MCMV infection (Fig. 2C). This observation suggests that Sca-1 may have an inhibitory function on activated cells. These findings are in concordance with the increased T cell responses observed in Sca-1−deficient mice (38). In addition, T cells from mice that overexpress Sca-1 have blunted responses to a variety of stimuli (56). Interestingly, similar observations were made regarding CD69. Although CD69 is highly expressed on activated NK cells, CD69-deficient mice manifest greater NK cell–dependent rejection of MHC class I–deficient tumor cells than do wt mice (57). These results support the conclusion that Sca-1 is an inhibitory receptor that marks nonselectively activated NK cells.

Although there are other reports of an inhibitory function for Sca-1 (38, 56), the mechanism of this inhibition remains unclear. Sca-1 is a GPI anchored protein of the Ly-6 gene family (58). Sca-1 may bind to CD22 on B cells (59), but it is unclear whether this is the only ligand for Sca-1. Various mechanisms for Sca-1 activity have been proposed (reviewed in Ref. 60). Sca-1 may act through association with an accessory signaling protein, such as the CD3 ζ chain (61, 62), allowing Sca-1 ligation to transduce a signal and potentially inhibit other cellular processes by competing for downstream signaling molecules. Alternatively, the function of Sca-1 might be dependent on its association with “lipid rafts” (63), specialized regions of the cell membrane that are enriched with saturated sphingolipids, cholesterol, and a variety of proteins, including GPI-anchored proteins (reviewed in Ref. 64). Lipid rafts and their associated proteins may regulate other protein–protein and protein–lipid interactions. For example, it was shown that ligation of Sca-1 with plate-bound Ab inhibits TCR-induced proliferation of T cells by preventing the translocation of the IL-2Ra-chain from lipid rafts into the soluble membrane fraction where IL-2Rα and γ-chains are located (65). Future work will be required to determine the mechanism of action of Sca-1 on NK cell function.

During the specific phase of NK cell activation during MCMV infection, Ly49H signaling following recognition of m157 results in selective activation of Ly49H+ NK cells. We showed that Ly49H signaling through DAP12 results in the differential expression of KLRG1, CD27, and Sca-1 on Ly49H+ NK cells compared with Ly49H− NK cells. Ly49H+ NK cells persistently express KLRG1 but downregulate CD27 and Sca-1 expression more rapidly than do Ly49H− NK cells (Fig. 3). By 8 d p.i. with MCMV, KLRG1+ CD27− Sca-1− NK cells are predominantly Ly49H+, whereas KLRG1+ CD27+Sca-1+ NK cells are primarily Ly49H− (Fig. 3E). We demonstrated that the differential expression of these markers is dependent on Ly49H recognition of m157 and the subsequent signaling via DAP12 (Fig. 4). Interestingly, Chen et al. (66) observed a similar expression pattern for Sca-1 on B cells: nonselective activation of B cells with IFN-γ results in Sca-1 upregulation, whereas stimulation through the BCR results in decreased expression of Sca-1. Additionally, there is increased expression of KLRG1 and decreased expression of CD27 on virus-specific T cells from both humans and mice (67, 68). Thus, the context-dependent regulation of activation markers is not unique to NK cells, rather it seems to be a more general phenomenon that is also observed in other immune cells.

Furthermore, we demonstrated that the differential expression of KLRG1, CD27, and Sca-1 on Ly49H+ and Ly49H− NK cells is not a direct consequence of preferential proliferation of the Ly49H+ NK cells (Fig. 5). In contrast with our in vitro experiments, two previous reports showed increased KLRG1 and CD27 expression on NK cells that had undergone homoeostatic proliferation in vivo following adoptive transfer into lymphopenic hosts (52, 53). This discrepancy may reflect the influence of increased levels of other cytokines in the RAG/common γ-chain–deficient recipients. Although our in vitro system does not fully mirror in vivo homoeostatic proliferation, it does provide a simplified system to be able to look directly at the effects of proliferation.

In summary, the identification of markers of nonselective and specific NK cell activation will significantly facilitate in vivo studies of NK cell activation during viral infections and has potential applications in other areas of NK cell research. For example, Sun et al. (69) suggested that increased expression of KLRG1 and decreased expression of CD27 are characteristic of memory NK cells. Although additional studies will be needed to determine how Sca-1 is related to memory NK cells, it appears that KLRG1 and CD27 expression correlate with both specific NK cell activation and memory response potential. The distinct expression patterns of KLRG1, CD27, and Sca-1 may also prove useful in isolating specifically activated NK cells during other viral infections and in identifying previously undiscovered activation receptors involved in responses to infections or other stimuli.

Disclosures

The authors have no financial conflicts of interest.

References


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Supplemental Figure 1. Marker expression on and IFN-γ production by hepatic NK cells following MCMV infection. A) Representative histograms showing Sca-1, KLRG1, or CD69 expression on hepatic NK cells from naïve B6 mice (thin line, grey fill) or from B6 mice 2 days p.i. wt MCMV (thick line, no fill). B) Percentage of hepatic NK cells that express Sca-1, KLRG1, or CD69 in naïve B6 mice (grey fill) or B6 mice 2 days p.i. MCMV (no fill). C) Representative histograms showing relative proportions of hepatic NK cells making IFN-γ 1.5 days p.i. MCMV based on expression of Sca-1 (left), KLRG1 (middle), or CD69 (right). D) Proportion of hepatic NK cells that make IFN-γ 1.5 days p.i. MCMV based on Sca-1, KLRG1, or CD69 expression. Marker negative cells (☐) and marker expressing cells (■). Statistical significance was determined using paired, two-tailed t-tests. ***p<0.001, **p<0.01 E) Frequency of IFN-γ+ splenic (◇, open) or hepatic (□, grey fill) NK cells from naïve mice or from mice 1.5 or 2 days p.i. MCMV. F) and G) Number of hepatic (F, grey) and splenic (G, black) NK cells that make IFN-γ 1.5 (diamonds, top row) or 2 (circles, bottom row) days p.i. MCMV based on the expression of Sca-1, KLRG1, and CD69. Marker negative cells (open symbols) and marker expressing cells (filled symbols). All panels show results from a 1-2 independent experiments with 3-4 mice/group. Hepatic leukocytes were prepared as previously described (14).
Supplemental Figure 2. Viral load does not affect the differential expression of KLRG1, CD27, and Sca-1 on Ly49H+ and Ly49H- NK cells during MCMV-Δm157 infection. A) Viral loads from spleens of wt B6 mice 4 days p.i. with 1 x 10^4 PFU MCMV-Δm157. Mice were injected i.p. daily with a control solution (△) or with 10 (◇), 20 (□), 40 (◇), or 80 (◇) mg/kg GCV. B) Proportion of Ly49H+ (filled symbol, solid line) or Ly49H- (open symbol, dashed line) splenic NK cells that express KLRG1 (left), CD27 (middle), or Sca-1 (right) from wt B6 mice 6 or 8 days p.i. with 1 x 10^4 PFU MCMV-Δm157. Mice were injected i.p. daily with either a control solution (△) of 80 mg/kg GCV (◇). n = 4 and 8 for days 6 and 8 p.i., respectively. C) Viral loads from spleens of wt B6 mice 2, 4, 6, or 8 days p.i. with 1 x 10^4 PFU MCMV-Δm157. Mice were injected i.p. daily with either a control solution (△) of 80 mg/kg GCV (◇). Symbols represent viral loads for an individual mouse. LOD is the limit of detection. GCV (Invivogen, San Diego, CA) was diluted according to manufacturer’s instructions. GCV was diluted in PBS to its final concentration and injected in a total volume of 200 uL.