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# Molecular definition of the identity and activation of natural killer cells

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**Using whole-genome microarray data sets of the Immunological Genome Project, we demonstrate a closer transcriptional relationship between NK cells and T cells than between any other leukocytes, distinguished by their shared expression of genes encoding molecules with similar signaling functions. Whereas resting NK cells are known to share expression of a few genes with cytotoxic CD8$^+$ T cells, our transcriptome-wide analysis demonstrates that the commonalities extend to hundreds of genes, many encoding molecules with unknown functions. Resting NK cells demonstrate a 'preprimed' state compared with naive T cells, which allows NK cells to respond more rapidly to viral infection. Collectively, our data provide a global context for known and previously unknown molecular aspects of NK cell identity and function by delineating the genome-wide repertoire of gene expression of NK cells in various states.**





The Immunological Genome (ImmGen) Project is a consortium of laboratories aimed at establishing a comprehensive database of gene expression in the mouse immune system[1]. As part of this collaboration, we have identified the gene-expression programs of natural killer cells (NK cells) and have analyzed this in the steady state and during the response to a viral infection to generate a resource for investigating NK cell biology. The immune system of vertebrates is classically divided into innate and adaptive branches. The innate immune system responds rapidly to infectious agents, whereas the adaptive response requires cell division and the differentiation of effector cells. NK cells and innate-like lymphocytes, which include γδ T cells, invariant NKT cells (*i*NKT cells), intestinal epithelial lymphocytes, B-1 cells and marginal-zone B cells, have both adaptive and innate features[2,3]. These innate B cells and T cells use receptors encoded by somatically rearranged genes to recognize specific structures from microbes and self antigens[2]. Functionally, innate-like lymphocytes mount quick effector responses such as cytolysis and the rapid secretion of cytokines, chemokines and antibodies.

Since the first description of NK cells[4,5], their relationship to lymphoid and myeloid cells has been a topic of debate. The ability of certain T cell populations, such as γδ T cells and some activated T cells bearing αβ T cell antigen receptors (TCRs), to mediate 'NK cell–like' cytolysis, as well as the shared expression by NK cells and T cells of many cell-surface antigens and effector molecules (such as CD2, CD7, CD90, perforin, granzyme A and interferon-γ (IFN-γ)), have led to the proposal that NK cells might simply represent a developmental or differentiation stage of T cells. However, the lack of productive rearrangement of TCR genes in mature NK cells and the development of NK cells in mice lacking a thymus or the recombinases required for TCR rearrangement unambiguously distinguish NK cells as a third, distinct lineage of lymphoid cells[6]. A relationship between NK cells and myeloid cells has been proposed on the basis of shared expression of cell-surface markers, such as CD11b and CD11c. However, subsequent studies defining the properties of hematopoietic progenitor populations have demonstrated that most NK cells are derived from progenitors shared with lymphocytes rather than with myeloid cells[7].

Global transcriptional analysis is a powerful approach that has yielded new insights into the biology of specific cellular subsets[8,9]. Early studies using this approach to analyze human and mouse NK cells identified sets of genes specifically expressed in NK cells, as well as transcriptional changes that occur during the activation of NK cells *in vitro*[10–12]. In this study, we have systematically defined the transcriptome of mouse NK cells in several contexts, including activation states and relative to all other lymphocyte and myeloid populations profiled by the ImmGen consortium. Our transcriptional profiling technique was multidimensional, which makes this study different from previous analyses because of the large number of data sets (such as conditions and cell types) compared simultaneously. The findings presented here provide molecular definitions of NK cell identity and function and provide both new insights into the nature of NK cells and a publicly available resource that documents the transcriptome of NK cells in various states.

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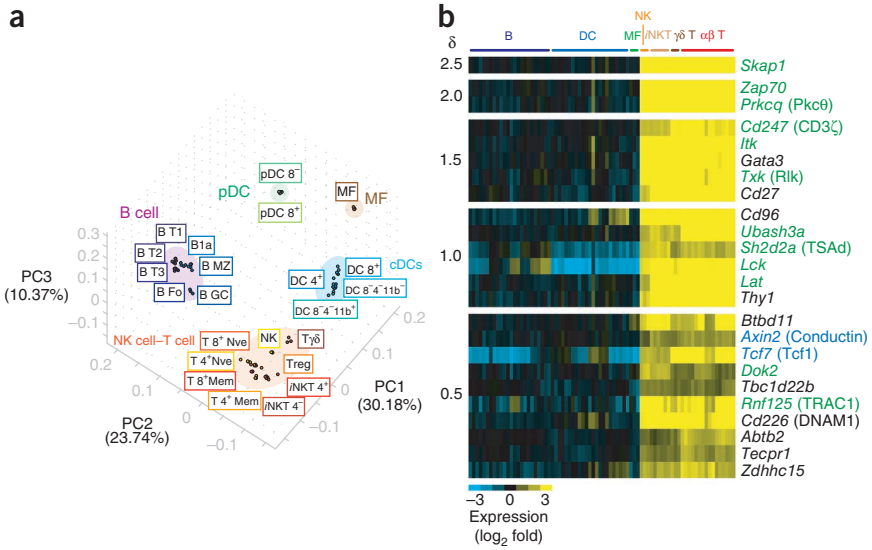


**Figure 1** NK cells and T cells show close similarity at the transcriptome level. (**a**) PCA of splenic leukocyte populations, showing the top three principal components (PC1–PC3) and their contribution to intersample variation (in parentheses). B T1–B T3, B cells of transitional types 1–3; B Fo, follicular B cells; B1a, B-1a cells; B MZ, marginal zone B cells; B GC, germinal center B cells; pDC 8− or pDC 8+, CD8− or CD8+ plasmacytoid DCs; MF, macrophage(s); DC 4+ or DC 8+, CD4+ or CD8+ DCs; DC 8−4−11b+, CD8−CD4−CD11b+ DCs; DC 8−4−11b−, CD8−CD4−CD11b− DCs; NK, NK cells; Tγδ, TCRγδ T cells; Treg, regulatory T cells; *i*NKT 4+ or *i*NKT 4−, CD4+ or CD4− *i*NKT cells; T 4+ Nve or T 8+ Nve, naive CD4+ or CD8+ T cells; T 4+ Mem or T 8+ Mem, memory CD4+ or CD8+ T cells. (**b**) Gene expression in the NK cell–T cell complex and in B cells, dendritic cells, and macrophages, presented as a heat map in decreasing order of significance (δ score; left margin); expression is relative to the median expression value of all analyzed populations. Green, molecules in the immunoreceptor tyrosine-based activation motif signaling pathway; blue, molecules in the Wnt–β-catenin pathway; protein designations in parenthesis. Data presented are based on the analysis of a minimum of three independent replicates per cell type.

## RESULTS

### Transcription-based organization of the main leukocyte subsets

To establish a molecular definition of NK cell identity, we investigated the relatedness of naive NK cells to other leukocyte populations using principal-component analysis (PCA), a method that identifies gene-expression patterns (principal components) that best explain variance across a data set. Delineation of population relationships with the three most informative principle components, as defined by the 15% of genes with the most variable expression across all splenic leukocyte populations, showed segregation of the populations into five discrete clusters (**Fig. 1a**). Lymphoid cells, including B cells, NK cells, *i*NKT cells, γδ T cells and αβ T cell subsets, formed groups distant from macrophages in the PCA plot. Whereas plasmacytoid dendritic cells grouped close to macrophages, conventional CD11c+ dendritic cell populations clustered between macrophages and lymphoid cell subsets. Among lymphoid cells, there was a distinct separation of a cluster containing B cell subsets and a cluster containing subsets of NK cells, *i*NKT cells, γδ T cells and αβ T cells (called the 'NK cell–T cell complex' here). Notably, NK cells and other innate-like T lymphocytes did not discernibly segregate from the adaptive T cell populations at this level of resolution.

Comprehensive pairwise comparison of each NK cell–T cell population with B cell populations and myeloid populations (macrophages, DCs and plasmacytoid DCs), followed by identification of the intersection of all pairwise comparisons, identified a 24-gene signature that distinguished subsets of resting NK cells, *i*NKT cells, γδ T cells and αβ T cells from other leukocyte populations (**Fig. 1b**). This group showed considerable enrichment for genes (in parentheses below) encoding the components of the immunoreceptor tyrosine-based activation motif signaling pathway, including signaling molecules known to regulate the activation of NK cells–T cells, such as Lck, Zap70, Tec kinases (*Itk* and *Txk*), PKC-θ (*Prkcq*)[13], STS-2 (*Ubash3a*), RNF125 (*Rnf125*), and adaptors (*Cd247*, *Lat*, *Skap1*, *Sh2d2a* and *Dok2*)[14]. The finding of such prominent enrichment for these signaling molecules by unbiased evaluation reflects the shared biology of NK cells and T cells and provides confirmation that this functional similarity in signaling constitutes a large component of the overall similarity between NK cells and T cells.

### Molecular organization of the NK cell–T cell complex

PCA of the 15% of genes with the most variable expression across the nine NK cell–T cell complex populations showed grouping of the populations of adaptive T cells (**Fig. 2a**). In contrast, the innate populations failed to group together, with only the *i*NKT cell subsets (CD4+ and CD4− *i*NKT cells) showing a close relationship. We observed a similar organization by hierarchical clustering (**Fig. 2b**). We hypothesized that despite the diversity of the innate populations of the NK cell–T cell complex, transcriptional commonalities would exist that would distinguish innate populations from adaptive T cells. To investigate these shared programs, we identified genes with significantly different expression in each innate population relative to their expression in adaptive T cells and assessed conservation across the four comparisons. The group of 112 genes with the most significant upregulation by resting NK cells and at least one other innate cell subset relative to their expression in αβ T cell subsets was enriched for genes encoding surface and signaling receptors and molecules (**Fig. 2c**). These included genes encoding activating and inhibitory NK cell receptors (NKRs; products encoded in parentheses), such as *Fcgr3* (CD16), *Ncr1* (NKp46), *Klrc2* (NKG2C), *Klrk1* (NKG2D), *Slamf7* (CRACC), *Klra5* (Ly49E), *Klra9* (Ly49I), *Klrc1* (NKG2A); genes encoding transmembrane proteins and other surface receptors, such as *Il12rb2*, *Fasl*, *Kit* and *Cd7*; genes encoding integrins, such as *Itga2* (CD11b) and *Itgax* (CD11c); genes encoding kinases, such as *Syk* and *Lyn*; and genes encoding adaptors, such as *Fcer1g* (FcRγ), *Tyrobp* (DAP12), *Lat2* (NTAL), *Sh2d1b1* (EAT-2) and *Clnk*. Many of these genes with different expression have been shown to have higher expression in innate lymphocytes, which provides confirmation of these data[8,15,16]. In addition, we found an additional set of genes (*Fgl2*, *Sulf2*, *Lrrk1*, *Aoah* and *Car2*) with a distinctive expression pattern in NK cells and innate lymphocytes, but the function of the gene products in these lineages is unknown. Staining for a representative set of cell-surface antigens and intracellular molecules showed that in most cases, the transcript measurements were reflected in the frequency of NK cells, *i*NKT cells or γδ T cells that stained positive for these markers (**Fig. 2d**,**e**).

Given the observation that unprimed NK cells, *i*NKT cells and γδ T cells respond rapidly to stimulation, it was not unexpected that resting NK cells had higher expression of genes encoding effector molecules,

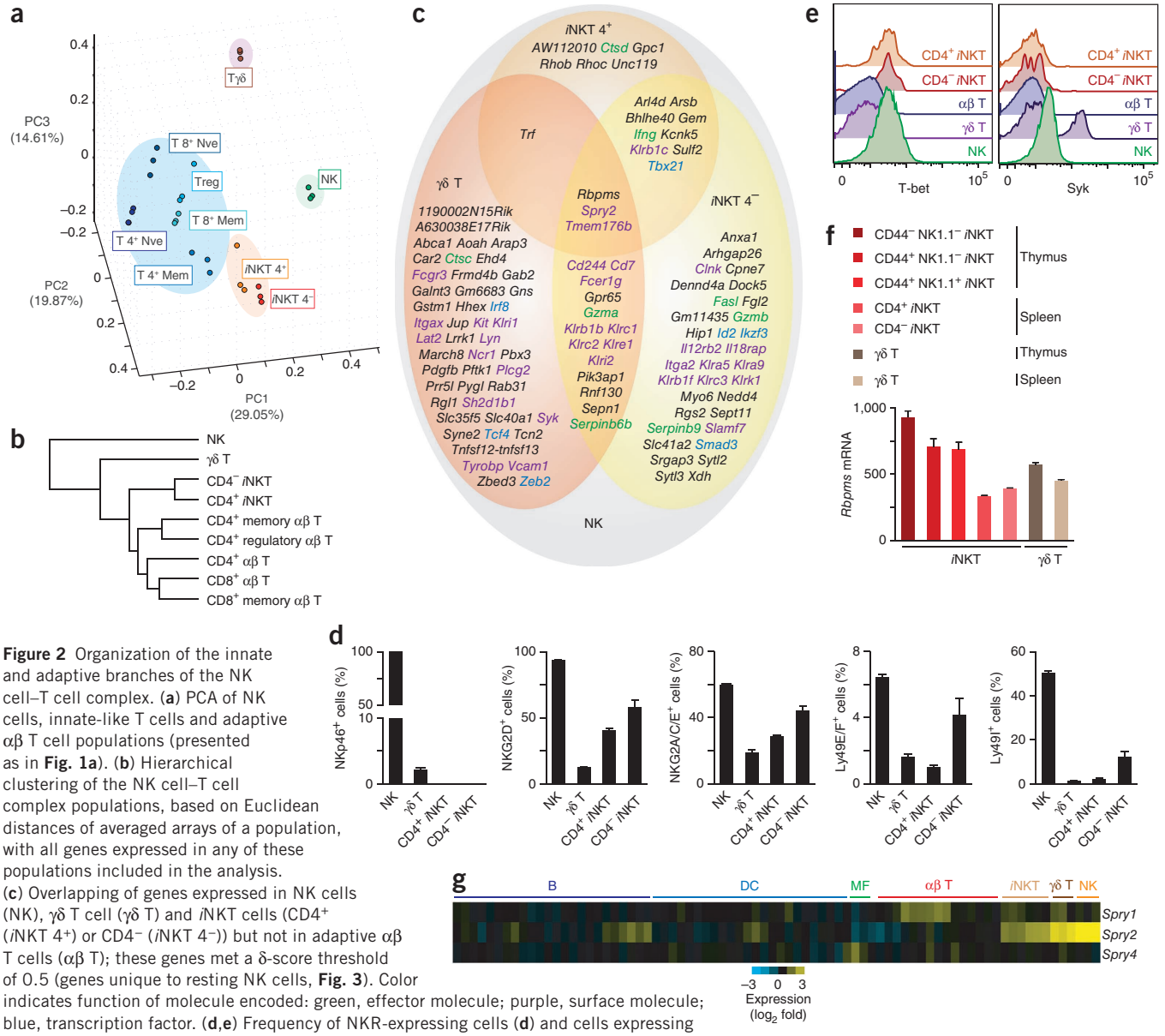


**Figure 2** Organization of the innate and adaptive branches of the NK cell–T cell complex. (**a**) PCA of NK cells, innate-like T cells and adaptive αβ T cell populations (presented as in **Fig. 1a**). (**b**) Hierarchical clustering of the NK cell–T cell complex populations, based on Euclidean distances of averaged arrays of a population, with all genes expressed in any of these populations included in the analysis. (**c**) Overlapping of genes expressed in NK cells (NK), γδ T cell (γδ T) and *i*NKT cells (CD4$^+$ (*i*NKT 4$^+$) or CD4$^-$ (*i*NKT 4$^-$)) but not in adaptive αβ T cells (αβ T); these genes met a δ-score threshold of 0.5 (genes unique to resting NK cells, **Fig. 3**). Color indicates function of molecule encoded: green, effector molecule; purple, surface molecule; blue, transcription factor. (**d**,**e**) Frequency of NKR-expressing cells (**d**) and cells expressing T-bet or Syk (**e**) among NK cells and innate-like lymphocytes (as in **c**) relative to their frequency among TCRαβ$^+$ T cells, assessed by flow cytometry (*x* axis, fluorescence intensity; *y* axis, percentage of maximum). (**f**) Abundance of *Rbpms* mRNA in *i*NKT cell and γδ T cell populations isolated from thymus or spleen, presented as a normalized microarray. (**g**) Expression of *Spry1*, *Spry2* and *Spry4* by splenic leukocyte populations (above), presented relative to the median expression value of all analyzed populations. *Spry3* expression was not above background in any population and is not presented here. Data are representative of two experiments (error bars (**d**–**f**), s.e.m.). Data presented are based on the analysis of a minimum of three independent replicates per cell type (**a**–**c**).



including IFN-γ (*Ifng*), proteases (*Gzmb*, *Gzma*, *Ctsc* (cathepsin C), and *Ctsd* (cathepsin D)) and protease inhibitors (*Serpinb6b* and *Serpinb9*). Expression of several genes encoding molecules involved in vesicle transport (such as *Rab31*, *Sytl2* and *Sytl3*) and in the regulation of the cytoskeleton (such as *Sept11* and *Myo6*) was higher in NK cells and/or *i*NKT cells and γδ T cells than in αβ T cells, which suggested that changes in vesicle trafficking or cytoskeletal rearrangements may affect the generation or release of lytic granules (**Fig. 2b**). In addition, genes encoding transcription factors, including *Tbx21* (T-bet) and *Id2*, had higher expression in the NK cell and *i*NKT cell lineages than in αβ T cells (**Fig. 2c**,**e**), consistent with reports indicating the requirement for these factors in the development and function of these cells[17]. Notably, the innate genes identified in this analysis did not merely represent genes characteristic of cell activation; only a fraction of genes with higher expression in NK cell and innate-like T cell populations than in αβ T cells corresponded to genes induced during cell activation and/or proliferation.

Consistent with our observation that the innate NK cell–T cell subsets were heterogeneous, only three genes (*Rbpms*, *Tmem176b* and *Spry2*) had significantly higher expression in resting NK cell, *i*NKT cell and γδ T cell populations than in αβ T cells (**Fig. 2c**). The transcriptional coactivator RBPMS (encoded by *Rbpms*) regulates transforming growth factor-β signaling[18]. Given that transforming growth factor-β is critical for development of the *i*NKT cell and γδ T cell lineages[19], it is notable that the more immature thymic *i*NKT cell and γδ T cell populations had the highest expression

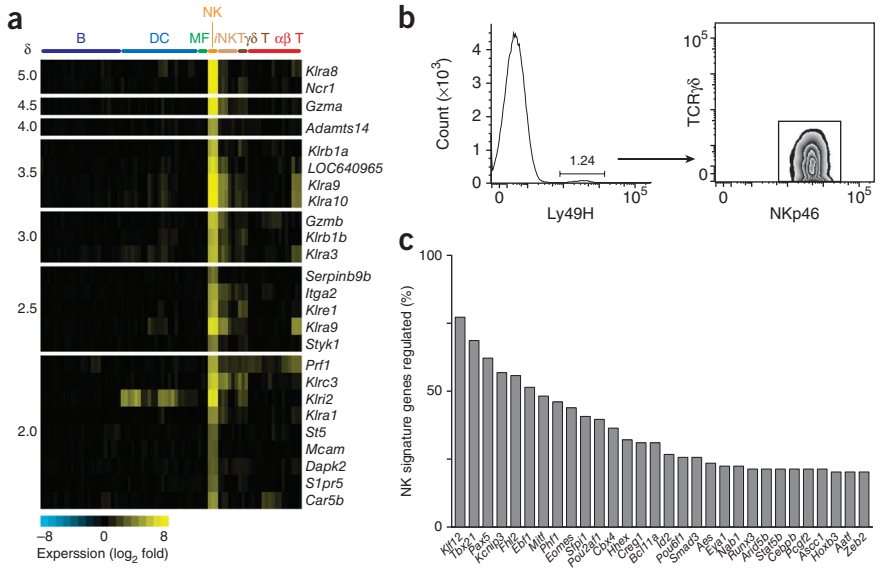


**Figure 3** Molecular uniqueness of resting NK cells. (**a**) Heat map of genes with the most significant difference in expression in NK cells relative to all other cell populations, presented (as in **Fig. 1a**) in decreasing order of significance (full list of genes, **Supplementary Table 1**). (**b**) Ly49H expression in splenic leukocytes (left; number above bracketed line indicates percent Ly49H$^+$ cells), and expression of NKp46 and TCRγδ by Ly49H$^+$ cells (right; outlined area indicates TCRγδ$^-$NKp46$^+$ cells). (**c**) Proportion of the 93 NK cell signature genes in **a** regulated by the transcriptional regulators (encoded by the genes along the horizontal axis) most 'strongly' predicted to do so (significantly linked), presented in decreasing degree of influence (left to right). Data presented are based on the analysis of a minimum of three independent replicates per cell type (**a**,**c**) or are representative of three independent experiments (**b**).

of *Rbpms* (**Fig. 2f**). Although expression of *Tmem176b* (which encodes the transmembrane protein Tmem176b) was specific to innate NK cell–T cell subsets (**Fig. 2c**), its expression is much lower in those subsets than in DCs. Sprouty homolog 2 (*Spry2*) is a member of the Sprouty family of transcriptional regulators; there are four such genes in higher vertebrates, but we found that only *Spry2* had high and specific expression in NK cell, *i*NKT cell and γδ T cell populations (**Fig. 2g**). Given that these proteins are involved in a negative feedback mechanism to limit antigen receptor–mediated signaling[20], Spry2 might represent an additional regulator of a developmental or activation program of innate lymphocytes. These analyses confirmed known functions of NK cells and innate-like T cells and identified previously unknown molecular components of innate-like lymphocyte populations.

### A transcriptional signature that defines resting NK cell identity

The shared repertoire of surface receptors, signaling molecules and transcription factors expressed by NK cells and other innate-like T cell populations blurs the distinctions among these cell types. We therefore defined a resting NK cell signature by identifying genes with higher expression in NK cells than in all other leukocyte populations. Nearly half of the 25 genes with significantly higher expression in NK cells than in all other leukocytes encoded NKRs, the most specific of which were *Klra8* (Ly49H) and *Ncr1* (NKp46; **Fig. 3a**). Although Ly49H was expressed in only 50% of NK cells in C57BL/6 mice, it was not detectable in any other leukocyte population (**Fig. 3b**). NKp46 has been shown to have selective expression in NK cells, with two exceptions: rare T cell subsets[21,22] (**Fig. 2d**), and a mucosal population of innate lymphoid cells that express the transcription factor RORγt[23]. Additional genes 'preferentially' expressed by NK cells that have been identified include those encoding a sphingosine 1-phosphate receptor (*S1pr5*)[24], adhesion molecules (*Mcam*[25] and *Itga2* (CD49b)[26]) and effector molecules (*Gzma*, *Gzmb* and *Prf1*)[27,28]. Among the genes whose expression was uniquely higher in NK cells, *Adamts14*, *Serpinb9b* and *Styk1* have not yet been reported to be expressed by this subset of lymphocytes, to our knowledge. The protease ADAM14 (*Adamts14*) processes extracellular matrix proteins[29], which we speculate may be important for the migratory ability of NK cells. The serine-protease inhibitor Serpinb9b (*Serpinb9b*) inactivates granzyme B in an irreversible manner[30] and is needed to protect cytotoxic lymphocytes from granzyme B–mediated cell death[31] and may protect NK cells from being killed by their own cytolytic molecules. The serine-threonine-tyrosine kinase Styk1 (*Styk1*) shares homology with receptors for platelet-derived growth factor and fibroblast growth factor and has been suggested to regulate cell proliferation and survival by activating both mitogen-activated protein kinases and phosphatidylinositol-3-OH kinase[32], but its function in NK cells is unknown, and it has not been identified as a potentially NK cell–restricted molecule expressed in the hematopoietic system. These molecules represent intracellular proteins now identified as distinguishing NK cells from other resting leukocytes.

We further sought to understand the regulatory control of NK cell 'uniqueness' and identified putative transcriptional regulators of the signature genes of resting NK cells through the use of a network modeling approach (**Supplementary Note 1**). To maximize regulator discovery, we used a lower threshold of statistical stringency to identify a total of 93 genes with 'preferential 'expression in NK cells (**Supplementary Table 1**). Of the predicted regulators of those genes, we identified many genes encoding molecules known to influence the development or function of NK cells, such as *Tbx21*, *Eomes*, *Mitf*, *Sfpi1*, *Id2*, *Smad3*, *Runx3* and *Stat5b*[17] (**Fig. 3c**). However, most of the transcriptional regulators identified have no known role in NK cell development, despite having strong associations with molecules encoded by genes with preferential expression in NK cells. For example, KLF12 was predicted to regulate 80% of the identified NK cell fingerprint (**Fig. 3c**); however, the role of this zinc-finger protein in NK cells is unknown. These data suggest that a rich biology related to the transcriptional definition of NK cell identity remains undiscovered.

### Transcriptional priming of effector functions of NK cells

NK cells are preprimed to allow rapid activation of some effector functions. We explored this at the genome level by identifying genes with high expression in naive NK cells and induced in effector CD8$^+$ T cells after infection with vesicular stomatitis virus (VSV) or *Listeria monocytogenes* relative to their expression in naive CD8$^+$ T cells. The expression programs shared by naive NK cells and effector CD8$^+$ T cells included genes encoding molecules with effector functions, NKRs, molecules involved in adhesion and homing, transcription factors, and signaling molecules (**Fig. 4a**,**b**). Killing of infected cells is a critical effector function of both NK cells and effector CD8$^+$ T cells and is mediated by the release of perforin and granzymes; accordingly, expression of *Gzma* and *Gzmb* was high in both naive NK cells and

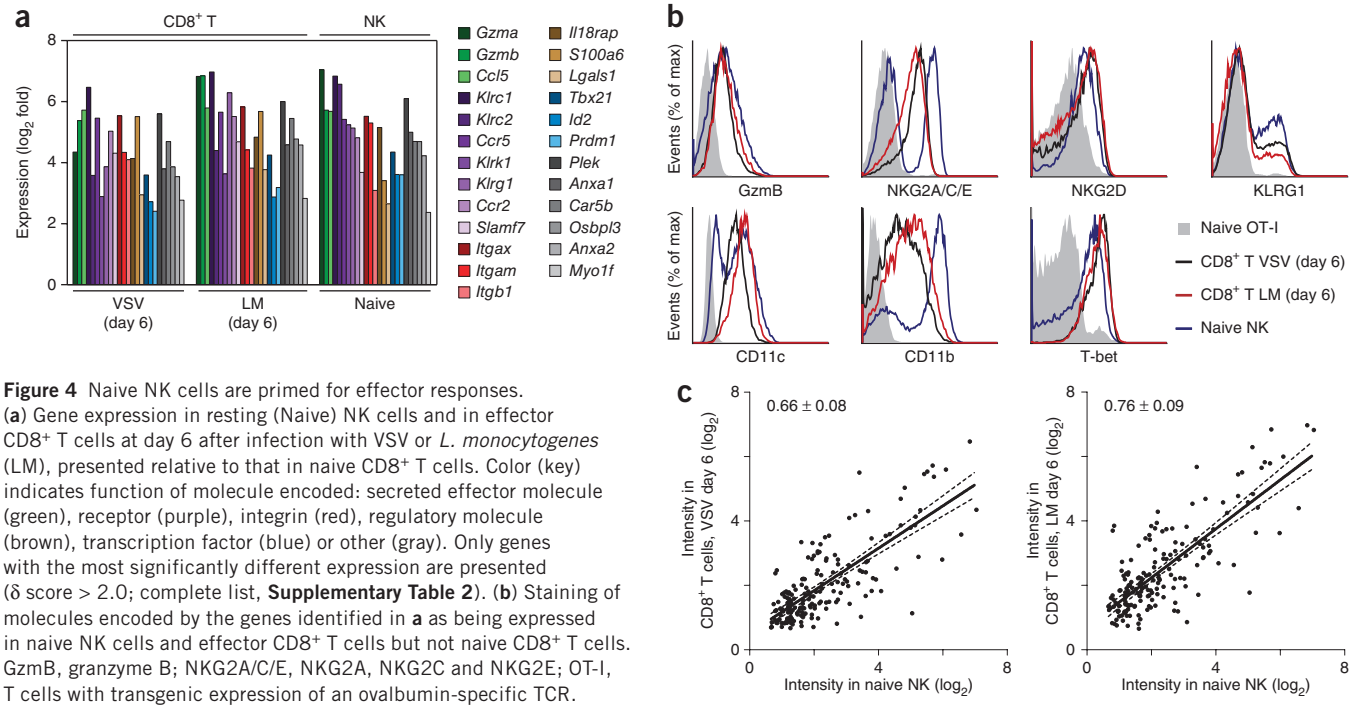


**Figure 4** Naive NK cells are primed for effector responses. (**a**) Gene expression in resting (Naive) NK cells and in effector CD8$^+$ T cells at day 6 after infection with VSV or *L. monocytogenes* (LM), presented relative to that in naive CD8$^+$ T cells. Color (key) indicates function of molecule encoded: secreted effector molecule (green), receptor (purple), integrin (red), regulatory molecule (brown), transcription factor (blue) or other (gray). Only genes with the most significantly different expression are presented ($\delta$ score > 2.0; complete list, **Supplementary Table 2**). (**b**) Staining of molecules encoded by the genes identified in **a** as being expressed in naive NK cells and effector CD8$^+$ T cells but not naive CD8$^+$ T cells. GzmB, granzyme B; NKG2A/C/E, NKG2A, NKG2C and NKG2E; OT-I, T cells with transgenic expression of an ovalbumin-specific TCR. (**c**) Gene expression in naive NK cells and in CD8$^+$ T cells at day 6 after infection with VSV (left) or *L. monocytogenes* (right), presented as normalized log values for genes with significantly different expression; dashed lines indicate 95% confidence intervals of the linear regression line (solid); numbers in plot indicate the slope of the regression line and the 95% confidence interval. Data presented are based on the analysis of a minimum of three independent replicates per cell type (**a**,**c**) or two independent experiments (**b**).



effector CD8$^+$ T cells. Naive NK cells and effector CD8$^+$ T cells also shared expression of genes encoding other effector molecules, such as *Ifng* and *Ccl5* (RANTES; **Fig. 4a** and **Supplementary Table 2**). Naive NK cells and effector CD8$^+$ T cells shared expression of genes encoding several activating NKRs (such as *Klrc2* (NKG2C), *Klrk1* (NKG2D) and *Slamf7* (CRACC)) and inhibitory NKRs (such as *Klrc1* (NKG2A) and *Klrg1* (KLRG1)); **Fig. 4a**,**b**), consistent with published observations[33]. For effector cells to respond to foreign invaders, they must acquire the ability to migrate to sites of infection, and this is largely attributed to an increase in the surface expression of chemokine receptors and adhesion molecules. We observed that naive NK cells had high basal expression of genes encoding the chemotactic proteins CCR2 (*Ccr2*) and CCR5 (*Ccr5*) and the cell-adhesion proteins CD11b (*Itgam*), CD11c (*Itgax*) and CD29 (*Itgb1*), rather than these genes requiring induction in effector CD8$^+$ T cells (**Fig. 4a**,**b**). Thus, the concerted action of these molecules may influence the appropriate tissue distribution of NK cells and effector CD8$^+$ T cells. Genes encoding transcription factors (such as *Tbx21* (T-bet), *Id2* (Id2) and *Prdm1* (Blimp-1)) were also expressed in naive NK cells and effector CD8$^+$ T cells (**Fig. 4a**,**b**), which suggested a common differentiation program. The higher expression of *Prdm1* (Blimp-1) in naive NK cells was notable, given the role of this transcription factor in regulating the differentiation of effector CD8$^+$ T cells[34,35].

A comparison of the expression of genes encoding effector molecules showed that on average, their expression was higher in naive NK cells than in effector CD8$^+$ T cells (**Fig. 4c**). This suggested that in terms of transcriptional prepriming, NK cells have maximal expression of these effector molecules as part of their persistently 'alerted' state. These findings demonstrate that the preprimed state described before for *Ifng* and genes encoding granzymes also applies transcriptome wide to many additional genes encoding putative effector molecules.

### Transcriptional profile of NK cells during infection

The transcriptional baseline defined above represents a single state (the resting state) of an NK cell's existence. To explore NK cell changes during pathogen-specific activation, we generated a kinetic portrait of gene expression by profiling Ly49H$^+$ NK cells as naive cells before infection with mouse cytomegalovirus (MCMV), and after MCMV infection as early effector cells (day 1.5 after infection), late effector cells (day 7 after infection) and memory cells (day 27 after infection). The largest changes occurred early during infection, as shown by the difference in expression of many genes in activated Ly49H$^+$ NK cells (upregulation of 875 genes; **Fig. 5a** and **Supplementary Table 3**). Most of this response was diminished by day 7 after infection, although expression of certain genes was sustained. On the whole, late effector NK cells at day 7 after infection and memory NK cells at day 27 after infection were more similar to each other in their gene-expression patterns than was any other population pair (**Fig. 5b**). Additionally, many genes were specifically upregulated in memory NK cells (**Fig. 5a**), which supported the proposal that NK cell memory reflects a unique state different from that of naive or effector NK cells[36].

The transcriptional profile of NK cells at day 1.5 after infection was clearly distinct from the transcriptional profile of NK cells at all other times points based on the Euclidean distance among the various NK cell populations (**Fig. 5b**). Genes upregulated at day 1.5 after infection included those encoding indicators of inflammation (such as *Cd69*, *Ifih1*, *Ifitm1* and *Ifitm3*), proliferation (such as *Il2ra* (CD25)) and effector function (such as *Ifng* and *GzmB*; **Fig. 5a** and **Supplementary Table 3**). We also confirmed higher expression of a set of these molecules by flow cytometry (**Fig. 5c**). Published studies have demonstrated that signaling by IL-12 through STAT transcription factors promotes IFN-$\gamma$ production[37]. The expression of genes encoding the IL-12 receptor (*Il12rb1*) and

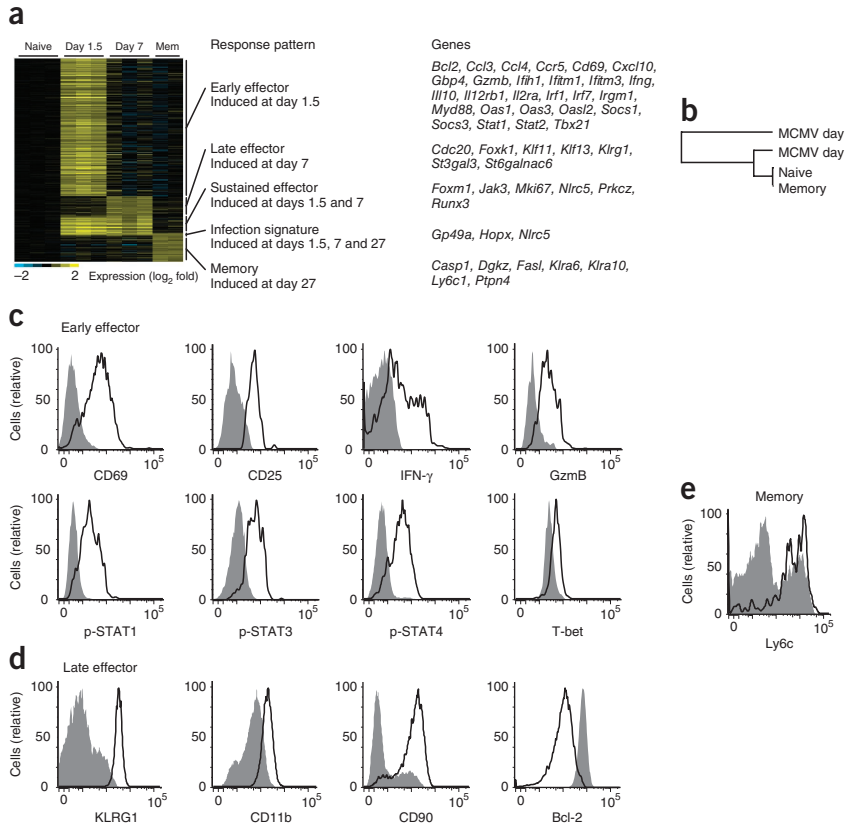


**Figure 5** The response of Ly49H$^+$ NK cells to MCMV infection is dominated by an early activation response, followed by effector and memory responses. (**a**) Heat map of all genes (subsets listed at right) significantly induced at any time point after infection relative to their expression in naive Ly49H$^+$ NK cells, grouped by hierarchical clustering. Mem, memory. Data presented are based on the analysis of a minimum of three independent replicates per cell type. (**b**) Hierarchical clustering of the transcriptome of Ly49H$^+$ NK cells before and at days 1.5 and 7 after infection with MCMV. (**c**–**e**) Surface expression of CD69 and CD25 and intracellular expression of IFN-γ, granzyme B (GzmB), phosphorylated (p-) STAT1, STAT3 and STAT4, and T-bet at day 1.5 after infection with MCMV (**c**); surface expression of KLRG1, CD11b and CD90, and intracellular expression of Bcl-2 at day 7 after infection with MCMV (**d**); and expression of Ly6c at day 27 after infection with MCMV (**e**), assessed by flow cytometry of Ly49H$^+$ NK cells (black lines); gray shaded curves indicate Ly49H$^+$ NK cells from uninfected mice. Data are representative of three independent experiments.

STAT1 and STAT2 (*Stat1* and *Stat2*) increased early after MCMV infection (**Fig. 5a**), which suggested that activated NK cells become sensitized to signaling via IL-12 and STAT proteins to mediate optimal production of effector cytokines such as IFN-γ. We found more phosphorylated STAT1, STAT3 and STAT4 at day 1.5 after infection than in naive NK cells (**Fig. 5c**). Although *Tbx21* (T-bet) was expressed in resting NK cells (**Fig. 2e**), both transcript and protein were further upregulated after infection (**Fig. 5a**,**c**); whether the higher expression of this transcription factor influences the effector function of NK cells remains to be determined. Because NK cells are such potent effector cells when activated, these cells must also be regulated immediately so that uncontrolled inflammation in the environment is not generated; this was reflected in the higher expression of the genes encoding the suppressors of cytokine signaling SOCS1 and SOCS3 at this early time point (**Fig. 5a**). Indeed, IFN-γ production by NK cells peaked at day 1.5 after infection but was rapidly abrogated (data not shown), probably because of the activity of SOCS proteins[38]. In addition, NK cells transcribed and expressed IL-10 early after MCMV infection (**Fig. 5a**), which would serve to regulate the magnitude of the immune response and limit pathology[39]. Thus, both proinflammatory and regulatory molecules exerted their influence on the activation and effector function of NK cells early after infection with MCMV.

Day 7 after MCMV infection marks the peak of clonal expansion for Ly49H$^+$ NK cells[40]. Consistent with the observation that Ly49H$^+$ NK cells are capable of population expansion of 100- to 1,000-fold during infection with MCMV[40], the expression of genes encoding regulators of the cell cycle (CDC (cell-division cycle) proteins) and a protein associated with cellular proliferation (MKI67) was higher at day 7 (**Fig. 5a** and **Supplementary Table 3**). The expression of genes encoding transcription factors shown to regulate the proliferation and survival of T lymphocytes, including *Foxm1* (ref. 41) and *Klf13* (ref. 42), was also higher in Ly49H$^+$ NK cells at day 7 than in naive Ly49H$^+$ NK cells (**Fig. 5a**). Additionally, we observed higher expression of *Klrg1* (KLRG1), *Itgam* (CD11b) and *Thy1* (CD90), as both transcript and protein, in Ly49H$^+$ NK cells at day 7 after infection than in resting NK cells (**Fig. 5a**,**d**). These data demonstrated that the transcriptional changes for these markers represented all Ly49H$^+$ NK cells rather than representing a subset.

After the peak of the response of effector Ly49H$^+$ NK cells to infection with MCMV, a contraction phase occurs in which most effector cells undergo cell death and leave behind a long-lived memory NK cell pool that persists for months after the initial infection[40,43]. Consistent with the beginning of the contraction phase of the NK cell response at approximately day 7 after infection, expression of the antiapoptotic protein Bcl-2 was lower at day 7 than at earlier times after infection (**Fig. 5d**). At day 27 after infection, expression of *Ly6c1* was higher in memory NK cells than in resting NK cells (**Fig. 5a**), a result we confirmed by cell surface staining (**Fig. 5e**). In summary, these data explored the transcriptional dynamics of NK cells during infection with MCMV and further suggested that many cellular processes are involved in the differentiation of naive NK cells into effector and memory cells, despite the prepriming of some effector mechanisms.

## Effector NK cell and CD8$^+$ T cell differentiation

The effector response to infection is characterized by the migration of effector cells from lymphoid tissues to nonlymphoid tissues, clonal expansion, the secretion of antiviral cytokines and the cytolysis of infected cells. Although some effector functions are preprimed, NK cells rely on migration to secondary lymphoid organs and dendritic cell–derived signals to become fully functional[24]. To define a transcriptional program central to effector differentiation, we examined changes in gene expression common to the differentiation of NK cell and CD8$^+$ T cells in response to infection. To identify the appropriate time points for comparison, we calculated correlations between the gene-expression changes in NK cells on days 1.5 and 7 after MCMV infection relative to that of CD8$^+$ T cells at each time point after infection with either

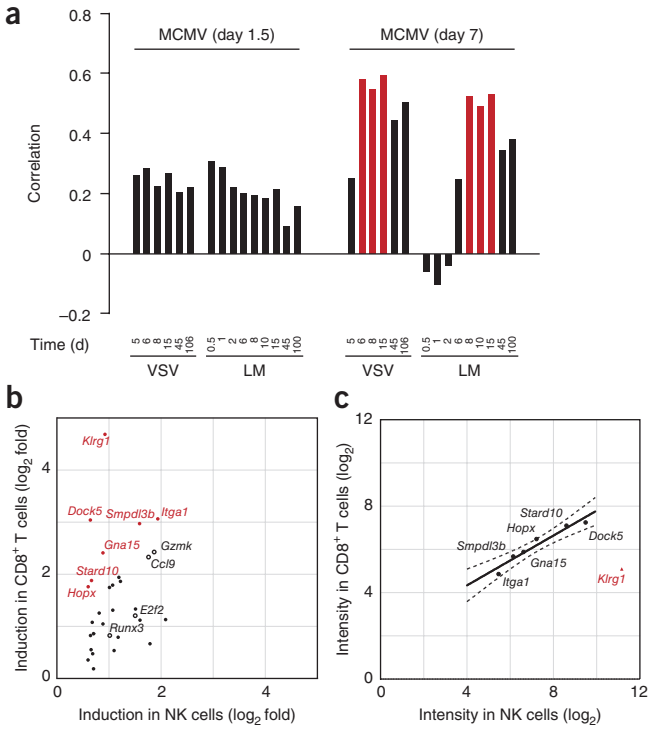


**Figure 6** Common effector mechanisms of NK cells and CD8$^+$ T cells. (**a**) Pearson correlation of the response of NK cells at day 1.5 and day 7 after infection with MCMV relative to the response of CD8$^+$ T cells to infection with VSV or *L. monocytogenes*. Red indicates responses with the strongest correlation. (**b**) Induction of genes encoding effector molecules in NK cells and CD8$^+$ effector cells, relative to their expression in naive cells. Red indicates genes with a difference in regulation of greater than twofold; black indicates additional selected genes. (**c**) Gene expression in NK cells and in CD8$^+$ T cells; dashed lines indicate 95% confidence intervals; red indicates 'preferential' induction of *Klrg1* in NK cells. Data presented are based on the analysis of a minimum of three independent replicates per cell type.

VSV or *L. monocytogenes* (**Fig. 6a**); this showed that NK cells on day 7 after infection were, on the whole, transcriptionally more similar to effector CD8$^+$ T cells than to NK cells on day 1.5 after infection. Furthermore, this response most closely resembled the CD8$^+$ T cell response at day 6 after infection with either VSV or *L. monocytogenes*. We therefore used the three time points after infection with the strongest correlation (VSV at days 6, 8 and 15, and *L. monocytogenes* at days 8, 10 and 15) for comparison with NK cells at day 7.

We identified 32 genes with significantly altered expression in effector NK cells and CD8$^+$ T cells relative to the expression of those genes in their naive counterparts (**Fig. 6b** and **Supplementary Table 4**). These included genes encoding transcription factors (such as *Runx3*, *E2f2*, *Hmgb2*, *Zmiz1* and *HopX*), migration and adhesion molecules (such as *Itga1* and *Ccl9*) and an effector molecule (*Gzmk*). We detected KLRG1 on the surface of both Ly49H$^+$ NK cells (**Fig. 5d**) and antigen-specific CD8$^+$ T cells undergoing clonal expansion (**Fig. 4b**); this may provide a means of regulating cells undergoing rapid cell division and of limiting collateral damage to host tissues.

Comparison of the effector responses of NK cells and CD8$^+$ T cells on a gene-by-gene basis showed that the magnitude of gene induction was lower in NK cells than in CD8$^+$ T cells (**Fig. 6b**); the median gene induction in NK cells was 85% of that in CD8$^+$ T cells ($P = 0.039$ (Student's two-tailed *t*-test assuming unequal variances)). Whereas seven genes had an induction in CD8$^+$ T cells of greater than twofold, no gene reached an induction greater than twofold in NK cells (**Fig. 6b**). That smaller magnitude of induction was due mainly to higher baseline expression of these genes in NK cells; their expression in naive CD8$^+$ T cells was (on average) 58% their expression in naive NK cells (**Fig. 6c**; slope = 0.58; 95% confidence interval, 0.36–0.79). *Klrg1* also followed this trend, but to a greater degree, with high expression in naive NK cells (67-fold higher than in naive CD8$^+$ T cells; **Fig. 6c**). We concluded that because NK cells are naturally primed for rapid responses to pathogens, the magnitude of their transcriptome-wide induction was generally smaller than the specific responses of effector CD8$^+$ T cells, as observed before for *Ifng* and *Gzmb*[44].

### A conserved program underlying memory

During the second phase of the immune responses of both NK cells and CD8$^+$ T cells, most effector cells die, but those that survive go on to seed a pool of long-lived memory cells that can subsequently acquire effector functions much more rapidly after reexposure to antigen[40,45]. To better understand the underlying program that establishes these functions, we identified the transcriptional changes that accompany memory-cell differentiation. Using a published adoptive-transfer system[40], we transferred purified NK cells from wild-type mice into Ly49H-deficient hosts (**Fig. 7a**). After infection with MCMV, Ly49H$^+$ NK cells underwent massive population expansion over the course of 7 d, followed by a contraction phase and the generation of long-lived memory NK cells isolated at day 27 after infection (**Fig. 7b**). In contrast to naive Ly49H$^+$ NK cells or endogenous Ly49H$^-$ NK cells, a higher frequency of memory NK cells expressed CD11b and a lower frequency expressed CD27 (**Fig. 7c**), consistent with published observations[40].

We compared the gene-expression profiles of memory Ly49H$^+$ NK cells (day 27 after infection with MCMV) and memory CD8$^+$ T cells (generated after infection with VSV or *L. monocytogenes*) with those of naive Ly49H$^+$ NK and CD8$^+$ T cells, respectively, and identified a common set of 47 genes that were coordinately regulated during the differentiation of naive cells into memory cells (**Fig. 7d** and **Supplementary Table 5**). Memory-specific transcripts included genes encoding molecules involved in signaling potential (such as *S100a6* and *Ptpn4*), effector function (such as *Gzmb*, *Fasl* and *Sytl2*), migration (such as *Sell* (CD62L) and *Itga1* (CD49a)) and apoptosis (such as *Casp1* and *Pmaip1*). A subset of those identified genes, including *S100a6*, *Casp1*, *Itga1*, *Ly6c1* and *Gzmb*, are also upregulated in memory CD8$^+$ T cells in the lymphocytic choriomeningitis virus model of the memory differentiation of CD8$^+$ T cells[46]. Flow cytometry confirmed upregulation of Ly6C and CD49a and downregulation of CD62L and CD55 in both memory NK cells (**Fig. 7e**) and memory CD8$^+$ T cells (**Fig. 7f**), with CD49a and CD55 being the newly identified cell-surface makers of memory NK cells and T cells. In addition, we identified a transcription factor, Hopx (*Hopx*), that was upregulated in memory cells. Published reports that *Hopx* is upregulated in induced regulatory T cells and effector memory T cells and is critical for the survival of activated mouse T helper type 1 effector-memory cells[47,48] suggest that this regulator may also promote the persistence of NK memory cells after infection. Analysis of expression intensity showed that similar to gene induction in effector populations, the magnitude of induction was generally greater in memory CD8$^+$ T cells than in memory NK cells (slope = 1.50; 95% confidence interval, 1.28–1.72) (**Fig. 6b**,**c**). Together these results demonstrated a common transcriptional program conserved in the memory differentiation of NK cells and CD8$^+$ T cells in response to infection.

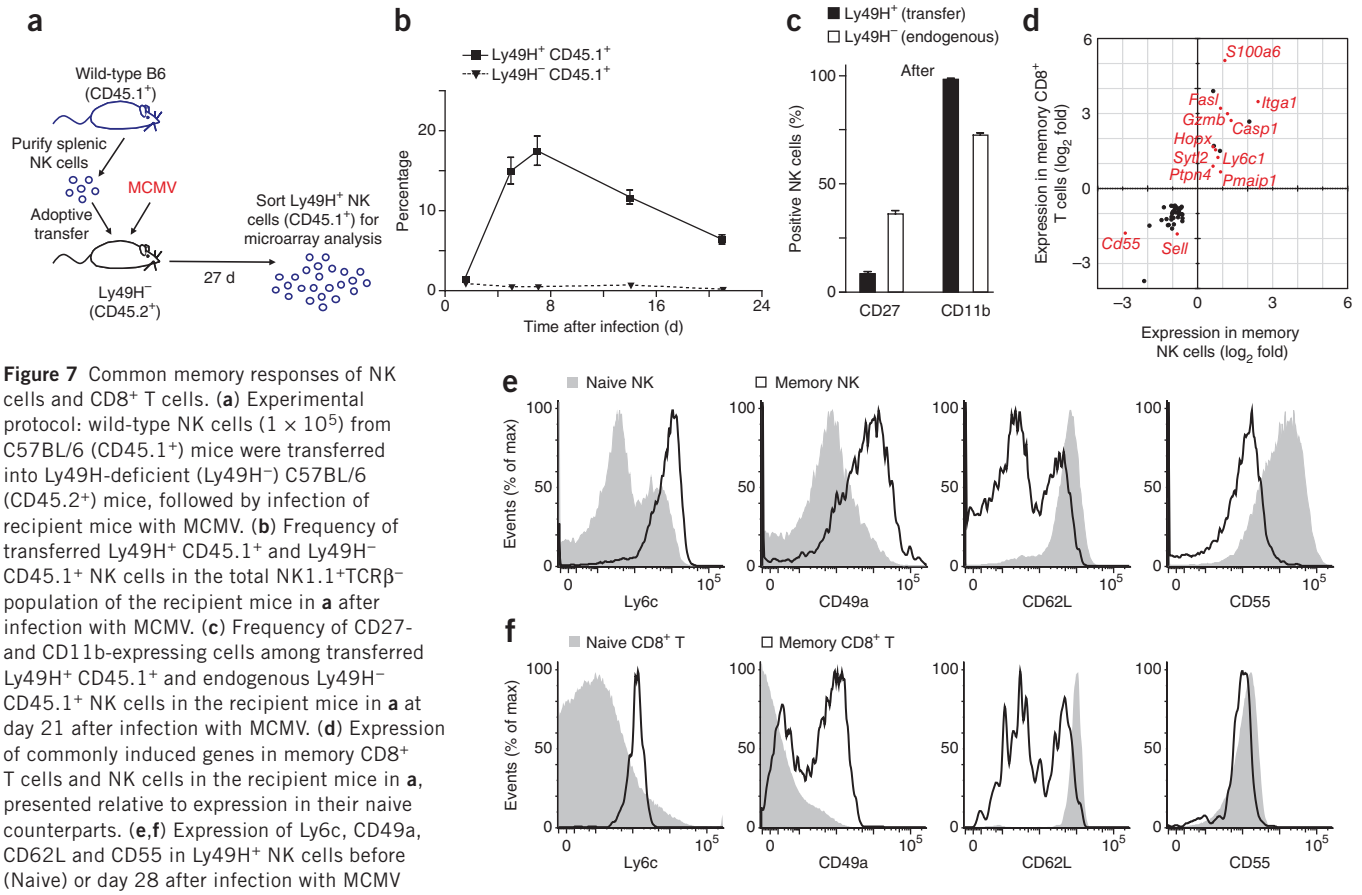


**Figure 7** Common memory responses of NK cells and CD8$^+$ T cells. (**a**) Experimental protocol: wild-type NK cells ($1 \times 10^5$) from C57BL/6 (CD45.1$^+$) mice were transferred into Ly49H-deficient (Ly49H$^-$) C57BL/6 (CD45.2$^+$) mice, followed by infection of recipient mice with MCMV. (**b**) Frequency of transferred Ly49H$^+$ CD45.1$^+$ and Ly49H$^-$ CD45.1$^+$ NK cells in the total NK1.1$^+$TCRβ$^-$ population of the recipient mice in **a** after infection with MCMV. (**c**) Frequency of CD27- and CD11b-expressing cells among transferred Ly49H$^+$ CD45.1$^+$ and endogenous Ly49H$^-$ CD45.1$^+$ NK cells in the recipient mice in **a** at day 21 after infection with MCMV. (**d**) Expression of commonly induced genes in memory CD8$^+$ T cells and NK cells in the recipient mice in **a**, presented relative to expression in their naive counterparts. (**e**,**f**) Expression of Ly6c, CD49a, CD62L and CD55 in Ly49H$^+$ NK cells before (Naive) or day 28 after infection with MCMV (Memory; **e**) or OT-I CD8$^+$ T cells before or 60 d (Ly6c, CD49a and CD55) or 100 d (CD62L) after infection with VSV (**f**). Data (**b**,**c**,**e**,**f**) are representative of at least two independent experiments (error bars (**b**,**c**), s.e.m.). Data presented are based on the analysis of a minimum of three independent replicates per cell type (**d**).



## DISCUSSION

A chief goal of this work was to define, from the transcriptome-wide perspective, NK cells in various states to generate new insights and a resource describing the specific genes involved with NK cell functions. We used the breadth of cell populations available from the ImmGen Project to explore transcripts that define the identity of NK cells in a more robust and in-depth perspective than provided by published analyses[10–12]. Our results have shown extensive transcriptional differences between NK cells and other leukocyte populations far beyond the few specific markers commonly used to identify these cells by flow cytometry. In addition, we found that few transcripts were uniquely specific to NK cells, with many being shared with other cell types of the immune system, particularly T cells. Our data provide a genome-wide context for the interpretation of NK cell functions and should accelerate the discovery of pathways that regulate the activation states of NK cells. We have demonstrated a close transcriptional relatedness between naive NK cells and innate-like T cells, which suggests that their gene-expression profiles reflect a functional similarity rather than a developmental similarity. The abundance of cell-surface receptors and signaling molecules expressed by NK cells and innate-like T cells is consistent with their role as primary sentinels and demonstrates a wealth of previously unknown signaling mechanisms for further targeted exploration.

Although NK cells are distinct in their innate properties, they also have properties associated with adaptive CD8$^+$ T cells, including cytotoxicity and memory. Our work has provided a systematic identification of the genes associated with these common activities and has identified hundreds of genes not previously known to be associated with these functions. We found elements of the effector and memory NK cell differentiation signature that were shared by effector and memory CD8$^+$ T cells, which suggests conservation between the NK cell and CD8$^+$ T cell lineages of some activation mechanisms. However, this must be interpreted in the context of dynamic changes in activation state, as shown by the distinct NK cell transcriptomes at each stage of differentiation from naive cell to early effector cell to late effector cell to memory cell. Although understanding the function of NK cell memory is in its infancy, one implication of a defined gene-expression signature that corresponds to memory differentiation is that specific genes could be useful as surrogate markers for memory NK cells with the greatest potential to confer immunological potential.

As for αβT cells, the memory NK cell differentiation signature included genes both unique to the memory state (such as *Casp1*, *Fasl*, and *Ly6c1*) and initially expressed in effector cells and maintained in memory cells (such as *Itga1* and *Hopx*), which represent genes that have not been previously appreciated as having a potential role in NK cell memory and are worthy of further study. This suggests that the transcriptome of memory NK cells represents a composite of genes uniquely expressed by these long-lived cells and those maintained from prior stages of differentiation, possibly to allow quick reexpression after secondary exposure to antigen. We speculate that the expression pattern of the memory repertoire, rather than the

individual genes themselves, is needed to define the memory NK cell state. In sum, our study has provided a comprehensive transcriptome perspective on various stages of NK cell function in the context of closely related T lymphocytes. Our data simultaneously support and extend published findings while providing a unique resource for the further investigation of NK cell biology.

## METHODS

Methods and any associated references are available in the online version of the paper.

**Accession codes.** GEO: microarray data, GSE15907.

*Note: Supplementary information is available in the online version of the paper.*

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### AUTHOR CONTRIBUTIONS

C.C.K. analyzed data; N.A.B. and J.C.S. sorted cell subsets, did follow-up experiments and analyzed data; G.M.-O., D.W.H. and Y.K. did experiments; J.A.B. and A.W.G. designed and did the T cell studies; and N.A.B., C.C.K., J.C.S. and L.L.L. designed studies and wrote the paper.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Heng, T.S. & Painter, M.W. The Immunological Genome Project: networks of gene expression in immune cells. *Nat. Immunol.* **9**, 1091–1094 (2008).
2. Bendelac, A., Bonneville, M. & Kearney, J.F. Autoreactivity by design: innate B and T lymphocytes. *Nat. Rev. Immunol.* **1**, 177–186 (2001).
3. Vivier, E. *et al.* Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44–49 (2011).
4. Herberman, R.B., Nunn, M.E., Holden, H.T. & Lavrin, D.H. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int. J. Cancer* **16**, 230–239 (1975).
5. Kiessling, R., Klein, E., Pross, H. & Wigzell, H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur. J. Immunol.* **5**, 117–121 (1975).
6. Lanier, L.L. & Phillips, J.H. Ontogeny of natural killer cells. *Nature* **319**, 269–270 (1986).
7. Di Santo, J.P. Natural killer cell developmental pathways: a question of balance. *Annu. Rev. Immunol.* **24**, 257–286 (2006).
8. Yamagata, T., Benoist, C. & Mathis, D. A shared gene-expression signature in innate-like lymphocytes. *Immunol. Rev.* **210**, 52–66 (2006).
9. Chambers, S.M. *et al.* Hematopoietic fingerprints: an expression database of stem cells and their progeny. *Cell Stem Cell* **1**, 578–591 (2007).
10. Obata-Onai, A. *et al.* Comprehensive gene expression analysis of human NK cells and CD8$^+$ T lymphocytes. *Int. Immunol.* **14**, 1085–1098 (2002).
11. Hanna, J. *et al.* Novel insights on human NK cells' immunological modalities revealed by gene expression profiling. *J. Immunol.* **173**, 6547–6563 (2004).
12. Dybkaer, K. *et al.* Genome wide transcriptional analysis of resting and IL2 activated human natural killer cells: gene expression signatures indicative of novel molecular signaling pathways. *BMC Genomics* **8**, 230 (2007).
13. Lanier, L.L. Up on the tightrope: natural killer cell activation and inhibition. *Nat. Immunol.* **9**, 495–502 (2008).
14. Bezman, N. & Koretzky, G.A. Compartmentalization of ITAM and integrin signaling by adapter molecules. *Immunol. Rev.* **218**, 9–28 (2007).
15. Pont, F. *et al.* The gene expression profile of phosphoantigen-specific human gammadelta T lymphocytes is a blend of αβ T-cell and NK-cell signatures. *Eur. J. Immunol.* **42**, 228–240 (2012).
16. Wilson, S.B. & Byrne, M.C. Gene expression in NKT cells: defining a functionally distinct CD1d-restricted T cell subset. *Curr. Opin. Immunol.* **13**, 555–561 (2001).
17. Hesslein, D.G. & Lanier, L.L. Transcriptional control of natural killer cell development and function. *Adv. Immunol.* **109**, 45–85 (2011).
18. Sun, Y. *et al.* Potentiation of Smad-mediated transcriptional activation by the RNA-binding protein RBPMS. *Nucleic Acids Res.* **34**, 6314–6326 (2006).
19. Li, M.O. & Flavell, R.A. TGF-β: a master of all T cell trades. *Cell* **134**, 392–404 (2008).
20. Akbulut, S. *et al.* Sprouty proteins inhibit receptor-mediated activation of phosphatidylinositol-specific phospholipase C. *Mol. Biol. Cell* **21**, 3487–3496 (2010).
21. Narni-Mancinelli, E. *et al.* Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor. *Proc. Natl. Acad. Sci. USA* **108**, 18324–18329 (2011).
22. Yu, J. *et al.* NKp46 identifies an NKT cell subset susceptible to leukemic transformation in mouse and human. *J. Clin. Invest.* **121**, 1456–1470 (2011).
23. Satoh-Takayama, N. *et al.* Microbial flora drives interleukin 22 production in intestinal NKp46$^+$ cells that provide innate mucosal immune defense. *Immunity* **29**, 958–970 (2008).
24. Walzer, T. *et al.* Natural killer cell trafficking *in vivo* requires a dedicated sphingosine 1-phosphate receptor. *Nat. Immunol.* **8**, 1337–1344 (2007).
25. Despoix, N. *et al.* Mouse CD146/MCAM is a marker of natural killer cell maturation. *Eur. J. Immunol.* **38**, 2855–2864 (2008).
26. Arase, H., Saito, T., Phillips, J.H. & Lanier, L.L. Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (α2 integrin, very late antigen-2). *J. Immunol.* **167**, 1141–1144 (2001).
27. Ewen, C.L., Kane, K.P. & Bleackley, R.C. A quarter century of granzymes. *Cell Death Differ.* **19**, 28–35 (2012).
28. Young, J.D., Hengartner, H., Podack, E.R. & Cohn, Z.A. Purification and characterization of a cytolytic pore-forming protein from granules of cloned lymphocytes with natural killer activity. *Cell* **44**, 849–859 (1986).
29. Colige, A. *et al.* Cloning and characterization of ADAMTS-14, a novel ADAMTS displaying high homology with ADAMTS-2 and ADAMTS-3. *J. Biol. Chem.* **277**, 5756–5766 (2002).
30. Hirst, C.E. *et al.* The intracellular granzyme B inhibitor, proteinase inhibitor 9, is up-regulated during accessory cell maturation and effector cell degranulation, and its overexpression enhances CTL potency. *J. Immunol.* **170**, 805–815 (2003).
31. Zhang, M. *et al.* Serine protease inhibitor 6 protects cytotoxic T cells from self-inflicted injury by ensuring the integrity of cytotoxic granules. *Immunity* **24**, 451–461 (2006).
32. Liu, L. *et al.* A novel protein tyrosine kinase NOK that shares homology with platelet-derived growth factor/fibroblast growth factor receptors induces tumorigenesis and metastasis in nude mice. *Cancer Res.* **64**, 3491–3499 (2004).
33. Kaech, S.M., Hemby, S., Kersh, E. & Ahmed, R. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* **111**, 837–851 (2002).
34. Kallies, A., Xin, A., Belz, G.T. & Nutt, S.L. Blimp-1 transcription factor is required for the differentiation of effector CD8$^+$ T cells and memory responses. *Immunity* **31**, 283–295 (2009).
35. Shin, H. *et al.* A role for the transcriptional repressor Blimp-1 in CD8$^+$ T cell exhaustion during chronic viral infection. *Immunity* **31**, 309–320 (2009).
36. Sun, J.C., Lopez-Verges, S., Kim, C.C., DeRisi, J.L. & Lanier, L.L. NK cells and immune "memory". *J. Immunol* **186**, 1891–1897 (2011).
37. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* **3**, 133–146 (2003).
38. Palmer, D.C. & Restifo, N.P. Suppressors of cytokine signaling (SOCS) in T cell differentiation, maturation, and function. *Trends Immunol.* **30**, 592–602 (2009).
39. Lee, S.H., Kim, K.S., Fodil-Cornu, N., Vidal, S.M. & Biron, C.A. Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection. *J. Exp. Med.* **206**, 2235–2251 (2009).
40. Sun, J.C., Beilke, J.N. & Lanier, L.L. Adaptive immune features of natural killer cells. *Nature* **457**, 557–561 (2009).
41. Xue, L., Chiang, L., He, B., Zhao, Y.Y. & Winoto, A. FoxM1, a forkhead transcription factor is a master cell cycle regulator for mouse mature T cells but not double positive thymocytes. *PLoS ONE* **5**, e9229 (2011).
42. Zhou, M. *et al.* Kruppel-like transcription factor 13 regulates T lymphocyte survival in vivo. *J. Immunol.* **178**, 5496–5504 (2007).
43. O'Leary, J.G., Goodarzi, M., Drayton, D.L. & von Andrian, U.H. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat. Immunol.* **7**, 507–516 (2006).
44. Sun, J.C. & Lanier, L.L. NK cell development, homeostasis and function: parallels with CD8 T cells. *Nat. Rev. Immunol.* **11**, 645–(2011).
45. Cui, W. & Kaech, S.M. Generation of effector CD8$^+$ T cells and their conversion to memory T cells. *Immunol. Rev.* **236**, 151–166 (2010).
46. Wherry, E.J. *et al.* Molecular signature of CD8$^+$ T cell exhaustion during chronic viral infection. *Immunity* **27**, 670–684 (2007).
47. Albrecht, I. *et al.* Persistence of effector memory Th1 cells is regulated by Hopx. *Eur. J. Immunol.* **40**, 2993–3006 (2010).
48. Hawiger, D., Wan, Y.Y., Eynon, E.E. & Flavell, R.A. The transcription cofactor Hopx is required for regulatory T cell function in dendritic cell-mediated peripheral T cell unresponsiveness. *Nat. Immunol.* **11**, 962–968 (2010).

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## ONLINE METHODS

**Mice and infection.** In accordance with standard operating protocols of the ImmGen Project, six- to eight week-old male C57BL/6 and B6.SJL-*Ptprc*$^{a}$ *Pepc*$^{b}$/BoyJ congenic CD45.1$^{+}$ mice (Jackson Laboratory) were maintained under specific pathogen–free conditions. C57BL/6 mice were infected by intraperitoneal injection of MCMV Smith strain ($5 \times 10^4$ plaque-forming units). An adoptive-transfer system was used for the generation of memory NK cells as described[40]. Purified NK cells from B6.SJL-*Ptprc*$^{a}$ *Pepc*$^{b}$/BoyJ mice were adoptively transferred into Ly49H-deficient C57BL/6 mice 1 d before viral infection. In the case of CD8$^{+}$ T cells, $5 \times 10^3$ CD45.1$^{+}$ OT-I T cells were transferred into C57BL/6 recipients. At 1 d after transfer, mice were infected with $5 \times 10^3$ colony-forming units of ovalbumin-expressing *L. monocytogenes* or $5 \times 10^3$ plaque-forming units of ovalbumin-expressing VSV. To obtain naive OT-I cells, $5 \times 10^6$ CD45.1$^{+}$ OT-I cells were transferred into C57BL/6 mice and were purified from mice 2 d after transfer. Experiments were done according to the Institutional Animal Care and Use Committee guidelines of the University of California, San Francisco, San Francisco.

**Cell sorting.** Cells were prepared according to standard operating protocols of the ImmGen Project. Naive NK or Ly49H$^{+}$ NK cells were isolated from spleens of uninfected C57BL/6 mice. Effector Ly49H$^{+}$ NK cells were isolated from C57BL/6 mice on days 1.5 and 7 after MCMV infection. Memory Ly49H$^{+}$ NK cells were isolated from spleens of reconstituted Ly49H-deficient mice on day 27 after MCMV infection. All samples were pooled from three mice and stained for cell-surface markers, and ~$1 \times 10^4$ to $3 \times 10^4$ cells (>99% pure) were double-sorted directly into Trizol (Invitrogen) with a FACSAria (BD). For each population, independent triplicate samples were sorted, except for memory Ly49H$^{+}$ NK cells, which independent duplicate samples were sorted from 16 mice.

**Microarray hybridization and analysis.** Isolated RNA was amplified and prepared for hybridization to the Affymetrix MoGene 1.0 ST array with the GeneChip Whole Transcript Sense Target Labeling Assay in accordance with manufacturer's instructions. Raw data were normalized with the robust multichip average algorithm in the 'Expression File Creator' module (Gene Pattern). Raw data from all ImmGen samples are available in the GEO database (GSE15907); processed data are available on the ImmGen website. The consortium-standardized post-normalization threshold of 120 was taken to indicate expression above background, and probes were included in comparisons only if they were expressed in all replicates of at least one population. Additional details[49] are provided as **Supplementary Notes 1 and 2**.

**Flow cytometry.** Fc receptors were blocked with mAb to CD16 and CD32 (10 μg/ml; 2.4G2; UCSF Antibody Core) before surface staining. The following antibodies to cell surface markers and intracellular proteins were used: antibody to NK1.1 (anti-NK1.1; PK136), anti-NKp46 (29A1.4), anti-TCRβ (H57-597), anti-CD4 (RM4-5 or GK1.5), anti-CD8 (53-6.7), anti-CD3 (145-2C11 or eBio500A2), anti-CD5 (53-7.3), anti-CD19 (MB19-1), anti-CD25 (PC61.5), anti-Gr-1 (RB6-8C5), anti-B220 (RA3-6B2), anti-Ter119 (Ter119), anti-Ly49E-Ly49F (CM4), anti-y49H (3D10), anti-Ly49I (YLI-90), anti-NKG2D (MI-6 or DX5), anti-NKG2A-NKG2C-NKG2E (20d5), anti-CD11b (M1/70), anti-CD69 (H1.2F3), anti-KLRG1 (2F1), anti-CD45.1 (A20), anti-CD45.2 (104), anti-IFN-γ (XMG1.2), anti-Bcl-2 (10C4), anti-CD45.1 (A20) and anti-CD45.2 (104; all from eBioscience); anti-CD11c (N418), anti-CD55 (RIKO-3), anti-CD69 (H1.2F3), anti-CD90 (30-H12) and anti-T-bet (4B10; all from BioLegend); anti-CD49a (Ha31/8), anti-CD62L (MEL-14), anti-Ly6C (AL-21), anti-TCRγδ (GL3), anti-granzyme B (GB11), antibody to phosphorylated STAT1 (4a), antibody to phosphorylated STAT3 (4/P-STAT3) and antibody to phosphorylated STAT4 (38/p-Stat4; all from BD); Live/Dead Fixable Near-IR dye (L10119; Invitrogen); and anti-Syk (5F5; provided by A. Weiss). Intracellular staining was done according to manufacturer's instructions (BD). All cells were analyzed on an LSR II (BD) with FloJo software (Tree Star).

49. Malhotra, D. *et al.* Transcriptional profiling of stroma from inflamed and resting lymph nodes defines immunological hallmarks. *Nat. Immunol.* **13**, 499–510 (2012).





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