NK cells developing *in vitro* from fetal mouse progenitors express at least one member of the Ly49 family that is acquired in a time-dependent and stochastic manner independently of CD94 and NKG2

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NK cells developing *in vitro* from fetal progenitors in the presence of IL-2 are phenotypically and functionally indistinguishable from mature adult NK cells, with the exception that they generally lack surface expression of any of the Ly49 molecules that have previously been examined. Using two recently developed anti-Ly49 mAb, we show here that most of these NK cells in fact express high levels of at least one previously uncharacterized member of the Ly49 family, most likely Ly49E. Detailed kinetic and clonal analysis revealed that these Ly49 molecules were acquired in a progressive and stochastic manner independently of CD94 and NKG2. CD94 and NKG2 were both expressed early in NK cell development, sometimes in the absence of NK1.1, with CD94 invariably being expressed at two different levels. IL-4 differentially inhibited the expression of CD94 and Ly49 receptors, but had little or no effect on the expression of NKRP1 molecules.

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1 Introduction

The discovery that the majority of progenitor cells present in the day 14 fetal thymus are able to develop autonomously into functional NK cells under defined *in vitro* conditions has provided a potentially powerful system for studying some of the critical events that occur during the differentiation of NK cells [1]. The NK cells that develop under these conditions show heterogeneous expression of several surface molecules involved in intercellular recognition, including Ly6, various CD45 isoforms, NKRP1 molecules, and receptors for the nonclassical class I molecule Qa1, each of which is acquired in a stochastic manner [2, 3]. As in the case of NK cells that develop from bone marrow progenitors in the absence of stromal cells [4], very few of the NK cells that develop autonomously from fetal thymus progenitors express any of several Ly49 molecules that have previously been examined, either at the cell surface or at the mRNA level. However, two exceptions to this have been noted. Firstly, some clones and lines that develop from fetal progenitors contain small numbers of cells that express Ly49C/I or G, suggesting that the Ly49 genes in these cells are in a potentially expressible state [2]. Secondly, abundant levels of mRNA for Ly49E have been found in these cells [5]. We report here that the majority of NK cells that develop *in vitro* from fetal thymus progenitors in fact express high levels of previously uncharacterized Ly49 molecules, most likely Ly49E, on their surfaces. We also show that these Ly49 molecules are acquired in a progressive and stochastic manner independently of CD94 and NKG2 molecules, and that the expression of Ly49 and CD94 receptors, but not NKRP1 molecules, is differentially regulated by IL-4.

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2 Results

2.1 NK cells developing from fetal progenitors frequently express at least one member of the Ly49 family

In previous studies it was shown that NK cells that developed from fetal progenitors in the presence of IL-2 generally failed to express certain members of the Ly49 family, in particular those recognized by the mAb A1, 5E6, 4D11, and 4D5 [2]. The data in Fig. 1 show that they also fail to express those members of the Ly49 family recognized by the mAb YE1/48, JR9, 4LO3311, SED85, HBF, Cwy3, 3D10, and YLI that collectively include Ly49A, C, D, F, G, H, and I. By contrast, many of the cells present in 4-week-old cultures of developing NK cells, and all of the cells present in a typical long-term cloned line derived from such cultures, stained strongly with the 4D12 mAb that recognizes Ly49C and E [6]. A similar pattern of staining was found with the 14B11 mAb that was previously found to recognize COS cells transfected with Ly49 C, I, F, or H [7], and, as shown in Fig. 2, also recognizes CHO cells transfected with Ly49E, albeit rather weakly. It therefore appears that a large proportion of the NK cells that develop from fetal progenitors under the conditions used here express at least one member of the Ly49 family that is not Ly49A, C, D, F, G, H, or I. Since these cells are known to express mRNA for Ly49E, it is highly likely that the Ly49 molecules detected by 14B11 and 4D12 on the surface of these cells are, or include, Ly49E.

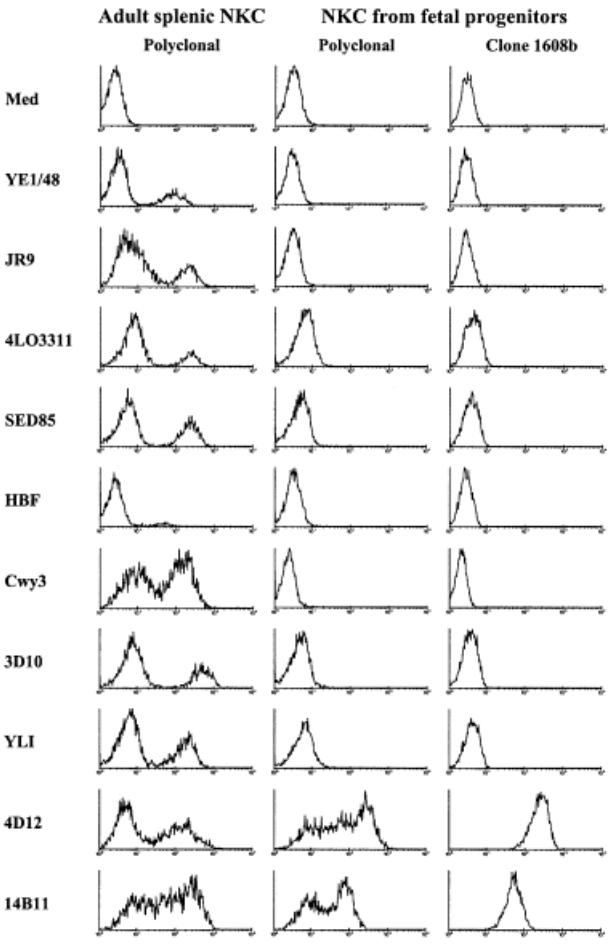

Fig. 1. Staining of NK cell populations with anti-Ly49 mAb. A polyclonal population of NK cells derived from fetal progenitors following 4 weeks of culture in IL-2, the 1608b NK clone derived from fetal progenitors and grown in continuous culture in IL-2 for >2 years, and adult splenic NK cells cultured for 2 weeks in IL-2 were stained with various anti-Ly49 mAb.

2.2 The Ly49 molecules detected by 14B11 and 4D12 are acquired progressively during NK cell development *in vitro*

Day 14 fetal thymocytes, either prior to culture or after the initial phase of culture in IL-4 + PMA, contained very few cells that stained with the 4D12 mAb (Fig. 3, top left). However, following a further 3 days of culture in IL-2, a small population of cells showing clear but weak staining with 4D12 became detectable. Both the proportion of cells that reacted with the 4D12 mAb and the level of staining per cell increased with time until the majority of cells were strongly stained (Fig. 3, Table 1). The expression of the Ly49 molecules identified by 4D12 appeared to be stable indefinitely, and most long-term lines and clones derived from such cultures displayed uniform strong staining. Interestingly, after ~2 weeks of culture in IL-2, the $4D12^{+}$ cells present amongst the developing NK cells often appeared to be composed of distinct subpopulations of $4D12^{lo}$ and $4D12^{hi}$ cells. Similar patterns of staining were obtained with the 14B11 mAb. Importantly, co-staining experiments revealed that throughout the period of culture in IL-2 the two mAb showed the same time-dependent changes in the pattern and level of staining, and identified the same subpopulations of cells (Fig. 4A). By contrast, co-staining of adult NK cells with the two mAb revealed patterns of great complexity, as expected from their known reactivity with several members of the Ly49 family.

2.3 CD94 and NKG2 are also acquired progressively during NK cell development

It has previously been shown that NK cells developing from fetal progenitors acquire receptors for the non-classical class I molecule, Qa1 [3, 8]. To examine in more detail the manner in which Qa1 receptors (Qa1R) are

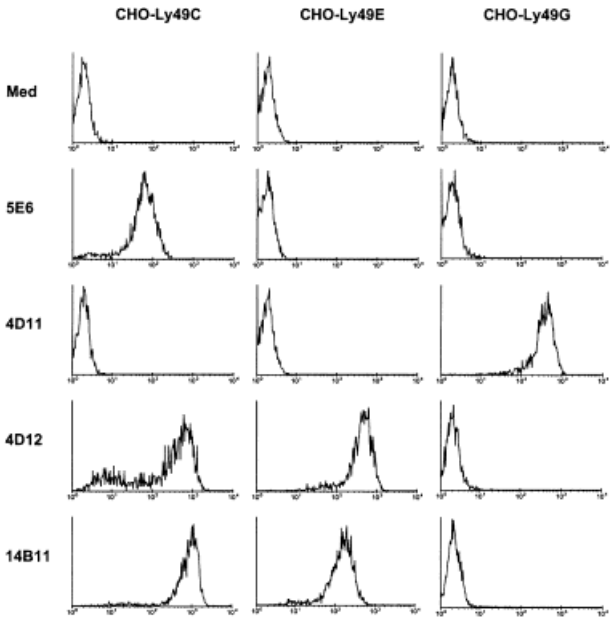

Fig. 2. Specificity of the 4D12 and 14B11 mAb. CHO cells transfected with plasmids encoding Ly49C, Ly49E, or Ly49G were stained with medium alone or the anti-Ly49 mAb 5E6, 4D11, 4D12, or 14B11.

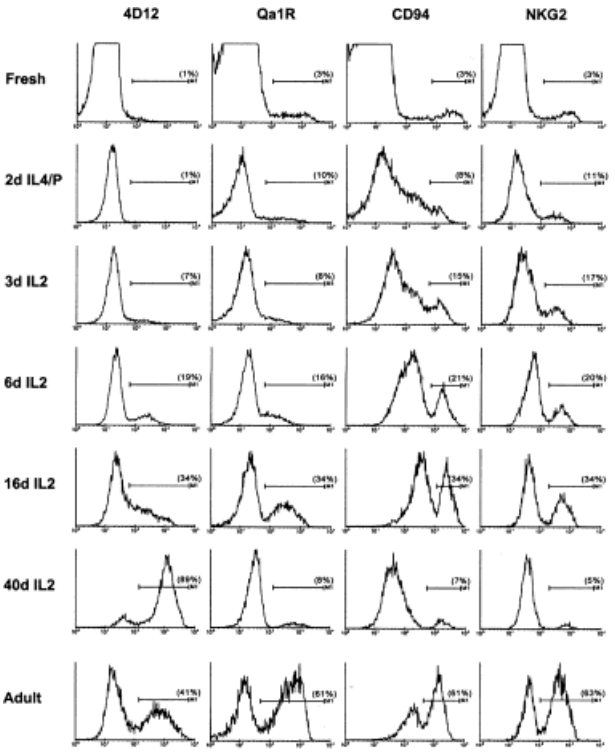

Fig. 3. Acquisition of NK cell receptors by developing NK cells. Day 14 fetal thymocytes were stained with the 4D12 anti-Ly49 mAb, the 18d3 anti-CD94 mAb, the 3S9 anti-NKG2 mAb, and Qa1 tetramers prior to culture (fresh), after 2 days culture in IL-4 + PMA (IL-4/P), and following further culture in IL-2 alone for the number of days shown. Adult splenic NK cells grown in IL-2 were stained in parallel. The results shown are typical of those obtained in three experiments with fresh thymocytes and in nine experiments with cultured cells. The histograms for fresh cells have been expanded to a y-axis maximum of 200 so as to make the small numbers of $CD94^+$, $NKG2^+$, and $Qa1R^+$ cells visible. For CD94 staining the marker positions shown were set to delineate the $CD94^{hi}$ subpopulation of $CD94^+$ cells.

acquired, we stained cells at various stages of development with Qa1 tetramers and with antibodies to CD94 and NKG2. In confirmation of previous findings, and in contrast to the situation for the Ly49 molecules identified by 14B11 and 4D12, fresh day 14 fetal thymocytes contain a small subpopulation of $Qa1R^+$ cells, readily visible on expanded histograms (Fig. 3, second column). They also contain a similar small subpopulation of $NKG2^+$ cells, and a somewhat larger subpopulation of $CD94^+$ cells. The latter are clearly heterogeneous, ~70% being $CD94^{hi}$ and ~30% $CD94^{lo}$, a ratio similar to that found for NK cells in adult spleen.

In further contrast to that found for the Ly49 molecules identified by 14B11 and 4D12, the proportion of cells expressing CD94 increased dramatically during the initial phase of culture in IL-4 + PMA, and there was also a substantial increase in the proportion of cells expressing Qa1R and NKG2 (Fig. 3). During subsequent culture in IL-2 the proportions of $CD94^{hi}$, $NKG2^+$, and $Qa1R^+$ cells increased further but, unlike the Ly49 molecules expressed on these cells, peaked after 2–3 weeks at ~35% and then usually declined slowly over a period of several weeks or months. In some experiments, such as the one illustrated, the proportion of $CD94^+/NKG2^+/Qa1R^+$ cells declined more abruptly. In addition, whereas the level of expression of Ly49 molecules increased substantially between days 3 and 40 of culture in IL-2, the level of expression of CD94 on CD94hi cells and the level of expression of NKG2 increased only slightly (Fig. 3, and Table 1). Interestingly, there seemed to be a disproportionate increase in the level of staining with Qa1 tetramers over this period. In all experiments and at all stages of development there was a striking correlation between the proportions of $CD94^{hi}$, $NKG2^+$, and $Qa1R^+$ cells. That the $CD94^{hi}$, $NKG2^+$, and $Qa1R^+$ cells were identical was indicated by the findings that (a) amongst both developing and mature NK cells the expression of NKG2 was confined to the $CD94^{hi}$ population of cells (Fig. 4B), and (b) antibody to NKG2 blocked staining with Qa1 tetramers (Fig. 4C).

Table 1. Acquistion of Ly49 and CD94/NKG2/Qa1 receptors by NK cells developing from fetal thymic progenitors

Source	Culture[a]	Time	Ly49		Qa1R		CD94hi		NKG2	
		(days)	+ ve	MFI[b]	+ ve	MFI	+ ve	MFI	+ ve	MFI
Day 14 fetal thymus	Fresh		< 1	nd	3.5	546	3.1	2,815	2.8	632
	IL-4/ PMA	2	< 1	nd	9.5	241	8.0	1,266	11	260
	IL-2	3	7.5	159	7.7	131	15	1,412	17	325
	IL-2	6	19	215	16	147	21	1,889	20	507
	IL-2	16	34	207	34	302	35	2,618	34	566
	IL-2	40	89	1,177	7.8	632	6.8	1,694	5.4	760
Adult spleen[c]	IL-2	14	41[d]	681[d]	61	507	61	1,362	63	471

a) Day 14 fetal thymocytes were examined fresh, after initial culture for 2 days in IL-4 + PMA, and following further culture in IL-2 for the number of days shown.
b) MFI: Median fluorescence intensity of positive cells after subtraction of background values.
c) Typical values obtained in > 10 experiments.
d) Includes all cells reacting with the 4D12 mAb.

2.4 Ly49 molecules are acquired by developing NK cells in a stochastic manner independently of CD94/NKG2/Qa1 receptors

Two-color staining with Qa1 tetramers and 4D12 revealed that, early in the development of NK cells, Qa1R and the Ly49 molecules recognized by 4D12 were initially expressed on largely separate subpopulations of cells in a close to random manner (Fig. 5, Table 2). For example, the ratio of $4D12^-$ to $4D12^+$ cells in the $Qa1R^-$ and $Qa1R^+$ subpopulations was very similar, being 1 in 4.09 and 1 in 5.73, respectively. However, in most experiments a noticeable deviation from the 'product rule' was found, with a bias against the expression of Ly49 molecules on cells that bore Qa1R, and this bias tended to become more pronounced with time. The experiment documented in detail here provided a particularly extreme example of this trend. Co-staining with the anti-CD94 and 14B11 mAb also revealed that at early stages of development there was a close to random partition of $14B11^+$ cells amongst $CD94^{hi}$ and $CD94^{lo}$ cells but with a similar bias against the expression of Ly49 molecules on $CD94^{hi}$ cells (Fig. 5, Table 2). In addition, there was a marked bias against the expression of Ly49 molecules on the $CD94^-$ cells present early in development (Fig. 5, Table 2). An exception to the generally close correlation between staining with the 4D12 and 14B11 mAb is also revealed in Fig. 5, namely that after 2 days of culture in IL-4 + PMA there was a markedly higher number of $14B11^+$ cells than $4D12^+$ cells despite the usually weaker staining by 14B11. The significance of this observation is unclear.

The existence of phenotypically distinct subpopulations of cells in bulk cultures of developing NK cells could be explained by there being a pre-existing heterogeneity amongst progenitor cells, such that different progenitors were committed to develop into NK cells bearing different combinations of receptors, or through the operation of a stochastic mechanism controlling the expression of receptors at later stages of development. To distinguish these possibilities, progenitor cells that had been grown in IL-4 + PMA for 2 days were cloned at an average of 0.1 cells/well. Of an estimated 144 cells seeded, 83 developed into colonies giving a cloning efficiency of 58% and a probability, calculated from the Poisson distribution, of 97% that any given clone had arisen from a single progenitor. Of 30 clones that were examined in detail, all 30 contained $4D12^-$ and $4D12^+$ cells. Furthermore, 27/30 clones contained $CD94^{hi}$ and $CD94^{lo}$ subpopulations, and many clones also contained $CD94^-$ cells. The staining patterns of representative clones are shown in Fig. 6. The following points should be noted: (1) The proportion of $4D12^+$ cells increased with time, whereas the proportion of $CD94^+$ cells generally did not. (2) The level of staining by 4D12 increased markedly between the two time points, whereas the level of staining by the anti-CD94 mAb did not. (3) At the later staining points, most of the clones contained $4D12^{hi}$ and $4D12^{lo}$ cells as well as $4D12^-$ cells. (4) The expression of CD94 and the Ly49 molecules recognized by 4D12 was largely independent and random, although often with a bias against the expression of Ly49 molecules on $CD94^{hi}$ cells and on $CD94^-$ cells (Table 1). (5) There was invariably close agreement between the proportion of $CD94^{hi}$,

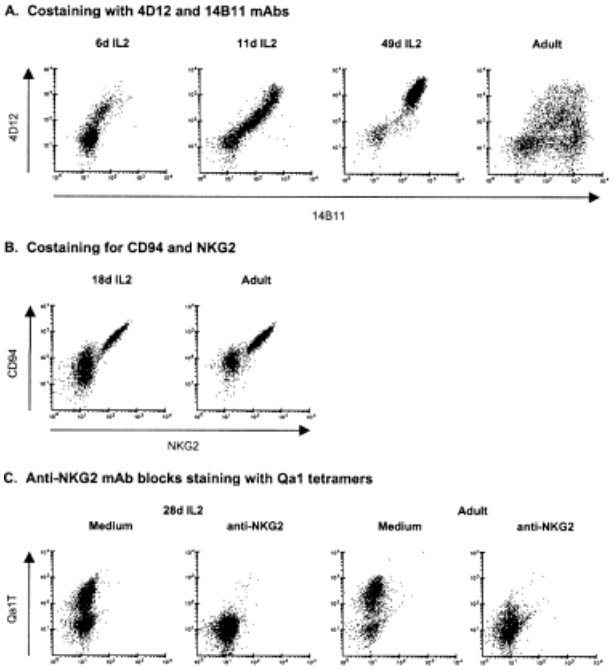

Fig. 4. Co-expression of CD94, NKG2, and Qa1R on developing NK cells. (A) NK cells at various stages of development were co-stained with the 4D12 and 14B11 anti-Ly49 mAb. (B) Developing NK cells were co-stained with the 18d3 anti-CD94 mAb and the 20d5 anti-NKG2 mAb. (C) Developing NK cells were preincubated with medium or the 3S9 anti-NKG2 mAb, then incubated with Qa1 tetramers. In each experiment mature adult NK cells grown in IL-2 were used as controls. The results are typical of those obtained in two to four experiments of each kind.

NKG2$^+$, and Qa1R$^+$ cells (data not shown). In all these respects, clones developing from single progenitors were indistinguishable from polyclonal bulk cultures.

2.5 Relationship between the expression of NK1.1, CD94/NKG2, and Ly49 molecules

The NK1.1 antigen is expressed by most if not all adult splenic NK cells and by all of the NK cells that develop *in vitro* from fetal progenitors in the C57 strain. To examine how the acquisition and expression of CD94/NKG2 receptors and Ly49 molecules is related to the expression of NK1.1, fresh fetal thymocytes and early cultures of developing NK cells were stained with antibodies to NK1.1, CD94, NKG2, and Ly49. To exclude the small numbers (1–2%) of T cells present, cells were co-stained with anti-TCR mAb and gated on TCR$^-$ cells.

Most of the very small number of Ly49$^+$ cells present amongst fresh day 14 thymocytes appeared to be NK1.1$^+$ (Fig. 7). By contrast, about half of the TCR$^-$ CD94^{hi} cells and most of the CD94^{lo} cells present in the day 14 fetal thymus were NK1.1$^-$. Similarly, ~40% of TCR$^-$ NKG2$^+$ cells were NK1.1$^-$. The day 14 fetal thymus therefore contains a population of non-T cells that expresses CD94 and/or NKG2 in the absence of NK1.1. The CD94$^+$ and NKG2$^+$ cells that were present following 2 days of culture in IL-4 + PMA also expressed little or no NK1.1. Yet within 24 h of transfer to IL-2, the majority of CD94$^+$ and NKG2$^+$ cells present in culture expressed NK1.1. That exposure to IL-2 rather than time is the critical factor was shown by the fact that most of the CD94$^+$ and NKG2$^+$ cells present after 2 days of culture in IL-4 + PMA did not acquire NK1.1 even after a further 3 days of culture in IL-4 + PMA; nor was there much, if any, increase in the numbers of CD94$^+$ and NKG2$^+$ cells. Similarly, although all of the Ly49$^+$ cells that developed in the presence of IL-2 expressed NK1.1, the few Ly49$^+$ cells present following 2 or 5 days of culture in IL-4 + PMA were mostly NK1.1$^-$.

2.6 IL-4 differentially inhibits the expression of CD94, NKG2, and Ly49 molecules but not NK1.1 during NK cell development

Not only does IL-4 on its own fail to support the efficient development of progenitor cells into NK cells, it also inhibits the acquisition of Qa1R during NK cell development in IL-2 [3]. To determine whether this effect is due to an inhibition of both CD94 and NKG2 expression and whether IL-4 also affects the expression of Ly49 and NK1.1 molecules, IL-4 was added at various times to cultures of developing NK cells. Fig. 8A shows that when progenitor cells that had been grown in IL-4 + PMA for 2 days were transferred to medium containing IL-4 in addition to IL-2, the acquisition of CD94, NKG2, and also of Ly49 molecules, was completely blocked by IL-4, whereas the acquisition of NK1.1 occurred relatively normally. Likewise, when cells that had already acquired these receptors were exposed to IL-4, the expression of CD94, NKG2, and Ly49 receptors was efficiently down-regulated, whereas the expression of NK1.1 was, if anything, slightly increased (Fig. 8B). As the NK cells became more mature, the down-regulation of receptor expression became less efficient and more selective for CD94/NKG2/Qa1 receptors. For example, exposure of the long-lived NK cell clone J1.1 to IL-4 resulted in the gradual *de novo* appearance of cells having either low or undetectable levels of CD94, NKG2, and Qa1R, but had little or no effect on the expression of Ly49 molecules. There was also little change in the expression of NK1.1 (Fig. 8C). Interestingly, down-regulation of Qa1R occurred more rapidly and more completely than the down-regulation of CD94 and NKG2. A similar difference

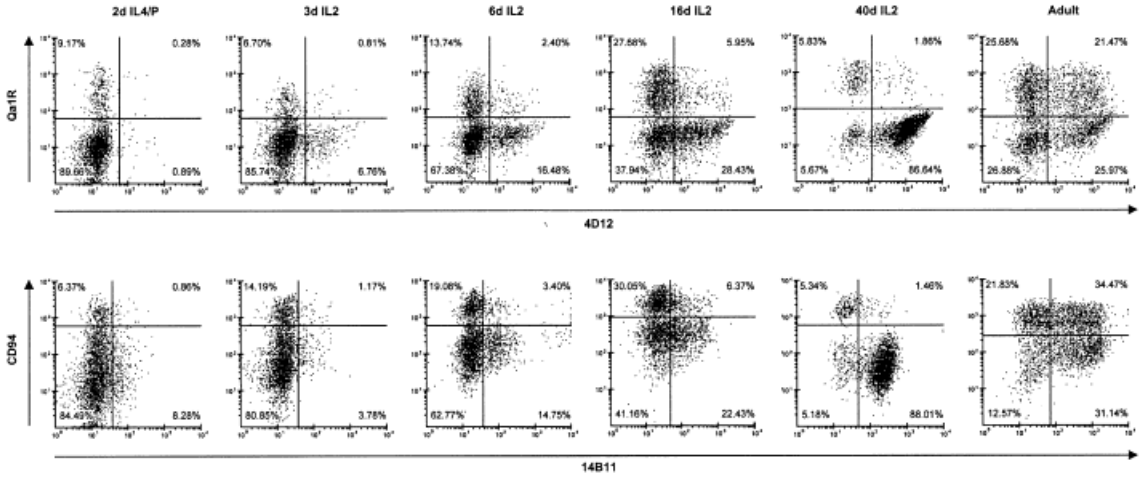

Fig. 5. Independent expression of CD94, Qa1R, and Ly49E on developing NK cells. NK cells at various stages of development were co-stained with 18d3 anti-CD94 and 14B11 anti-Ly49 mAb or Qa1 tetramers and 4D12 anti-Ly49 mAb. In each experiment mature adult NK cells grown in IL-2 were used as controls, and one example of the staining of adult NK cells is included. The results are typical of those obtained in six experiments.

Table 2. Analysis of receptor co-expression on developing NK cells

		Percentage of cells with phenotype:						
Population	Culture	A	B	C	D	A/B	C/D	Bias[a)]
		$Qa1R^-$ $4D12^-$	**$Qa1R^-$ $4D12^+$**	**$Qa1R^+$ $4D12^-$**	**$Qa1R^+$ $4D12^+$**			
Polyclonal	3-day IL-2	85.74	6.76	6.70	0.81	12.68	8.27	1.53
	6-day IL-2	67.38	16.48	13.74	2.40	4.09	5.73	0.71
	16-day IL-2	37.94	28.43	27.68	5.95	1.33	4.65	0.29
	40-day IL-2	5.67	86.64	5.83	1.86	0.07	3.13	0.02
		$CD94^{lo/-}$ $14B11^-$	**$CD94^{lo/-}$ $14B11^+$**	**$CD94^{hi}$ $14B11^-$**	**$CD94^{hi}$ $14B11^+$**			
Polyclonal	3-day IL-2	80.85	3.78	14.19	1.17	21.39	12.13	1.76
	6-day IL-2	62.77	14.75	19.08	3.40	4.26	5.61	0.76
	16-day IL-2	41.16	22.43	30.05	6.37	1.84	4.72	0.39
	40-day IL-2	5.18	88.01	5.34	1.46	0.06	3.66	0.02
		$CD94^-$ 14 $B11^-$	**$CD94^-$ $14B11^+$**	**$CD94^{lo/hi}$ $14B11^-$**	**$CD94^{lo/hi}$ $14B11^+$**			
Polyclonal	3-day IL-2	48.00	0.83	32.86	2.95	57.83	11.14	5.19
	6-day IL-2	13.72	1.41	49.05	13.35	9.73	3.67	2.65
		$CD94^{lo/-}$ $4D12^-$	**$CD94^{lo/-}$ $4D12^+$**	**$CD94^{hi}$ $4D12^-$**	**$CD94^{hi}$ $4D12^+$**			
Clone TC9.4	25-day IL-2	14.58	69.21	6.63	9.58	0.21	0.69	0.30
Clone TC9.13	18-day IL-2	35.16	61.11	1.41	2.32	0.58	0.61	0.95
Clone TC9.14	25-day IL-2	0.29	15.79	2.86	81.06	0.02	0.04	0.52
Clone TC9.17	18-day IL-2	14.21	15.55	37.41	32.83	0.91	1.14	0.80
		$CD94^-$ $4D12^-$	**$CD94^-$ $4D12^+$**	**$CD94^{lo/hi}$ $4D12^-$**	**$CD94^{lo/hi}$ $4D12^+$**			
Clone TC9.4	11-day IL-2	6.19	2.60	65.07	26.14	2.38	2.49	0.96
Clone TC9.13	11-day IL-2	27.04	0.12	69.14	3.70	225.33	18.69	12.06
Clone TC9.17	11-day IL-2	6.52	1.18	54.19	38.11	5.53	1.42	3.89

a) Calculated as the ratio of A/B to C/D. A value of 1.00 represents completely random expression of the two receptors, and exact compliance with the 'product rule'. A value of >1.00 represents a bias towards co-expression of the two receptors (*i. e.* a higher than expected proportion of double-positive cells), while a value of <1.00 represents a bias against co-expression of the two receptors (*i. e.* a lower than expected proportion of double-positive cells). Values >2.0 and <0.5 are considered to indicate a significant deviation from random co-expression.

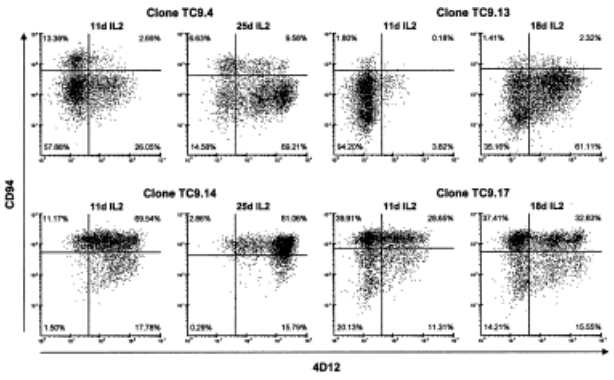

Fig. 6. Acquisition of NK cell receptors by developing NK cell clones. Fetal thymocytes were cultured for 2 days in IL-4 + PMA then cloned at an average of 0.1 cells/well in medium containing IL-2. Clones were co-stained with 18d3 anti-CD94 and 4D12 anti-Ly49 mAb. A total of 30 clones were analyzed. Results for four typical clones stained on two separate occasions are shown.

between the expression of Qa1R and CD94/NKG2 receptors on cells early in development was noted above. These discrepancies suggest either that the binding of Qa1 tetramers to NK cells is highly sensitive to the surface density of CD94/NKG2 heterodimers or that at different stages of development and following exposure to IL-4 qualitative changes in the composition, structure, or distribution of these receptors occur.

3 Discussion

Using two recently developed Ly49-specific mAb we report here that NK cells developing *in vitro* from fetal thymic progenitors express at least one member of the Ly49 family that is not Ly49A, C, D, F, G, H, or I. The fact that both of these Ly49-specific mAb recognize cells transfected with Ly49E, that developing NK cells express abundant levels of mRNA for Ly49E, and that Ly49E mRNA can clearly be translated into protein that is expressible at the cell surface, provides compelling evidence that the molecules detected by these mAb on developing NK cells are, or include, Ly49E. However, the cross-reactivity of anti-Ly49 mAb in general [9], and of 14B11 and 4D12 in particular [6, 7], coupled with the likelihood that not all members of the Ly49 family have yet been identified, precludes a formal assignment that may ultimately require complete sequencing of immunoprecipitated material. Although the patterns of staining obtained with the 4D12 and 14B11 mAb were generally very similar, and co-staining experiments demonstrated that they generally identified identical subpopulations of developing NK cells, no competition for binding was observed between the two mAb, indicating that if they recognize the same molecules they do so via non-overlapping epitopes. Occasional exceptions to the patterns of identity were noted. For example, the 14B11 mAb stained a larger proportion of the cells present after 2 days of culture in IL-4 + PMA than did the 4D12 mAb. In addition, both mAb frequently identified strongly positive and weakly positive subpopulations of developing NK cells. Although this most likely represents heterogeneous acquisition of a single Ly49 molecule, the possibility that one or both mAb recognize more than one Ly49 molecule expressed on developing NK cells cannot be ruled out.

Expression of the Ly49 molecules recognized by 14B11 and 4D12 arose in a time-dependent and stochastic manner, being close to the limits of detection at the time cells were transferred to IL-2 but constituting the majority of cells after 4–6 weeks of culture. Most, but not all, long-term lines and clones displayed uniform high staining of 100% of the cells with 14B11 and 4D12. Small numbers of $4D12^+$ cells (generally $<1\%$) were found amongst fresh day 14 fetal thymocytes. However, the possibility that these were the source of the Ly49-expressing cells found in NK cell cultures can be ruled out because (a) the NK cells that develop *in vitro* arise from the majority population of fetal thymocytes, not from a minor subpopulation [1], (b) the vast majority of colonies developing at high efficiency from individual progenitor cells expressed Ly49 molecules, and (c) the acquisition of Ly49 molecules in colonies derived from individual progenitors occurred in the same progressive and stochastic manner as in bulk cultures. The gradual increase in the level of expression per cell of the Ly49 molecules detected by 14B11 and 4D12 contrasted with the situation for three other NK-specific receptor molecules, NK1.1, CD94, and NKG2, each of which was expressed at close to maximal levels from the point of their initial appearance. Whether this unusual pattern of acquisition applies to other Ly49 molecules remains to be determined. Although Ly49 molecules are generally believed to bind to class I molecules, the ligand for Ly49E is currently unknown and indirect evidence suggest that it may not be a classical class I molecule because: (1) in contrast to cells transfected with various other Ly49 molecules, cells transfected with Ly49E show no significant binding to cells expressing a wide spectrum of classical class I molecules [10, 11]; (2) Ly49E-transfected cells also show no detectable binding to a range of soluble class I molecules [11]; (3) the expression of Ly49E is no higher in class I deficient mice than in class I-sufficient mice [6]; and (4) NK cells that express Ly49E but lack CD94/NKG2 receptors and other members of the Ly49 family lyse class I-sufficient and class I-deficient target cells equally well [3]. Due to the presence of an ITIM in the cytoplasmic domain, it would be

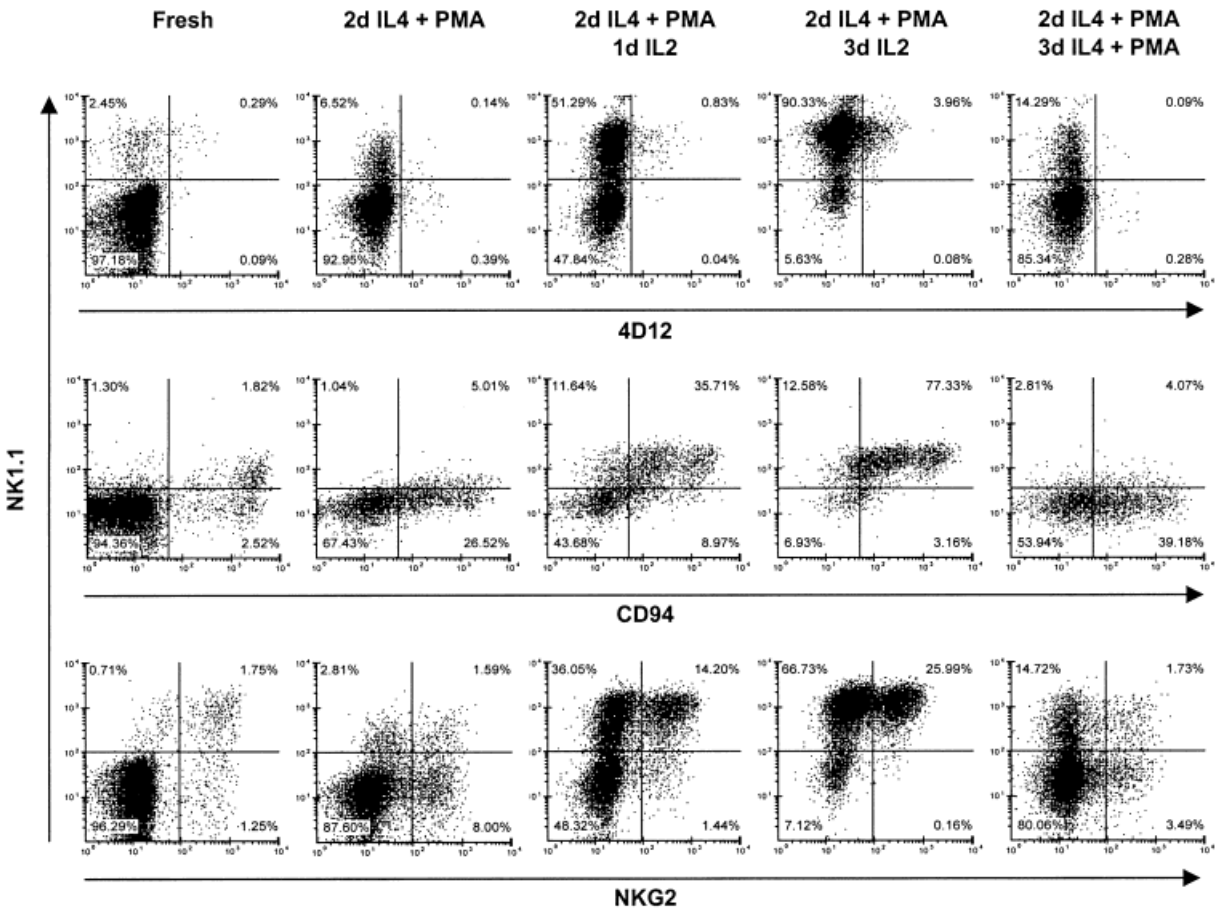

Fig. 7. Acquisition of NK1.1, Ly49E, and CD94 during the early stages of NK development. Day 14 fetal thymus cells were stained with PK136 anti-NK1.1, 4D12 anti-Ly49, 18d3 anti-CD94, 3S9 anti-NKG2, and anti-TCR mAb immediately following their removal from animals (fresh), after 2 days culture in IL-4 + PMA, or following a further 1 or 3 days of culture in IL-2 or IL-4 + PMA. The dot plots show the staining of TCR^- cells for NK1.1 together with 4D12, CD94, or NKG2. The dot plots in the top and bottom rows together with the left-hand plot in the middle row show data from 25,000 acquired cells, the remaining dot plots from 5,000 acquired cells. Note that the biotin-conjugated anti-NK1.1 mAb preparation used for co-staining with 4D12 and anti-NKG2 mAb gave higher levels of fluorescence than the FITC-conjugated anti-NK1.1 mAb preparation that had to be used for co-staining with anti-CD94. The results are representative of those obtained in three experiments.

expected that Ly49E is an inhibitory receptor, although this has not been formally demonstrated. Interestingly, the Ly49E gene occupies the most centromeric position of all of the Ly49 genes that have so far been mapped [12]. Taken together, these observations suggest that Ly49E may play a special role in NK cell development. It is also possible that it is specifically associated with a separate lineage of NK cells derived from embryonic progenitors. Indeed, most of the apparently mature NK cells present in fetal and neonatal mice express Ly49E [6]. Whether these cells can go on to express other Ly49 molecules remains to be determined. The NK cells that develop from fetal progenitors *in vitro* clearly can [2, 13], albeit inefficiently under the culture conditions used here.

Limiting dilution cloning showed that the vast majority of individual fetal progenitors could give rise to both $CD94^{hi}$ and $CD94^{lo}$ cells. Thus, whether an NK cell is $CD94^{hi}$ or $CD94^{lo}$ is determined not by the precursor from which it arises, but by stochastic events later in differentiation. At all stages of development, and in all the clones examined, the proportion of $CD94^{hi}$ cells was very similar to the proportion of cells that clearly expressed NKG2. Co-staining experiments revealed that the $CD94^{hi}$ and $NKG2^+$ subpopulations of developing NK cells were identical. The stochastic event that determines the level of surface expression of CD94 on NK cells might therefore be the expression of NKG2. However, evidence that all NK cells express mRNA for NKG2 [3, 6], and at least low levels of NKG2 at the surface [6], suggests that

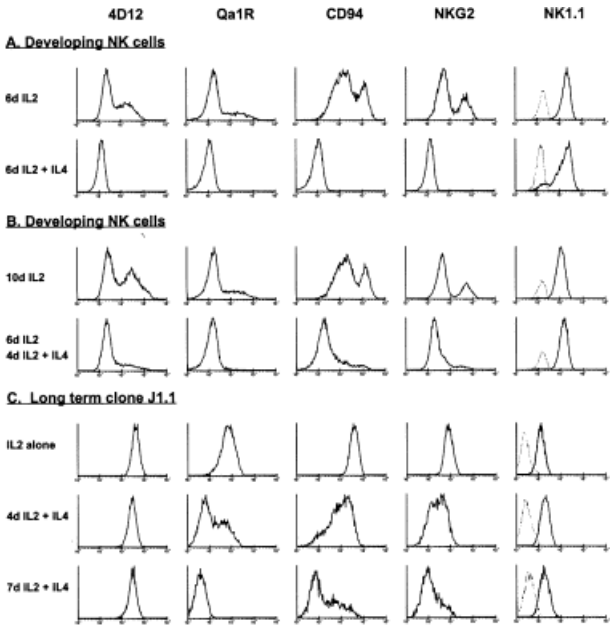

Fig. 8. IL-4 selectively inhibits the expression of NK cell receptors. (A) Following culture for 2 days in IL-4 + PMA, fetal thymic progenitors were cultured for a further 6 days in either IL-2 alone or IL-2 and IL-4. (B) Following culture for 2 days in IL-4 + PMA and 6 days in IL-2 alone, developing NK cells were cultured for a further 4 days in IL-2 alone or IL-2 and IL-4. (C) The NK clone J1.1 grown continuously in IL-2 was exposed to IL-4 for the last 4 or the last 7 days prior to staining. The results shown are representative of three to six experiments of each type. In the plots showing NK1.1 staining, control unstained cells are also shown (left-hand peak).

another factor might be responsible. One possibility would be that $CD94^{hi}$ cells express a different isoform of NKG2. Studies in man have shown that most $CD94^{hi}$ NK clones express inhibitory CD94/NKG2 receptors that contain NKG2A or B chains, whereas most $CD94^{lo}$ NK clones express activating CD94/NKG2 receptors that contain NKG2 isoforms other than NKG2A/B [14–16].

During NK cell development in medium containing IL-2, the expression of CD94 and NKG2 was strongly linked to the expression of NK1.1. Surprisingly, a substantial proportion of fetal thymocytes that had been cultured in IL-4 + PMA for 2 days were found to express CD94 and/or NKG2 in the absence of NK1.1. The existence of cells expressing CD94 and/or NKG2 in the absence of NK1.1 was not a culture artifact because a similar population of cells was found amongst the TCR^- cells present in the day 14 fetal thymus. These cells might represent a subpopulation of immature T cells, a subpopulation of NK cells that does not express NK1.1, or immature NK cells that have not yet acquired NK1.1. Contrary to general belief, it is unclear whether all mature NK cells in C57 mice express NK1.1, and whether NK1.1 is invariably the first NK-specific receptor acquired during development. Indeed, recent studies have indicated that developing NK cells may acquire Ly49A prior to NK1.1 [17]. The potential for independent regulation of the expression of NK receptors was further revealed in the present study through the finding that IL-4 could block the acquisition of CD94, NKG2, and Ly49 receptors but not NK1.1 during NK cell development *in vitro*. Similarly, IL-4 could reverse the expression of CD94, NKG2, and Ly49, but not NK1.1, on cells that had recently acquired these receptors. Preliminary results indicate that IL-4 is unable to down-regulate the expression of the NKRP1A and/or NKRP1B molecules recognized by the mAb 10A7, indicating that IL-4 is a selective regulator of different classes of NK cell receptors encoded in the NK complex. On more mature cells the down-regulation of receptor expression required more extended exposure to IL-4, and became more selective for CD94 and NKG2, with now little effect on the expression of either NK1.1 or Ly49 molecules. Whether IL-4 can exert similar effects *in vivo*, and whether its effects *in vitro* are, like those of IL-2, due to its being able to substitute for another cytokine present in the developmental environment *in vivo* are important questions that remain to be answered.

4 Materials and methods

4.1 Culture media and reagents

Cells were cultured in a 10% CO_2 atmosphere at 37°C in DMEM (52100-039; Life Technologies, Paisley, GB) made up in highly purified water and supplemented with 2× nonessential amino acids, 5×10^{-5} M 2-ME, and 10% FBS (F-7524; Sigma, Poole, GB). Human rIL-2 was obtained from Cetus (Emeryville, CA). Mouse rIL-4 was obtained as the supernatant of X6310 cells transfected with the mouse IL-4 gene [18], kindly provided by Dr. F. Melchers (Basel Institute for Immunology, Switzerland).

4.2 Generation of NK cells

Adult NK cells were prepared from C57BL/6 mice as described previously [19]. The culture system that supports the development of NK cells from fetal thymic progenitors has been described in detail elsewhere [1, 20]. Briefly, thymocytes were prepared from the day 14 embryos of timed-mated C57BL/6 mice (day of vaginal plug = day 0), cultured for 2 days in medium containing 10 U/ml IL-4 and 10 ng/ml PMA, then transferred to medium containing 10^4 U/ml IL-2.

4.3 Immunofluorescence and flow cytometry

The following mAb were used. YE1/48 that recognizes Ly49A [9] was kindly provided by Dr. F. Takei (University of British Columbia, Vancouver, Canada). JR9 that recognizes Ly49A [21] was kindly provided by Dr. J. Roland (Pasteur Institute, Paris). 4LO3311 that recognizes Ly49C [22] was kindly provided by Dr. S. Lemieux (University of Quebec, Laval, Canada). Cwy3 that recognizes Ly49G [23] was kindly provided by Dr. K. Kane (University of Alberta, Edmonton, Canada). 4D11 that recognizes Ly49G [24] was kindly provided by Drs. L. Mason and J. Ortaldo (National Cancer Institute, Frederick, MD). 3D10 that recognizes Ly49H [25] was kindly provided by Drs. H. Smith and W. Yokoyama (Washington University School of Medicine, St. Louis, MI). The SED85 mAb that recognizes Ly49D, the HBF mAb that recognizes Ly49F, the YLI mAb that recognizes Ly49I, and the 14B11 mAb that recognizes Ly49C, F, I, and H have been described previously [7], as has the 4D12 mAb that recognizes Ly49C and E [6]. The CD94 and NKG2 mAb used were 18d3 anti-CD94, 20d5 anti-NKG2, and 3S9 anti-NKG2 that have been described previously [6, 26]. Antibodies were used either unconjugated followed by staining with FITC- or PE-conjugated secondary antibodies, or directly following conjugation to FITC or biotin. Biotinylated $Qa1^b$-Qdm tetramers refolded using human β2-microglobulin were obtained from the NIH tetramer facility [27]. FITC- and biotin-conjugated antibodies to NK1.1, and PE-conjugated antibodies to TCRβ and TCRδ chains were obtained from PharMingen (San Diego, CA). All biotinylated reagents were stained with Red670-streptavidin (19543–024; Life Technologies).

Aliquots of $\sim 2\times10^5$ cells were incubated with appropriate combinations of reagents in Hanks' BSS supplemented with 2% FBS and 0.2% sodium azide at room temperature, except for staining with the Qa1 tetramers that requires incubation at 37°C [28]. Staining was analyzed on a FACScan (Becton Dickinson, San Jose, CA), using forward and side scatter to gate on single viable cells. Compensation for spectral overlap of dyes was set by running mixtures of unstained cells and cells stained with each fluorochrome singly. To permit comparison between the levels of staining in different experiments, the same reagent stocks and FACScan acquisition parameters were used throughout. Consistency was confirmed by the finding that the median fluorescence level of control beads (FluoroSpheres; Dako, Glostrup, Denmark) did not vary by more than 10% between experiments. Data were collected using Lysis II software (Becton Dickinson), converted to PC format using Lifutil, analyzed using FCS Express software [29], and compiled in Microsoft Excel.

4.4 Transfection

CHO cells were co-transfected with Ly49 cDNA cloned into the pME-18S vector together with the pSV2-neo plasmid and Lipofectamine (18324-012; Life Technologies) according to the manufacturer's instructions. Transfected cells were selected with G418 (Novagen, Madison, WI) at a nominal 1 mg/ml, and cloned by limiting dilution.

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