



Reduced release and binding of perforin at the immunological synapse underlies the age-related decline in natural killer cell cytotoxicity

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Summary

Physiological aging is accompanied by a marked reduction in natural killer (NK) cell cytotoxicity (NKCC) at the single cell level, but the underlying mechanisms are unknown. To address this issue, we isolated NK cells from healthy young (≤ 35 years) and old (≤ 60 years) subjects and examined the effect of age on events fundamental to the process of NKCC. Simultaneous assessment of NKCC and NK cell–target cell conjugate formation revealed a marked age-associated decline in NK cell killing but comparable conjugate formation, indicating a post-target cell binding defect was responsible for impaired NKCC. Despite a reduction in the proportion of NK cells expressing the activatory receptor NKp46, NK cells from old donors were not hyporesponsive to stimulation, as no age-associated difference was observed in the expression of the early activation marker CD69 following target cell coculture. Furthermore, intracellular levels of the key cytotoxic effector molecules perforin and granzyme B, and the fusion of secretory lysosomes with the NK cell membrane were also similar between the two groups. However, when we examined the binding of the pore-forming protein perforin to the surface of its target cell, an event that correlated strongly with target cell lysis, we found the percentage of perforin positive target cells was lower following coculture with NK cells from old subjects. Underlying this reduction in binding was an age-associated impairment in perforin secretion, which was associated with defective polarization of lytic granules towards the immunological synapse. We propose that reduced perforin secretion underlies the reduction in NKCC that accompanies physiological aging.

Key words: aging; apoptosis; cell death; cellular immunology; human.

Introduction

Physiological aging is accompanied by marked alterations in immune function, a phenomenon termed immunosenescence. Observed in both the innate and adaptive arms of the immune system, these changes are thought to contribute in part to the increased incidence and the severity of infection reported by older adults (Gavazzi & Krause, 2002). Adaptive immunity has been the primary focus of immunogerontological studies (Aw *et al.*, 2007), and consequently, adaptive immunosenescence was

thought until recently to be primarily responsible for the increased susceptibility of older individuals to infection. However, evidence is now accumulating to indicate that the innate arm of the immune system is also subject to considerable age-related modification (Gomez *et al.*, 2008; Panda *et al.*, 2009).

Characterized phenotypically as CD3⁺CD56⁺, natural killer (NK) cells are a heterogeneous subset of innate lymphocytes that offer front-line protection against virus-infected, stressed and malignant cells. On the basis of the differential surface expression of CD56, NK cells are divided into one of the two major subsets: CD56^{DIM} or CD56^{BRIGHT} (Cooper *et al.*, 2001a). CD56^{DIM} NK cells are primarily responsible for the direct elimination of virus-infected and transformed cells, whilst CD56^{BRIGHT} cells are the principal source of NK cell–derived immunoregulatory cytokines (e.g. IFN γ , TNF α) and chemokines [e.g. macrophage inflammatory protein 1 alpha (MIP-1 α), MIP-1 β] (Cooper *et al.*, 2001b; Jacobs *et al.*, 2001). Regulating the induction of NK cell cytotoxic activity is an array of surface-expressed germline-encoded activatory and inhibitory receptors (Lanier, 2005).

Studies to date that have investigated the effect of age on NK cell function have reported conflicting observations, which arise primarily from interstudy differences in subject inclusion criteria and methodological approaches. Nevertheless, the general consensus is that aging is accompanied by marked alterations in NK cell numbers, phenotype and function (Solana *et al.*, 1999; Mocchegiani & Malavolta, 2004). Age-related increases in the number and/or proportions of CD56^{DIM} NK cells have been frequently documented (Krishnaraj, 1997; Almeida-Oliveira *et al.*, 2011; Lutz *et al.*, 2011), as has a decrease in NK cell cytotoxicity (NKCC) at the single cell level (Facchini *et al.*, 1987; Mariani *et al.*, 1990; Miyaji *et al.*, 1997). For CD56^{BRIGHT} NK cells, their number and/or proportions decrease with age (Krishnaraj, 1997; Le Garff-Tavernier *et al.*, 2010; Almeida-Oliveira *et al.*, 2011), as does their capacity to produce cytokines and chemokines upon stimulation (Krishnaraj & Bhooma, 1996; Mariani *et al.*, 2002). Importantly, in a prospective study, Ogata *et al.* (2001) demonstrated low NKCC to be associated with an increased susceptibility to infection and death because of infection in a cohort of older subjects.

Given the clinical significance of these data, it is surprising that no study to date has identified the mechanism(s) behind the decline in NKCC that accompanies physiological aging. For example, whilst age-associated changes in the surface expression of NK activatory and inhibitory receptors have been reported (Le Garff-Tavernier *et al.*, 2010; Almeida-Oliveira *et al.*, 2011), results are often inconsistent, and the changes observed are not always accompanied by a concomitant decline in NKCC (Le Garff-Tavernier *et al.*, 2010; Almeida-Oliveira *et al.*, 2011). In addition, although Mariani *et al.* (1998) observed an age-related delay in NK cell phosphoinositide signalling following target cell recognition, only one study has reported a decrease in NK degranulation with age (Le Garff-Tavernier *et al.*, 2010), which is surprising given the importance of calcium signalling in this process. Thus, in an effort to elucidate the underlying cause(s) of the age-associated reduction in NKCC, we isolated NK cells from healthy young and old donors and examined the effect of age on each of the events fundamental to the process of NKCC.

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Accepted for publication 15 May 2012

Results

Age-related alterations in the composition of the circulating NK cell pool and NKCC

Immunostaining of peripheral blood mononuclear cells (PBMCs) and multicolour flow cytometry confirmed the age-associated changes in NK cell proportions and subset distribution previously reported in the literature. Compared with young adult subjects, older individuals had greater proportions of both CD3⁺56⁺ and CD3⁺56⁺57⁺ NK cells within their lymphocyte pool, which was accompanied by a decline in the percentage of CD3⁺ cells (Table 1). A differential effect of age on NK cell subsets was also observed, as older individuals presented with a significantly higher percentage of CD56^{DIM} cells but a significantly lower proportion of CD56^{BRIGHT} cells, culminating in an increased CD56^{DIM} to CD56^{BRIGHT} ratio (Table 1). Further analysis based on coexpression of CD56 and the low affinity Fc receptor CD16 revealed the accumulation of CD56^{DIM} cells to be the result of an increased proportion of CD3⁺16⁺56^{DIM} NK cells, whilst a significant decline in the percentage of CD3⁺16⁺56^{BRIGHT} cells accounted for the reduction in the CD56^{BRIGHT} subset (Table 1).

Through the use of an immunostaining and flow cytometric protocol that allowed for the simultaneous assessment of NKCC and NK cell–target conjugate formation, we found that when compared to younger controls, NK cells from older adults exhibited reduced cytotoxicity against tumour cell targets (Fig. 1A). Conjugate formation was however comparable between the two groups (Fig. 1B).

NK cell receptor expression and activation status

Natural killer cell activity is governed by signals transmitted through an array of germline-encoded activatory and inhibitory receptors (Lanier, 2005). Because reduced expression of activatory receptors had previously been shown to be associated with impaired NK cell cytotoxicity against tumour cells (Epling-Burnette *et al.*, 2007; Garcia-Iglesias *et al.*, 2009), we examined the effect of age on the surface phenotype of cytotoxic CD56^{DIM} NK cells (Table 2). Whilst no difference in either the density or percentage of NK cells positive for the activatory receptor NKG2D was found (Table 2), a marked decline in the percentage of cells positive for the natural cytotoxicity receptor Nkp46 was observed with age (young 81.95% vs. old 67.05%, *P* = 0.005). Expression of CD94, a protein that

Table 1 Age-associated changes in the circulating lymphocyte pool

	Young (<i>n</i> = 21)	Old (<i>n</i> = 21)	<i>P</i> -value
CD3 ⁺ 56 ⁺	10.60 ± 0.9	17.21 ± 1.2	0.0001
CD3 ⁺	64.44 ± 1.8	58.40 ± 1.8	0.02
CD3 ⁺ 56 ⁺ 57 ⁺	55.62 ± 2.9	66.10 ± 2.1	0.03
CD56 ^{DIM}	10.40 ± 0.9	16.75 ± 1.2	<0.0001
CD56 ^{BRIGHT}	0.56 ± 0.1	0.40 ± 0.04	0.03
CD3 ⁺ 16 ⁺ 56 ^{DIM}	85.7 ± 1.5	91.8 ± 1.0	0.001
CD3 ⁺ 16 ⁺ 56 ^{DIM}	3.8 ± 0.5	3.8 ± 0.8	0.537
CD3 ⁺ 16 ⁺ 56 ^{BRIGHT}	1.6 ± 0.2	1.5 ± 0.3	0.392
CD3 ⁺ 16 ⁺ 56 ^{BRIGHT}	8.3 ± 1.1	2.6 ± 0.4	<0.0001
CD56 ^{DIM} :BRIGHT	23:1 ± 3.54	51:1 ± 5.72	0.0001

Values are presented as mean ± standard error of mean (SEM).
†Data for CD16 and CD57 expression reflect the proportion of positively stained cells within the natural killer cell pool. All other data relate to the percentage of positive cells within the lymphocyte pool. Significant differences are indicated in bold font.

associates with both activatory and inhibitory receptors of the C-type lectin family was also altered with age. Compared with young controls, both the proportion of NK cells expressing CD94 and the number of molecules expressed on the surface of each cell was lower in the peripheral pool of older donors, a pattern that was also observed for the terminal oligosaccharide CD57 (Table 2).

To determine the significance of these changes in surface phenotype, we examined the activation status of NK cells following a 4 h coculture with K562 target cells, by measuring surface expression of CD69, a marker of early activation. We observed no difference with age in either the percentage of CD69 positive cells at baseline (Fig. 2A) or in the up-regulation of this activation marker postculture (Fig. 2B).

NK cell degranulation and cytotoxic effector molecule expression

Granule exocytosis is the predominant mechanism utilized by NK cells to eliminate their targets. Through the release of the pore-forming protein perforin and a family of serine proteases termed granzymes, NK cells induce caspase-dependent and caspase-independent target cell death (Smyth *et al.*, 2005). Measuring intracellular levels of these effector

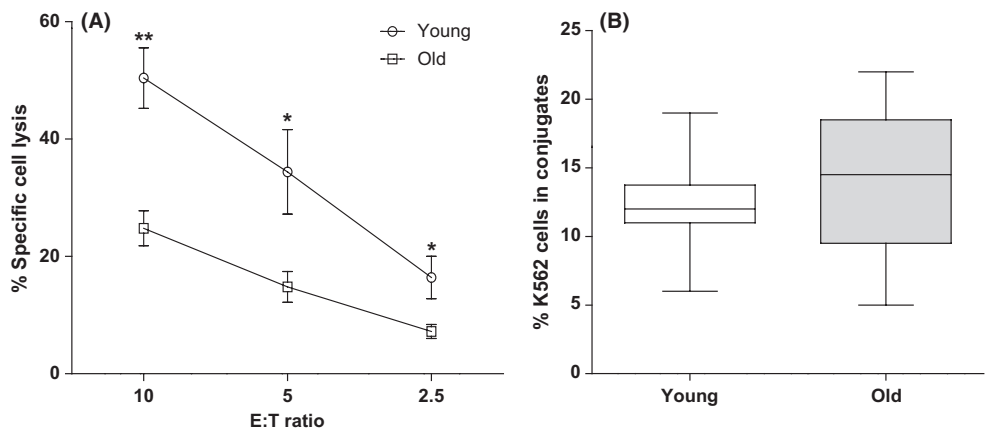


Fig. 1 Effect of age on NKCC and conjugate formation. (A) Cytotoxicity of resting natural killer (NK) cells towards K562 cells over a range of effector to target (E:T) ratios in young and old donors (*n* = 5). Data points are mean ± SD and * indicates *P* < 0.05, ***P* < 0.005; (B) the ability of NK cells to form conjugates with K562 target cells at an E:T ratio of 10:1 (*n* = 12). Horizontal bar within box and whisker plots depicts the median value. NKCC, natural killer cell cytotoxicity.

Table 2 Natural killer (NK) cell receptor expression

	Percentage positive cells			Surface density (MFI)		
	Young	Old	<i>P</i>	Young	Old	<i>P</i>
NKG2D	92.10 ± 1.5	91.71 ± 1.2	0.512	42.86 ± 2.7	39.62 ± 2.8	0.406
NKp46	81.95 ± 1.9	67.05 ± 4.6	0.005	10.57 ± 0.7	8.52 ± 1.2	0.148
CD16	96.71 ± 0.4	96.76 ± 0.6	0.496	146.3 ± 9.3	120.2 ± 11.1	0.080
CD94	62.29 ± 3.8	43.52 ± 2.9	0.0003	30.05 ± 5.1	14.19 ± 2.4	0.006
CD57	61.52 ± 2.3	70.05 ± 1.9	0.006	137.9 ± 43.8	214.0 ± 35.6	0.014

Expression of activatory and inhibitory receptors on the surface of CD3⁺CD56^{Dim} NK cells isolated from young (*n* = 21) and old (*n* = 21) donors. Values are presented as mean ± standard error of mean (SEM). Significant differences are indicated in bold font. MFI, median fluorescence intensity.

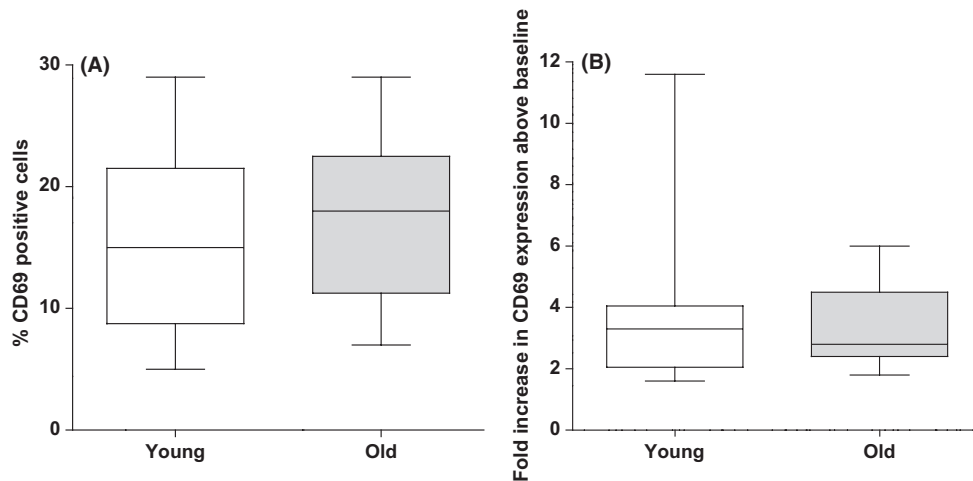


Fig. 2 Natural killer (NK) cell activation status. Surface expression of the activation marker CD69 was examined on isolated CD3⁺CD56⁺ NK cells obtained from young (*n* = 14) and old (*n* = 13) subjects at rest (A) and following a 4-h coculture with K562 target cells (B). Horizontal bar within box and whisker plots depicts the median value.

proteins in resting NK cells revealed no differences with age in either the percentage of cells positive for these molecules (data not shown) or in the level of expression of these proteins (Fig. 3A), suggesting that NK cells from older subjects are armed to a similar degree with the key cytotoxic proteins needed to induce target cell lysis. Moreover, no age-related impairment was observed in the fusion of secretory lysosomes with the NK cell membrane, as expression of CD107a, a lysosomal protein whose surface expression is considered a sensitive marker of NK cell degranulation (Alter *et al.*, 2004), was comparable on NK cells obtained from young and old donors (Fig. 3B).

Age-associated reduction in perforin binding to the target cell surface

Binding of perforin to the target cell plasma membrane is a fundamental step in granzyme-mediated apoptosis. As our data had suggested age to have no effect on perforin expression or fusion between perforin containing secretory lysosomes and the membrane of NK cells, we questioned whether the decline in NK cell killing, which was observed with age, was attributable to reduced perforin binding to the tumour cell surface. Using a FITC-conjugated antiperforin antibody, we found the percentage of K562 target cells exhibiting positive FITC fluorescence after a 4-h coculture with resting NK cells was significantly lower when effector cells were obtained from old individuals (Fig. 4A). To determine whether this reduction was related to impaired NK cell killing, perforin binding and NK

cytotoxicity assays were performed simultaneously. As shown in Fig. 4B, we found perforin binding to the surface of K562 cells correlated strongly with target cell death.

Impaired secretion of perforin into the immunological synapse (IS) by NK cells from old donors

In an effort to unearth the mechanism behind the reduced percentage of perforin positive K562 cells found in cocultures that contained NK cells from older donors, we hypothesized that despite age having no effect on perforin expression or secretory lysosome fusion, NK cells from older adults released less perforin into the IS. To address this proposal, we measured the concentration of perforin in supernatants by enzyme-linked immunosorbent assay (ELISA) post-4-h NK cell–K562 cell coculture and found the levels present to be significantly lower when NK cells had been isolated from aged donors (Fig. 5A). To further confirm reduced perforin release at the IS, we immunostained NK cell–K562 conjugates for perforin. Immunofluorescence imaging of NK cells confirmed similar levels and punctate distribution of perforin in NK cells from young and old donors (Fig. 5B, upper panels). The images also show the well-documented autofluorescence of K562 cells (Fig. 5B, bottom left panel), but also revealed a pronounced age-related impairment in perforin mobilization to the NK–K562 contact point (Fig. 5B), suggesting that defective polarization to the IS underlies the reduction in perforin secretion we observed with age.

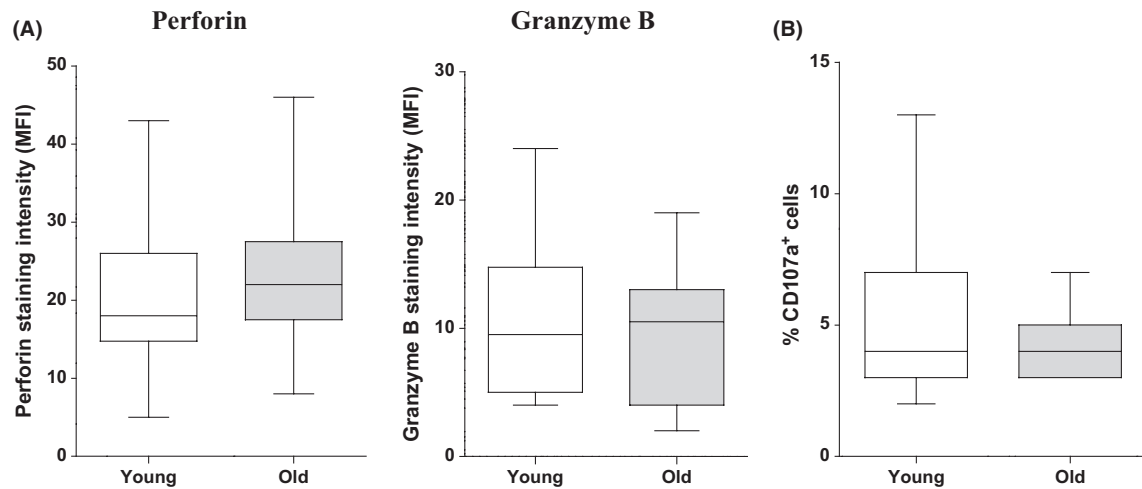


Fig. 3 Effect of age on the granule exocytosis pathway. (A) Intracellular expression of the cytotoxic effector molecules perforin and granzyme B was studied in resting natural killer (NK) cells obtained from 10 young and 10 old donors by flow cytometry. (B) NK cell degranulation was assessed by surface CD107a expression after a 4-h coculture of isolated NK cells and K562 target cells at an E:T ratio of 10:1 ($n = 11$). Horizontal bar within box and whisker plots depicts the median value.

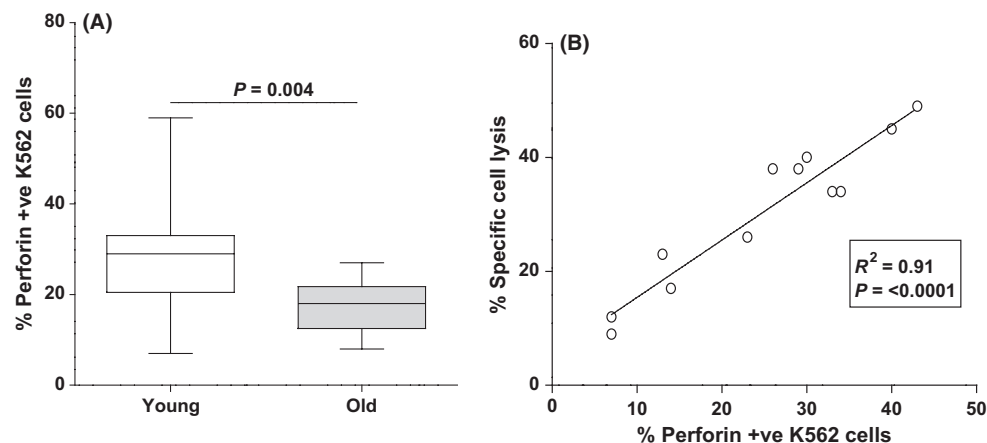


Fig. 4 Age-related reduction in the binding of perforin to the surface of target cells. Natural killer (NK) cells isolated from young and old subjects were cocultured for 4 h (E:T, 10:1) with K562 cells, after which, perforin binding to the target cell surface was assessed by flow cytometry. (A) Percentage of perforin positive K562 cells (young, $n = 16$; old, $n = 16$). (B) Correlation between perforin binding and NK cell cytotoxicity in young individuals ($n = 12$). Horizontal bar within box and whisker plots depicts the median value.

Discussion

Decreased killing at the single cell level is a well-documented feature of NK cell immunosenescence. As the front-line defence against virally infected and malignant cells, this impairment in NK cell function is thought to contribute in part to the increased incidence of infectious disease and cancer reported by older adults (Gavazzi & Krause, 2002). Yet, despite a number of *in vitro* studies reporting defective NK cell-mediated killing (Facchini *et al.*, 1987; Mariani *et al.*, 1990; Miyaji *et al.*, 1997), our understanding of the mechanisms that underlie this impairment is at best equivocal. Here, in addition to reporting marked alterations in the composition of the peripheral NK cell pool with age, we show for the first time that NK cells from older adults exhibited impaired perforin secretion upon target cell contact. This defect led to decreased perforin binding to the surface of target cells and a reduction in NKCC. Thus, we propose defective perforin secretion in to the IS mediates the reduction in NKCC that accompanies physiological aging.

In agreement with the previous studies (Almeida-Oliveira *et al.*, 2011; Lutz *et al.*, 2011; Miyaji *et al.*, 1997), we found the peripheral lymphocyte pool of older individuals contained a greater proportion of both CD3⁺56⁺ and CD3⁺56⁺57⁺ NK cells when compared to younger adults. As a marker of NK cell maturity, CD57 is found predominantly on the surface of NK cells belonging to the more mature CD56^{DIM} subset, where its expression is associated with distinct functional behaviour (Lopez-Verges *et al.*, 2010). For example, CD56^{DIM} CD57⁺ NK cells produce lower levels of IFN- γ and undergo fewer rounds of cell division following cytokine and/or target cell stimulation when compared to their negative counterparts (Lopez-Verges *et al.*, 2010). Because these features of CD57⁺ NK cells mirror previously described defects in NK cells isolated from aged individuals (Krishnaraj & Bhooma, 1996; Krishnaraj, 1997; Borrego *et al.*, 1999), an accumulation of this subset may contribute to the functional deterioration in NK cell function that is observed with age. Supporting this are the results of the previous studies that have demonstrated an age-associated increase in CD56^{DIM} CD57⁺ NK cells with age (Lutz *et al.*,

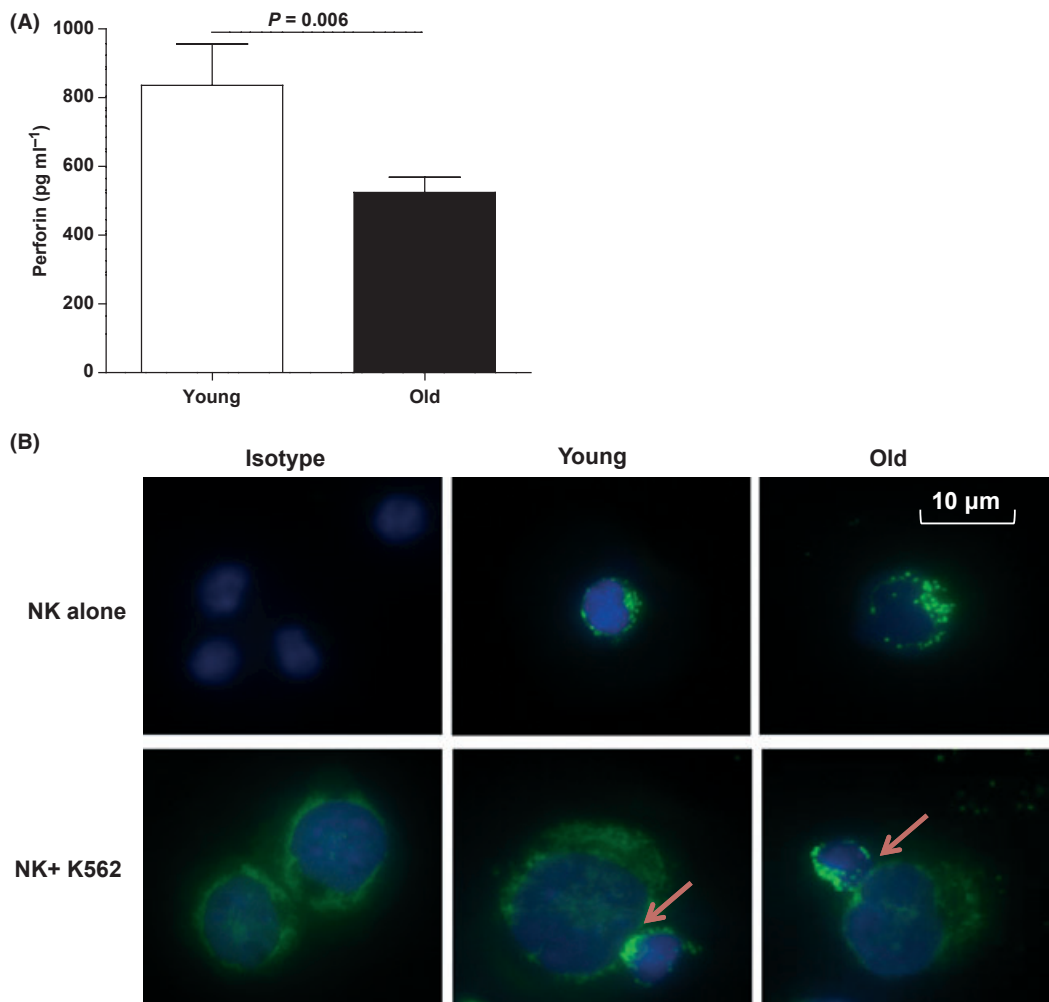


Fig. 5 Impaired secretion of perforin into the immunological synapse (IS) by natural killer (NK) cells from older adults. (A) natural killer (NK) cells isolated from young ($n = 13$) and old ($n = 15$) subjects were cocultured for 4 h (E:T, 10:1) with K562 cells, after which, the concentration of perforin in cell-free supernatants was assessed by ELISA. Data are presented as mean \pm SEM. (B) NK cell:K562 cell conjugates prepared from young and old donors were immunostained after a 30 min coculture to examine perforin localization (FITC-conjugated antibody) at the IS. Nuclei were counterstained with DAPI. Fluorescence seen in the K562 cells is autofluorescence. The confocal image is representative of five separate experiments performed.

2011), a finding confirmed in this report. Whilst less responsive to stimulation by cytokines and target cells, CD57⁺ NK cells exhibit greater lytic activity after stimulation through the low affinity Fc receptor CD16 (Lopez-Verges *et al.*, 2010). Even with a greater proportion of CD56^{DIM}CD57⁺ NK cells in their peripheral pool, older subjects exhibit a level of antibody-dependent cell cytotoxicity (ADCC) that is comparable with younger individuals (Fernandes & Gupta, 1981), suggesting that similar to natural cytotoxicity, ADCC may also be impaired at the single cell level.

On the basis of the differential surface expression of CD56, NK cells are divided into one of the two major subsets: CD56^{DIM} or CD56^{BRIGHT} (Cooper *et al.*, 2001a). We found that with age, the percentage of CD56^{DIM} NK cells increased whilst the percentage of CD56^{BRIGHT} decreased. These observations are in line with the previous studies (Krishnaraj, 1997; Le Garff-Tavernier *et al.*, 2010; Almeida-Oliveira *et al.*, 2011; Lutz *et al.*, 2011) and culminated in a significantly higher CD56^{DIM} to CD56^{BRIGHT} ratio in older subjects. More detailed examination of NK subsets based on the coexpression of CD56 and CD16 revealed the increase in CD56^{DIM} cells with age was attributable to an accumulation of

CD56^{DIM}CD16⁺ NK cells, whilst the loss of CD56^{BRIGHT} cells reflected a decline in the CD56^{BRIGHT}CD16⁻ subset. An abundance of CD56^{DIM} NK cells in the periphery may serve to compensate for the decrease in NKCC that is observed with age at the single cell level (Facchini *et al.*, 1987; Mariani *et al.*, 1990; Miyaji *et al.*, 1997). The significance of an age-related decline in blood CD56^{BRIGHT} cells is however unclear. Until recently, this subset was considered to be the sole source of NK cell-derived immunoregulatory cytokines and chemokines, and thus, an age-related decline in CD56^{BRIGHT} cells would be expected to impact upon the capacity of NK cells to amplify an ongoing immune response. However, recent studies have shown that in addition to mediating potent cytotoxicity against transformed cells, CD56^{DIM} NK cells upon activation rapidly produce cytokines (e.g. IFN- γ , TNF- α) and chemokines (e.g. MIP1 α/β) at a level greater than that reported for the CD56^{BRIGHT} subset (Fauriat *et al.*, 2010; Juelke *et al.*, 2010; De *et al.*, 2011). Thus, CD56^{DIM} NK cells could amplify inflammatory responses and activate/recruit other immune cells, a role previously thought to be exclusive to the CD56^{BRIGHT} subset. Whether cytokine and chemokine production by CD56^{DIM} NK cells is reduced with age is currently unknown.

In accordance with the previous studies (Facchini *et al.*, 1987; Mariani *et al.*, 1990; Miyaji *et al.*, 1997), we found aging to be associated with a reduction in resting NKCC at the single cell level. Simultaneous assessment of conjugate formation revealed this step in NK-mediated cell death was comparable between young and older subjects, suggesting that a postbinding defect is responsible for the age-related deterioration in NK cell killing. NK cell activity is regulated by signals transmitted through a multitude of germline-encoded activatory and inhibitory receptors (Lanier, 2005). Activatory receptors, which recognize both viral and self-proteins, include the natural cytotoxicity receptors NKp30, NKp44 and NKp46 and the C-type lectin family member NKG2D. Upon ligand recognition, these receptors initiate diverse signalling pathways, which, provided they overcome signals derived from inhibitory receptors, result in NK cell activation (MacFarlane & Campbell, 2006). Reduced expression of activatory receptors has been shown to be associated with impaired NKCC against K562 target cells (Epling-Burnette *et al.*, 2007; Garcia-Iglesias *et al.*, 2009). We therefore investigated whether alterations in the surface phenotype of cytotoxic CD56^{DIM} NK cells could account for the age-related decline in NKCC. In support of previous work, we found no change in the expression of NKG2D with age, but observed a marked decline in the proportion of NK cells expressing NKp46 (Le Garff-Tavernier *et al.*, 2010; Almeida-Oliveira *et al.*, 2011). Nevertheless, it is unlikely that this deficiency is responsible for the impaired NK killing we noted with age as blocking NKp46 signalling has been shown to have no effect upon lysis of K562 cells by resting NK cells (Epling-Burnette *et al.*, 2007). However, as a receptor capable of recognizing viral hemagglutinin (Mandelboim *et al.*, 2001), reduced expression of NKp46 may contribute to the increased incidence of influenza infection in older individuals, a major cause of morbidity and mortality in this group. Further confirming that changes in activatory receptor expression were not responsible for the decline in NKCC was the comparable levels of the early activation marker CD69 we observed on the surface of NK cells from young and older subjects following target cell contact. As reported by others (Hayhoe *et al.*, 2010; Almeida-Oliveira *et al.*, 2011), we also noted a marked reduction in the expression of CD94, a protein that associates with both activatory (CD94-NKG2C/E/H) and inhibitory (CD94-NKG2A/B) receptors of the C-type lectin family (Iwaszko & Bogunia-Kubik, 2011). Recently, Hayhoe *et al.* (2010) proposed a decline in CD94 would lead to unregulated NK cell lysis, the result presumably of reduced CD94-NKG2A/B expression. However, given the fact that the inhibitory receptor CD94-NKG2A has a greater affinity than CD94-NKG2C for their shared ligand, the non-classical MHC class I molecule HLA-E (Vales-Gomez *et al.*, 1999; Iwaszko & Bogunia-Kubik, 2011), and can override the activatory signals emanating from this complex (Beziat *et al.*, 2011), we feel that reduced CD94 expression would actually lead to more stringent regulation of NK cell activity and possibly a reduction in NK cell killing.

Granule exocytosis is the predominant mechanism utilized by NK cells to eliminate their targets. Through the release of the pore-forming protein perforin and a family of serine proteases termed granzymes, NK cells rapidly induce both caspase-dependent and caspase-independent target cell death (Smyth *et al.*, 2005). We found no difference with age in the expression levels of either perforin or granzyme B, suggesting that NK cells from older adults are suitably armed with the effector molecules needed to induce target cell lysis. Similar data for perforin expression were reported previously (Mariani *et al.*, 1996), although one group has reported reduced perforin expression with aging (Rukavina *et al.*, 1998). However, in the latter study, the group of old subjects was small ($n = 7$), and they were all care home residents, suggesting they may be a more frail elderly grouping. Similar expression of the degranulation marker CD107a was also observed on NK cells from young and older adults

post-target cell contact suggesting that there is no age-related defect in secretory lysosome fusion with the NK cell membrane. This finding is in agreement with one study (Hayhoe *et al.*, 2010) but contrasts that of another (Le Garff-Tavernier *et al.*, 2010), which reported an age-related reduction in CD107a expression following target cell stimulation. This impairment was observed when comparing young donors to those considered very old (≥ 80 years). Our older cohort contained few individuals meeting this criterion, which may explain these discordant findings.

Currently, there are two schools of thought as to how perforin mediates granzyme entry into the target cell cytoplasm. The first proposes this pore-forming protein works directly upon the plasma membrane of target cells, acting as a conduit through which granzymes passively diffuse (Stewart *et al.*, 2012). The second suggests that following interaction with the target cell surface, perforin and granzyme are coendocytosed, and that perforin facilitates granzyme entry to the cytoplasm by lysing endosomal membranes (Thiery *et al.*, 2010, 2011). Whilst it is currently unclear which model is correct, an event critical to both is the interaction of perforin with the target cell membrane. In this report, we have shown that the percentage of K562 cells with perforin bound on their surface was markedly lower when these cells were cocultured with NK cells from older donors. Critically, perforin binding correlated strongly with target cell death, suggesting that reduced perforin binding contributes to the age-related decline we and others have observed in NK cell killing. Underlying this reduction in perforin binding was reduced availability. ELISA analysis revealed the concentration of perforin to be markedly lower in supernatants harvested from NK-K562 cocultures that contained NK cells from aged individuals, which was found to be associated with an age-associated impairment in perforin mobilization to the IS. To our knowledge, we are the first group that has directly examined the effect of human aging on the polarization and release of perforin by NK cells following target cell stimulation. Other groups have favoured the CD107a assay that measures secretory lysosome fusion with the NK cell membrane rather than studying the outcome of this interaction, which may explain why impaired perforin secretion by aged NK cells has not been described previously.

An area of future study arising from this work is to address the reason(s) why NK cells from older adults secrete less perforin upon target cell contact than those of younger individuals. The impaired mobilization of perforin towards the IS that we observed in NK cells from aged donors points to defective intracellular signalling. The activation of mitogen-activated protein kinases (MAPK) is critical for lytic granule polarization, granule exocytosis and NKCC (Trotta *et al.*, 1998; Li *et al.*, 2008), and it is possible that these proximal signalling events are compromised by aging.

Experimental procedures

Subjects

In total, 67 young (mean age, 26.9 ± 0.6 years; range, 20–35 years) and 98 old (mean age, 72.2 ± 0.7 years; range, 61–91 years) volunteers were enrolled in this study, which was approved by the local research ethics committee. Blood samples were collected after obtaining written informed consent from donors, who at the time of participation were in good health, free of immunological illness and significant comorbidity and not taking any medication known to interfere with immunity.

Cell isolation and culture

Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation and resuspended in complete media (RPMI 1640

with 2 mM L-glutamine, 100 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin (Sigma-Aldrich, Dorset, UK) supplemented with 10% (vol/vol) heat-inactivated foetal calf serum (Sera Laboratories International, Sussex, UK) at a concentration of $1-2 \times 10^6$ per mL for functional and phenotypic analysis. NK cells were obtained from PBMC samples by negative selection using MACS[®] technology (Human NK Cell Isolation Kit; Miltenyi Biotec, Gladbach, Germany) and resuspended in complete media at 1×10^6 per mL. Routinely, NK cell purity was $\geq 96\%$. The MHC class I deficient cell line K562 (American Type Culture Collection, Middlesex, UK) was used as the target population in cytotoxicity assays. Cells were maintained in complete media at 37 °C in a humidified 5% CO₂ atmosphere and washed once in PBS prior to use.

Phenotypic analysis

Natural killer cell phenotyping was carried out using immunostaining and multicolour flow cytometry, performed using a CyAn_{ADP}[™] bench top cytometer (Dako, Cambridgeshire, UK) with data analysed using SUMMIT v4.3 software (Dako, Fort Collins, CO, USA). Protocols were established using isotype controls and compensated electronically using cells stained with individual fluorochromes. For PBMC staining, lymphocytes were gated and NK cells identified as CD3⁻CD56⁺. PBMCs (2×10^5 in PBS/1%BSA) were incubated on ice for 20 min with the combinations of the following fluorochrome-conjugated mouse monoclonal antibodies or their relevant concentration-matched isotype controls: CD3-FITC (Clone UCHT1; Dako) CD3-PcB (Clone UCHT1; BD Biosciences, Oxford, UK) CD16-FITC (Clone DJ130c; Dako), CD56-PE (Clone C5.9; Dako), CD57-FITC (Clone HCD57; BioLegend, Cambridge, UK), CD94-FITC (Clone DX22; eBioscience, Hatfield, UK), NKG2D-PECy7 (Clone 1D11; BioLegend), Nkp46-PcB (Clone 9E2; BioLegend). Postincubation, samples were washed once in PBS/1%BSA, resuspended in 100 µL PBS and transferred to cytometric tubes for analysis. NK cell percentages were calculated after acquiring 15 000 events within the lymphocyte gate, whilst activatory and inhibitory receptor expression was analysed on 5000 CD56^{DIM} NK cells. Data are presented either as the percentage of antigen positive cells or as median fluorescent intensity (MFI), after subtracting the MFI values of samples stained with isotype controls.

Intracellular staining

After a single wash in PBS, isolated NK cells (2×10^5) were fixed for 30 min at room temperature in 50-µL fixation medium (Invitrogen, Paisley, UK). Cells were then washed once in PBS, resuspended in 50 µL of permeabilization medium (Invitrogen) containing either 10 µg mL⁻¹ anti-perforin-PE (Clone δG9; Ancell, Bayport, MN, USA), 20 µL of antigranzyme B-FITC (Clone GB11; BioLegend Europe) or concentration-matched isotype controls and incubated in the dark for 30 min at room temperature. Poststaining, cells were washed once in PBS, transferred to cytometric tubes and analysed by flow cytometry, where both the percentage of positive cells and MFI values for 10 000 NK cells were recorded.

Functional analyses

NK cytolytic activity and assessment of conjugate formation

Natural killer cell cytotoxicity and conjugate formation was assessed simultaneously by two-colour flow cytometry, using an adapted version of the protocol described by Godoy-Ramirez *et al.* (2000). Briefly, NK cells and K562 target cells were incubated at a range of effector/target (E:T) cell ratios (10:1; 5:1; 2.5:1) for 4 h at 37 °C in a humidified 5% CO₂

atmosphere. Postincubation, cells were pelleted and resuspended in PBS/1%BSA containing 0.3 µg anti-CD56-PE. After 20 min of labelling on ice, cells were washed once in PBS/1%BSA, stained for 5 min with 125 nM of sytox[®] blue dead cell stain (Invitrogen) and transferred into flow cytometric tubes for analysis. To determine NKCC, the number of lysed K562 target cells (defined as sytox blue positive) within a total population of 2000 was recorded and percentage-specific lysis calculated as follows: $(TL - SL/2000) \times 100$, where TL is the number of lysed target cells in NK:K562 cell cocultures, and SL is the number of lysed targets in the absence of effector cells. From these data, an E:T ratio of 10:1 was selected for subsequent assays. To assess conjugate formation, conjugates were defined as K562 cells exhibiting positive PE fluorescence. The number of these conjugates within a total K562 population of 2000 was recorded.

Assessment of NK cell activation by CD69 staining

Freshly isolated NK cells (1×10^6 per mL) were incubated for 4 h at 37 °C in the absence or presence of K562 cells at an E:T of 10:1. Postincubation, samples were pelleted and resuspended in PBS/1%BSA containing 0.3 µg mL⁻¹ anti-CD56-PE and either 10 µg mL⁻¹ FITC-conjugated anti-CD69 (Clone L78; BD Biosciences) or its concentration-matched isotype control. Following 20-min staining on ice, cells were washed once in PBS/1%BSA, resuspended in 100 µL PBS and transferred to cytometric tubes. For analysis, 15 000 NK cells were acquired and the percentage expressing CD69 recorded.

Assessment of secretory lysosome fusion with the NK cell membrane

Freshly isolated NK cells (1×10^6 per mL) were incubated for 1 h at 37 °C either alone or with K562 target cells (1×10^5 per mL) in the presence of 1.25 µg mL⁻¹ FITC-conjugated CD107a antibody (clone eBIOH4A3; eBioscience) or isotype control. Postincubation, 6 µg mL⁻¹ of monensin (diluted in 100% ethanol; Sigma-Aldrich) was added and samples incubated for an additional 3 h, after which, cells were pelleted and stained on ice for 20 min in PBS/1%BSA containing 0.3 µg mL⁻¹ anti-CD56-PE. After a single wash in PBS/1%BSA, CD107a expression was examined using flow cytometry. A total of 10 000 NK cells were gated and the percentage displaying positive FITC fluorescence recorded. CD107a expression on NK cells cultured alone was considered to reflect spontaneous degranulation, and therefore, the value was subtracted from the test sample.

Detection of perforin binding to K562 cells

Perforin binding to the surface of K562 target cells was assessed using a modified version of a previously described protocol (Lehmann *et al.*, 2000). Following a 4-h coculture of NK and K562 cells at an E:T ratio of 10:1, samples were pelleted and stained in PBS/1%BSA containing 0.3 µg anti-CD56-PE and either 5 µg mL⁻¹ of antiperforin-FITC (Clone dG9; BioLegend) or its concentration-matched isotype control. After 20 min incubation on ice, cells were washed once in PBS/1%BSA, resuspended in 100 µL PBS and transferred to flow cytometric tubes for analysis. The percentage of K562 cells to which perforin had bound was determined by gating on PE negative K562 cells and recording the number within a total population of 2000 that displayed positive FITC fluorescence.

Assay for the detection of perforin in cell-free supernatants

Following a 4-h coculture of NK and K562 cells at an E:T ratio of 10:1, cell-free supernatants were harvested, snap-frozen in liquid nitrogen and stored at -80 °C prior to analysis. Perforin concentrations were determined using a commercially available solid-phase sandwich ELISA according to manufacturer's instructions (Abcam, Cambridge, UK).

Immunostaining for perforin at the IS

Natural killer cells (1×10^6 per mL) were incubated for 30 min at 37 °C either alone or with K562 target cells (1.5×10^5 per mL), after which, samples were centrifuged onto microscope slides (300 rpm for 5 min at RT; Shandon Cytospin 2), and fixed in cold acetone (Sigma-Aldrich) for 20 min at 4 °C. After air-drying the slides for 5 min at room temperature (RT), cells were rehydrated in PBS and permeabilized by exposure to 0.2% Triton x-100 (Sigma-Aldrich) in PBS for 3 min at RT. After 3×5 min washes in PBS, cells were incubated in a humidified chamber for 10 min at RT with 10% goat serum (Sigma-Aldrich) in PBS/2% BSA, after which, slides were subjected to a single 5-min wash in PBS. Cells were then stained in a humidified chamber for 1 h at RT with $2.5 \mu\text{g mL}^{-1}$ of a FITC-labelled antiperforin antibody or its concentration-matched isotype control. Postincubation, slides were washed three times in PBS (5 min per wash) before a 30-min treatment at RT with 100 μL of an anti-FITC goat IgG fraction Alexa Fluor® 488 conjugate (Invitrogen) that had been prediluted 1:100 from stock in PBS. Following 3×5 min washes in PBS, cells were stained for 2 min at RT with the DAPI fluorescent stain (Invitrogen), after which, slides were washed once in PBS for 5 min before cover slips were adhered using fluoromount medium (Sigma-Aldrich). Cells were visualized using a LEICA DMI 6000 B microscope and an x63 objective (Leica Microsystems, Milton Keynes, UK).

Statistics

Statistical analyses were performed using GRAPH PAD PRISM® software (GraphPad Software Ltd, La Jolla, CA, USA). Data distribution was checked using the Kolmogorov–Smirnov test. For data with a normal distribution, group differences were examined by an unpaired Student's *t*-test. For non-normally distributed data, a Mann–Whitney *U*-test was used. In both incidences, differences were considered statistically significant at $P \leq 0.05$. To assess the relationship between perforin binding and target cell lysis, a Pearson's rank correlation coefficient was performed. For box and whisker plots, the central line represents the median value, whilst the whiskers depict the minimum and maximum values.

Acknowledgments

JH is funded by a PhD studentship from the UK Biotechnology and Biological Sciences Research Council and PH is funded by Research into Ageing (AgeUK). We thank Dr E. Sapey for help with subject recruitment.

Author contributions

JH performed the experimental work, data analysis and wrote the manuscript. PH helped with subject recruitment and blood cell isolation, and JL designed the study and also contributed to data interpretation and writing the manuscript.

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