

## Characterization of NKR<sup>+</sup> T-cell subsets in human bone marrow: implications for immunosurveillance of neoplasia

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### Abstract

In addition to hematopoietic progenitors, human bone marrow contains mature T/NK lymphocytes. V $\alpha$ 24V $\beta$ 11 NKT-cells, a subset of NK receptor<sup>+</sup> (NKR<sup>+</sup>) T-cells in humans, are rare in bone marrow, suggesting the presence of other NKR<sup>+</sup> T-cells which may contribute to tumor surveillance. NKR<sup>+/-</sup> T-cells were examined in blood (PB), and bone marrow from donors (DM) and patients with active hematopoietic malignancy (PM), or in remission (PR). T-cells in PR & PM were enriched for CD56<sup>+</sup> and CD57<sup>+</sup> subsets, compared to DM. All marrow NKR<sup>+/-</sup> T-cell subsets were more activated than PB. PM and, surprisingly, PR marrow contained more activated cells than DM. CD8<sup>+</sup> cells were significantly increased in all patient marrows and there was evidence of the formation of an effector/memory pool in malignant marrow. These data suggest that NKR<sup>+</sup> T-cell enrichment in human bone marrow that has been exposed to neoplastic transformation is compatible with a role in localized tumor surveillance/eradication.

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**Keywords:** Bone marrow; Immunosurveillance; Neoplasia

### Introduction

The bone marrow is primarily regarded as a hematopoietic organ and is largely composed of stem/progenitor cells and immature cells from all hematopoietic lineages. However, it is also known to possess a significant population of mature immune cells. Around 46% of CD45<sup>+</sup> cells within the bone marrow are fully differentiated T- and NK cells, which, unlike the pre-B-cells present, are phenotypically and functionally mature [1–7]. The presence of such cells may be critically important in fighting malignancy, as both cell types are known to play a role in the attack of cancerous cells [8–10].

It is now recognized that local, specialized populations of immune cells—particularly lymphocytes—reside within tissues outside the circulatory and lymphatic systems that

experience specific immunological demands [11,12], contrasting the classical belief that immune cells were merely recruited in response to chemotactic stimuli [13]. Regional immune systems have been identified in sites where the body encounters both harmful antigens that must be rejected, harmless antigens that must be tolerated, and/or where high cell turnover increases the risk of neoplastic transformation. In gut epithelium, localized populations of T-lymphocytes with unique phenotypic and functional characteristics have been identified. They include cells that express both CD4 and CD8, or neither surface protein, as well as large proportion of  $\gamma\delta$  T-cell receptor (TCR)-positive cells [14] and T-cells expressing NK cell receptors (NKR) [15]. Similar populations have been found in human liver, where up to 50% of all T-lymphocytes co-express NKR, such as CD56 [11]. Distinct lymphocyte subsets have been described in many other sites of primary antigen encounter, including the uterus [16], skin [17] and respiratory mucosae [18].

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Although the bone marrow differs from the gut and skin in that it is not directly exposed to foreign antigen, it does share with these sites a high level of cellular turnover, resulting in a higher probability of somatic mutation and consequent cellular transformation into a malignant state. Hence, it is likely that there are local mechanisms for identifying and suppressing neoplastic cells before they can become established. Murine bone marrow contains large numbers of cells that share phenotypic and functional characteristics of both T- and NK cells [19] that have been shown to play important roles in the regulation of the tolerance/rejection of engrafted allogeneic tissue [20]. They are believed to be among the most important anti-cancer cell populations in the mouse, causing rejection of malignant cells *in vivo* [21–23]. Principal among these effector cells are the NKT cells—T-cells that express NK cell surface antigens, possess a highly restricted T-cell receptor repertoire [24,25] and can produce large quantities of polarizing cytokines, such as interferon-gamma (IFN- $\gamma$ ) and interleukin-4 (IL-4), without the need for prior antigenic stimulation [26–28]. Human studies have shown an homologous population to exist at a number of sites [25,29] which, like their murine counterparts, exist as a subset of NKR<sup>+</sup> T-cells and exert their function through recognition of the non-polymorphic MHC class I-like molecule, CD1d. However, CD1-restricted NKT-cells are not abundant anywhere in the human, and are only present in the bone marrow at levels similar to those in the periphery [30]. This may be due to the existence of several different forms of the CD1 molecule in humans (CD1a, b, c and d), compared to the single, CD1d-homologous form found in mice. The human immune system may, therefore, ‘compensate’ for any apparent lack of these important effector cells by utilizing T-cells with specificities to molecules other than CD1d, thus resulting in a broader spectrum of NKT-like cells.

With this fact in mind, the present study set out to examine human bone marrow for cells that may be involved in response to local (i.e., hematopoietic) malignancy. While conventional T- and NK cell subsets are well studied in human bone marrow, little is known about “non-conventional”, NKR<sup>+</sup> subsets in this context. Given the situation in the mouse, where large numbers of NKR<sup>+</sup> T-cells are known to be present and actively participating in anti-cancer surveillance in the bone marrow [21–23], the aim of this study was to investigate the phenotypic and functional composition of the NKR<sup>+/−</sup> T-lymphoid component of human bone marrow.

## Materials and methods

### *Patients and biopsy samples*

Bone marrow aspirates and trephine biopsies were taken from the posterior iliac crest of forty-five patients

attending the hematology clinic at St Vincent’s Hospital, in remission following ( $n = 21$ ), or with currently active ( $n = 24$ ) hematopoietic malignancy. Of the patients in remission, five had previously been diagnosed with acute myelocytic leukemia (AML), five with non-Hodgkin’s Lymphoma (NHL), four with myeloma, three with acute lymphocytic leukemia (ALL), two with Hodgkin’s Disease and one each with lymphoma and acute promyelocytic leukemia (ApML). Those patients with active malignancy comprised eight with myeloma, three with malignant myelodysplasia, three with lymphoma, two each with chronic myelocytic leukemia (CML), AML and malignant T-cell clones, and one each with chronic lymphocytic leukemia (CLL), ALL, megaloblastic leukemia and Waldenstrom’s macroglobinemia. Donor samples ( $n = 10$ ) were obtained from bone marrow donors at the time of harvest in the National Centre for Bone Marrow Transplantation in St James’ Hospital, while peripheral blood samples ( $n = 12$ ) were obtained from healthy volunteers. Ethical approval for this study was received from both Hospital Ethics Committees.

### *Mononuclear cell isolation*

Peripheral blood and bone marrow mononuclear cells (PBMC and BMMC, respectively) were isolated by density gradient centrifugation. Blood or marrow aspirate was carefully layered over 5 ml Lymphoprep<sup>®</sup>, then centrifuged at  $250 \times g$  for 25 min. The buffy layer of mononuclear cells was removed and washed twice with Hanks Balanced Salts Solution (HBSS). Finally, cells were resuspended in ‘complete’ RPMI 1640 medium (supplemented with 10% FCS, L-glutamine, penicillin and streptomycin).

### *Flow cytometric staining*

PBMC/BMMC were prepared as described previously. Fluorescently conjugated monoclonal antibodies (mAbs) directed against human CD3, CD4, CD8, CD45, CD45RA, CD45RO, CD56, CD57, CD69, CD161, TCR  $\alpha\beta$ , TCR  $\gamma\delta$  and HLA-DR, as well as appropriate isotype controls, were obtained from Becton Dickinson Flow Cytometry Systems (Oxford, UK). Antibodies recognizing the V $\alpha$ 24 (PE) and V $\beta$ 11 (FITC) TCR chains were obtained from Immunotech (Marseille, France). Three-color flow cytometry was performed by staining approximately  $10^5$  PBMC/BMMC for 10 min at room temperature with 5  $\mu$ l of the relevant mAbs. After washing twice with PBS/BSA/Azide, cells were fixed in 500  $\mu$ l 1% paraformaldehyde (PFA) prior to acquisition.

Intracellular staining was performed by first incubating the cells at 37°C for 4 h with 10 ng/ml PMA, 1  $\mu$ g/ml ionomycin and 10  $\mu$ g/ml Brefeldin-A. The cells were washed once with PBS/BSA/Azide before cell

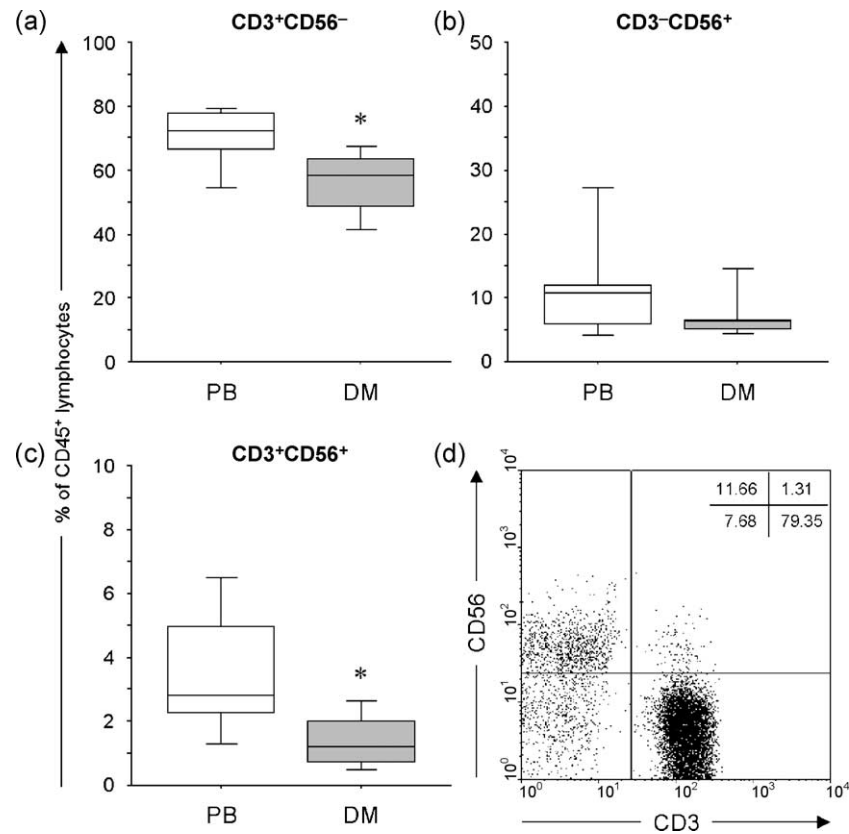


Fig. 1. Median percentage of CD45<sup>+</sup> lymphoid cells, along with range and interquartile range, accounted for by conventional T-cells (CD3<sup>+</sup>CD56<sup>-</sup>; a), NK cells (CD3<sup>-</sup>CD56<sup>+</sup>; b) and CD3<sup>+</sup>CD56<sup>+</sup> NKR<sup>+</sup> T-cells (c) in peripheral blood ( $n = 17$ ) and donor bone marrow ( $n = 11$ ). The flow cytometry dot plot (d) shows representative data for one peripheral blood sample and illustrates the delineation of the individual populations being examined. Numbers in the upper right-hand corner indicate the relative proportions of CD45<sup>+</sup> lymphoid cells in each of the four quadrants.

surface staining (see previous) and fixation in 500 ml 4% PFA. Permeabilization was achieved by addition of 2 ml of 0.2% saponin in PBS/BSA/Azide for 30 min, after which the cells were resuspended in approximately 100  $\mu$ l 0.2% saponin and 5  $\mu$ l of the appropriate anti-cytokine mAbs added for 60 min. Finally, the cells were washed with PBS/BSA/Azide and fixed in 500  $\mu$ l 1% paraformaldehyde (PFA) prior to acquisition.

#### Flow cytometric analysis

Flow cytometric data was analyzed using CellQuest software (Becton Dickinson Flow Cytometry Systems; San Diego). Electronic gating was used to select the lymphoid cells on the basis of forward and side scatter parameters (size and granularity, respectively). Similarly, CD45<sup>+</sup> or CD3<sup>+</sup> cells were gated and expression of other markers within a combination of the lymphoid and surface marker-positive gates was determined.

#### Statistics

Differences between groups of non-parametric data were analyzed using the Mann–Whitney  $U$  statistic, with a  $P$  value of <0.05 taken as significant.

## Results

### T/NK composition of blood and bone marrow

Multi-parameter flow cytometry was used to determine the T- and NK/NKT-cell composition of mononuclear cell preparations from peripheral blood (PB) and donor marrow (DM) samples. T-cells (CD3<sup>+</sup>CD56<sup>-</sup>) comprised a significantly lower proportion of CD45<sup>+</sup> lymphocytes in bone marrow compared to PB (median 56.10% in DM versus 75.95% of CD45<sup>+</sup> lymphocytes in PB;  $P < 0.005$ ). There was no difference in the levels of NK (CD3<sup>-</sup>CD56<sup>+</sup>) cells in DM compared to PB, however, levels of CD3<sup>+</sup>CD56<sup>+</sup>NKR<sup>+</sup> T-

Table 1  
Conventional T-cell subsets in peripheral blood and donor bone marrow

Population	PB % positive	DM % positive
CD4	58.40 (33.89–83.30)	53.80 (20.64–71.76)
CD8	36.27 (14.82–53.91)	42.14 (26.96–74.94)
DP	1.35 (0.54–4.63)	0.62 (0.00–2.92)
DN	4.60 (0.56–12.24)	2.41 (1.27–5.69)
TCR $\alpha\beta$	93.04 (87.35–97.68)	95.89 (91.92–98.56)
TCR $\gamma\delta$	6.26 (1.85–11.85)	3.44 (1.34–5.25)

Values indicate the median proportion of each subset, and interquartile range, expressed as a percentage of total CD3<sup>+</sup> cells.

cells were significantly reduced in DM (1.22% DM, 2.82% PB,  $P < 0.01$ , Fig. 1). T-cells expressing the V $\alpha$ 24V $\beta$ 11 TCR were present in donor marrow at levels not significantly different from those seen in peripheral blood, with a median of 0.09% of DM T-cells (interquartile range 0.05–0.18) expressing this TCR, compared to 0.03% of T-cells in the periphery (IQR 0.02–0.11%;  $P > 0.2$ ).

*Conventional T-cell subsets in donor marrow*

We further examined the phenotype of T cells in DM with respect to CD4/CD8 co-receptor and  $\alpha\beta/\gamma\delta$  TCR

expression. No differences were detected, with the exception of a significant decrease in the proportion of T cells expressing both CD4 and CD8 (DPs) in donor marrow (0.62% versus 1.35%,  $P < 0.03$ , Table 1).

*NK marker expression by T-cells*

Next, we examined the expression of key NK surface molecules on CD3<sup>+</sup> populations from DM and PB. Significantly lower proportions of CD3<sup>+</sup>CD56<sup>+</sup> NKR<sup>+</sup> T cells were present in donor marrow (1.52% of total CD3) compared to PB (5.30%,  $p < 0.001$ ). No difference was

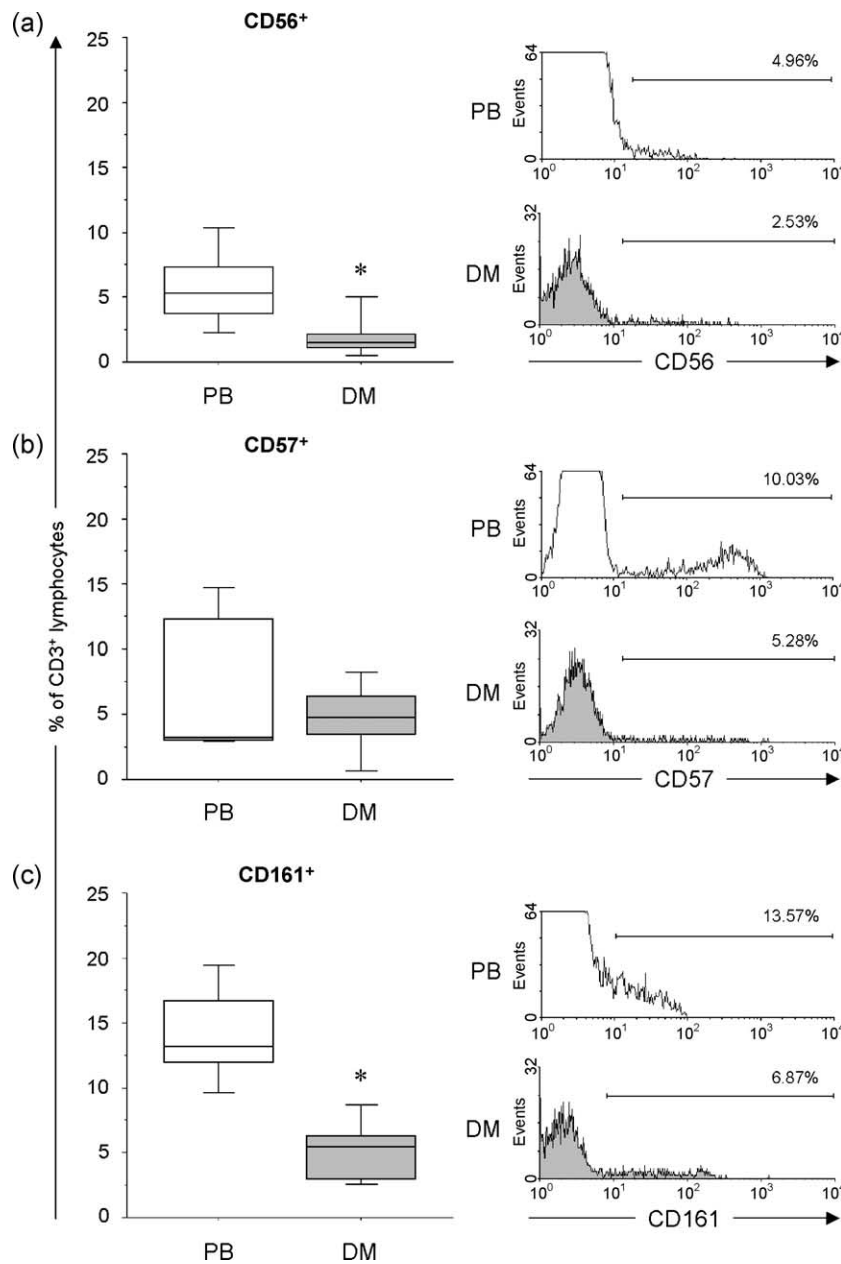


Fig. 2. NKR expression by peripheral blood ( $n = 7$ ) and donor marrow T-cells ( $n = 10$ ). Proportions of CD3<sup>+</sup> lymphocytes expressing the NK cells marker molecules CD56 (a), CD57 (b) and CD161 (c) were calculated and the medians, ranges and interquartile ranges plotted. Representative histograms of each marker are shown for one peripheral blood and one donor marrow sample (upper and lower panel for each of CD56, CD57 and CD161, respectively). Numbers indicate the proportion of T-cells expressing each marker.

observed in the CD3<sup>+</sup>CD57<sup>+</sup> subpopulation. However, the CD3<sup>+</sup>CD161<sup>+</sup> subset of NK-receptor positive T cells was significantly diminished in DM (5.48%) compared to PB (13.16%,  $P < 0.003$ , Fig. 2).

#### Activation status

The activation status of lymphocytes was assessed by surface expression of early (CD69) and late (HLA-DR) activation antigens by NKR<sup>+/-</sup> cells in normal blood and marrow, and in all three patient marrow groups. Overall, there is an increase in the expression of CD69, but not in HLA-DR, in donor marrow, compared to peripheral blood, and also in the marrow of patients with, or in remission following, hematopoietic malignancy compared to donors. This increase in CD69 was due to increased expression of this early activation marker on total CD3<sup>+</sup> cells and CD3<sup>+</sup> subsets examined. CD69 expression was also increased on DM NK cells, however, in contrast to T cells, a significant increase was also observed for HLA-DR, compared to PB (Table 2).

#### Cytokine production

Production of IFN- $\gamma$  and IL-4 by NKR positive and negative T-cell subsets marrow and peripheral blood was compared. Although the majority (>60%) of cells from both sources predominantly produced IFN- $\gamma$ , and a minority (<10%) produced IL-4, no significant difference in the proportions of cells producing either cytokine was detected (data not shown).

#### Effect of malignancy on marrow lymphocyte composition

As marrow NK/NKR<sup>+</sup> T populations have been postulated to play a role in tumor surveillance, we next examined the lymphocyte composition of bone marrow aspirates (BMAs) from patients with active, or in remission following, hematopoietic malignancy. No difference was observed in T, NK, NKR<sup>+</sup> T-cell frequency as a percent of total lymphocytes in marrow from pa-

Table 3

T-cell subsets in bone marrow aspirate samples from patients in remission (PR) or with active malignancy (PM)

Population	PR % positive	PM % positive
CD3 <sup>+</sup> CD56 <sup>-</sup>	53.68 (28.49–64.51)	53.02 (33.39–60.89)
CD3 <sup>-</sup> CD56 <sup>+</sup>	12.55 (3.83–18.60)	10.04 (6.18–13.63)
CD3 <sup>+</sup> CD56 <sup>+</sup>	1.62 (1.10–3.60)	1.70 (0.90–3.30)
TCR $\alpha\beta$ <sup>+</sup>	95.70 (94.75–97.58)	94.97 (90.66–97.51)
TCR $\gamma\delta$ <sup>+</sup>	3.19 (1.91–4.53)	4.97 (3.03–7.40)

Values indicate median percentage of CD45<sup>+</sup>(or CD3<sup>+</sup> for TCR  $\alpha\beta/\gamma\delta$ ) lymphoid cells expressing the relevant markers, while numbers in parentheses indicate the interquartile ranges.

tients in remission or with active malignancy when compared to donor marrow. Expression of the  $\alpha\beta/\gamma\delta$  TCR isoforms was also similar in all groups (Table 3). However, CD4 co-receptor expression is significantly decreased, with a concomitant increase in CD8, in remission marrows but not in active malignancy, when compared to donor marrow. DPs and DNs were unchanged (Figs. 3a and b). We further examined the phenotype of CD3<sup>+</sup> cells with respect to NK antigen expression. CD3<sup>+</sup>CD56<sup>+</sup> and CD3<sup>+</sup>CD57<sup>+</sup> populations were detected at the same frequency in remission and active malignant marrows. Surprisingly, these populations were both significantly higher than those in DM. CD3<sup>+</sup>CD161<sup>+</sup> populations were similar in all marrow types (Figs. 3c–e). Significantly more T cells in both remission and active malignancy express the early (CD69) and late (HLA-DR) activation markers (Figs. 3f and g).

#### Memory and naïve T cells in marrow

Because both remission and active malignancy had a similar activation profile, which was increased compared to donors, we wanted to see if activation status corresponded with the acquisition of memory/effector phenotype. Although both patient groups show higher levels of activation, this is not reflected in their CD45RORA phenotype, which is similar in DM and in remission. Malignant marrow, on the other hand, had a

Table 2

Activation marker expression by CD3- and CD56- expressing lymphoid subsets in peripheral blood (PB) and donor bone marrow (DM)

Marker	Population	PB % positive	DM % positive	<i>P</i> value
CD69	All CD3 <sup>+</sup>	1.33 (1.00–1.43)	6.30 (4.94–14.08)	<0.005
	All CD56 <sup>+</sup>	5.54 (5.23–6.50)	26.86 (24.20–27.59)	<0.007
	CD3 <sup>+</sup> CD56 <sup>-</sup>	1.15 (0.82–1.19)	6.06 (4.33–13.11)	<0.005
	CD3 <sup>-</sup> CD56 <sup>+</sup>	5.26 (4.04–6.32)	19.88 (15.26–23.03)	<0.02
	CD3 <sup>+</sup> CD56 <sup>+</sup>	5.43 (3.91–7.75)	38.46 (31.6–40.72)	<0.005
HLA-DR	All CD3 <sup>+</sup>	5.39 (5.25–5.96)	6.05 (2.85–8.23)	ns
	All CD56 <sup>+</sup>	6.39 (6.32–7.87)	19.27 (17.89–23.24)	<0.007
	CD3 <sup>+</sup> CD56 <sup>-</sup>	5.39 (5.01–5.99)	5.98 (2.78–7.90)	ns
	CD3 <sup>-</sup> CD56 <sup>+</sup>	7.49 (7.19–8.57)	17.24 (14.27–24.80)	<0.007
	CD3 <sup>+</sup> CD56 <sup>+</sup>	5.55 (4.80–7.32)	8.24 (7.19–20.28)	ns

Values indicate the median percentage of each subset expressing CD69 or HLA-DR, while numbers in parentheses indicate the interquartile range. *P* values were generated using the Mann–Whitney *U* test. (ns: not significant).

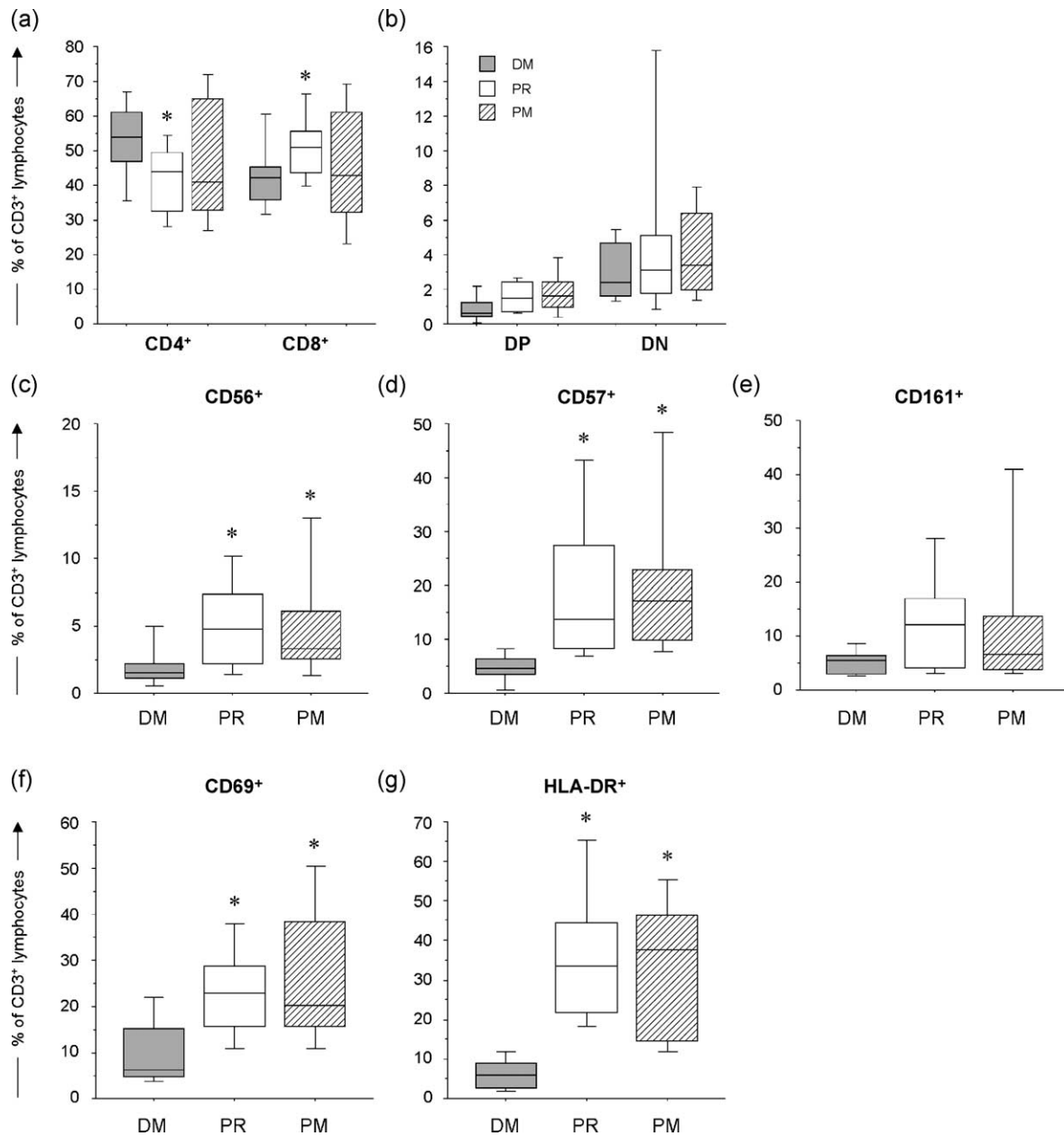


Fig. 3. Co-receptor, NKR and activation marker expression by T-cells in donor marrow (DM,  $n = 10$ ), and marrow from patients in remission following (PR,  $n = 12$ ), or with currently active hematopoietic malignancy (PM,  $n = 15$ ). Cells expressing the CD4 and CD8 co-receptors (a), or both/neither molecule (b) were detected, as were the NKR molecules CD56 (c), CD57 (d), and CD161 (e). Early (CD69; f) and late (HLA-DR; g) activation marker expression by CD3<sup>+</sup> cells was also examined. Values are expressed as median percentage of CD3<sup>+</sup> cells (with interquartile range) in each case. Asterisks are indicative of statistical significance for patient samples (PR and PM) compared to donor marrow.

higher proportion of memory T cells, as evidenced by increased RO expression (Fig. 4).

### Discussion

Although the primary role of the bone marrow is as a hematopoietic organ, the presence of mature, functional NK and T-lymphocytes within the marrow suggests that some additional immunological process or processes are occurring at this site. Murine studies

have described populations of T-cells with potent capacity to act as anti-cancer effectors [22,23] and that are enriched at a number of sites, including the bone marrow [31,32]. These cells, comprising a subset of NKR-expressing T-cells, are further characterized by a restricted T-cell receptor (TCR) repertoire, predominantly expressing an invariant V $\alpha$ 14J $\alpha$ 18  $\alpha$ -chain, and preferentially pairing with a limited number of V $\beta$  elements [25]. Unlike conventional T-cells, this population reacts to lipid antigens being presented by the MHC class I-like molecule CD1 [29]. Equivalent cells have been

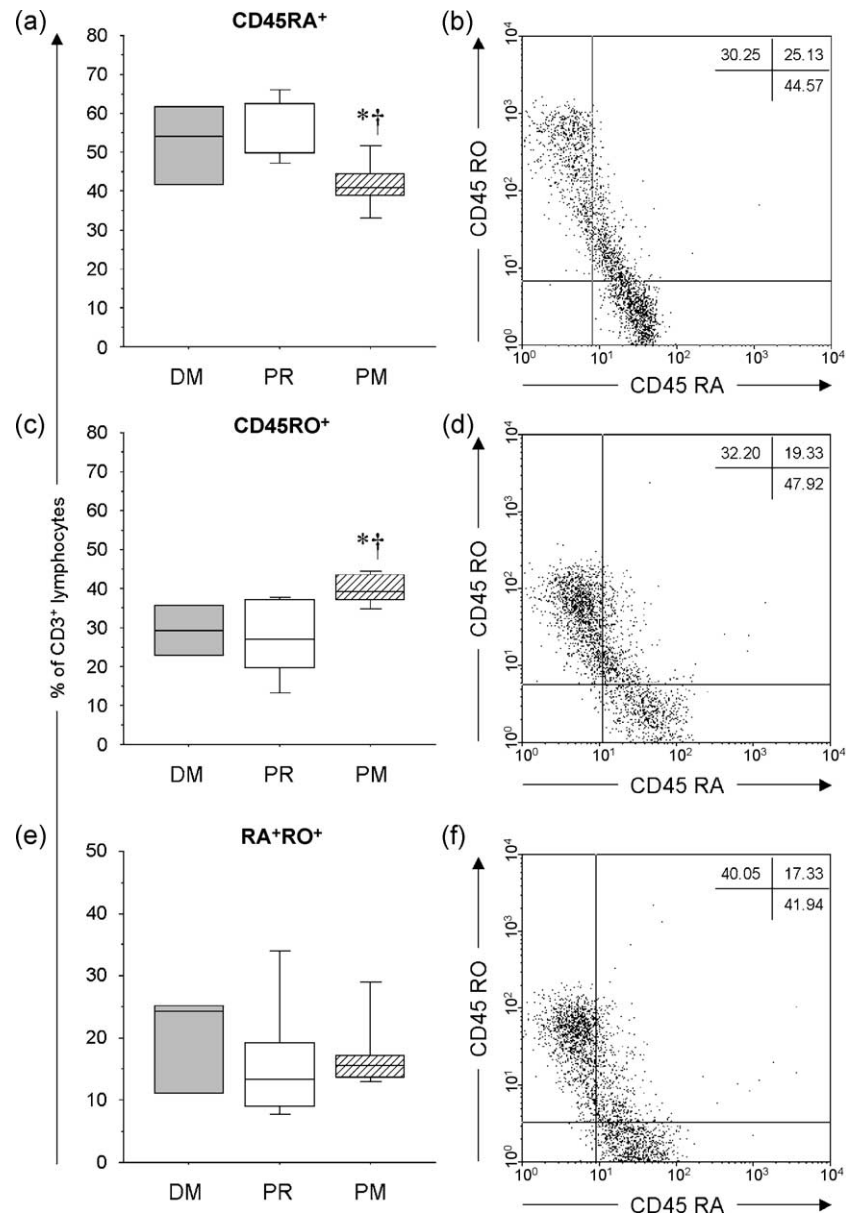


Fig. 4. Naïve/memory status of T-cells in donor bone marrow (DM,  $n = 6$ ) and bone marrow aspirate samples obtained from patients in remission (PR,  $n = 7$ ) or with active malignancy (PM,  $n = 6$ ). CD45RA and CD45RO single-positive cells (a and c, respectively) and cells positive for both isoforms (e) were detected. Dot plots show representative data for one donor marrow (b), one remission patient (d) and one malignant patient marrow sample (f). Numbers in the upper right hand corner of each plot show relative percentage of T-cells falling into the upper left, upper right and lower right quadrants, corresponding with RA<sup>-</sup>RO<sup>+</sup>, RA<sup>+</sup>RO<sup>+</sup>, and RA<sup>+</sup>RO<sup>-</sup> cells, respectively. Asterisks are indicative of statistical significance for patient samples compared to donor marrow, while the dagger symbol indicates statistical significance between patient sample types.

identified in humans, where they express an homologous TCR  $\alpha$ -chain, comprised of V $\alpha$ 24 and J $\alpha$ 18, paired with V $\beta$ 11, and are also restricted by antigen presented in the context of CD1 [24,25,29]. Human liver is enriched for NKR<sup>+</sup> T-cells [33,34], but, unlike their V $\alpha$ 14<sup>+</sup> murine counterparts, human V $\alpha$ 24<sup>+</sup> T-cells are rare throughout the body, perhaps due to the existence of four functional human CD1 isoforms (CD1a, b, c and d) [35–37], each of which is capable of antigen presentation. There is already some evidence that CD1-reactive cells other than V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup> T-cells exist in humans [38], and these may perform the

immunosurveillance role attributed to NKT cells in the mouse. CD56 has been found to represent a good surrogate marker for a broader range of NKR molecules when expressed on human T-cells [34] and, although CD161 is an exception to this, it is the only identified human member of the NKR-P1 family of c-type lectins to which NK1.1 the predominant antigenic marker used in murine studies belongs [39]. Furthermore, CD56<sup>+</sup> and CD57<sup>+</sup> T-cells have been seen to increase in some malignancies [40–43]. This study aimed to examine the NKR positive and negative T-cells in human bone marrow for such candidate populations.

As expected, T-cells represent a smaller proportion of all CD45<sup>+</sup> cells in bone marrow (BM), compared to peripheral blood (PB), as myeloid precursors and other immature cells predominate in the marrow compartment. Although CD4<sup>+</sup>CD8<sup>+</sup> double positive T-cells were significantly reduced among BM T-cells compared to those in PB, marrow T-cells showed no significant difference in the composition of conventional T-cell subsets such as the CD4<sup>+</sup>, CD8<sup>+</sup> or TCR  $\alpha\beta/\gamma\delta$  populations.

Our data indicate that a variety of NKR<sup>+</sup> T-cell populations can be identified in human bone marrow. However, the CD56<sup>+</sup> and CD161<sup>+</sup> subsets are both depleted among BM T-cells, compared to PB, even though NKR<sup>+</sup> T-cells are enriched in murine bone marrow [32] and other sites of primary antigen encounter or high cellular turnover in humans, such as the liver and gut [11,15,34]. Despite being diminished, human bone marrow NKR<sup>+</sup> T-cell populations possess an activated phenotype, expressing the early activation marker CD69 with a frequency significantly higher than their counterparts in the peripheral blood, a trait associated with other organ-specific immune compartments [14,15,34]. This suggests the presence of a localized stimulus, or ‘priming’ of the NKR<sup>+</sup> T-cells in readiness for immediate response in the future. In donor marrow, only NK cells were seen to express the late activation marker HLA-DR at levels higher than their PB equivalents. This may indicate an expansion of NK cells in response to local stimulus, as CD3<sup>-</sup>CD56<sup>+</sup> NK cells are not depleted in the marrow, unlike T-cells or NKR<sup>+</sup> T-cells. However, functionally, NKR<sup>+/-</sup> T-cells in the bone marrow do not appear to be any different from those in the PB, as no subset tested demonstrated altered cytokine expression upon pharmacological stimulation. This, again, contradicts expectations, given that NKR<sup>+</sup> T-cells at other sites in the human rapidly produce IFN- $\gamma$  and IL-4 upon stimulation [26,33,44].

While NKR<sup>+</sup> T-cells are diminished in donor bone marrow, our data suggest that there is some form of response to malignancy within bone marrow by NKR<sup>+</sup> T-cells. Cytotoxic CD8<sup>+</sup> T-cells are increased in the bone marrow of patients in remission following hematopoietic malignancy, with a concomitant decrease in CD4<sup>+</sup> T-cell levels. This may reflect the role in control of hematopoietic malignancies attributed to CD8<sup>+</sup> T-cells [45,46]. Our data also show that the CD56<sup>+</sup> T-cell population responds to malignancy, as it is expanded in both active malignancy and in remission states. CD57<sup>+</sup> T-cells are also enriched in these patients, suggesting antigen-specific activation events have occurred, as upregulation of the CD57 molecule by CD8 cells is associated with activation-induced cell death (AICD) processes. However, CD161, the only human member of the NKR-P1 family of cell surface lectins to which murine NK1.1 belongs [39], showed no difference in expression in the bone marrow and PB. This emphasizes the differences between

the human and murine bone marrow, as NK1.1 T-cells are identified as the principle effectors in a number of anti-cancer models in the mouse [21–23].

As the CD57<sup>+</sup> T-cell data has already suggested, NKR<sup>+</sup> T-cells, and T-cells on the whole, appear to become activated by some stimulus within the BM environment. In normal marrow (donors), heightened CD69 expression by donor marrow NKR<sup>+/-</sup> T-cells makes it unlikely that the further expansion of activated cells in malignancy is due solely to increased trafficking from other lymphoid organs [47]. In addition, these malignancy-associated marrows show enhanced expression of the late activation marker HLA-DR, suggesting that the stimulus is more prolonged. The possession of a CD45RO<sup>+</sup>, effector/memory phenotype by T-cells in actively malignant marrows further supports this idea and implicates the malignancies themselves as the stimulus.

In conclusion, we describe a range of NKR<sup>+/-</sup> T-cell populations within human bone marrow. While we cannot exclude the possibility that differences in the immunophenotypic composition of the aspirated marrow samples used have been masked by peripheral blood contamination, our data illustrate a number of significant differences between bone marrow and the circulation. Bone marrow NKR<sup>+/-</sup> T-cells comprise expanded groups of activated, cytotoxic, effector/memory cells in remission following hematopoietic malignancy, and show evidence of the formation of a memory pool during active disease in the marrow. These data indicate that T-cells, involved in cancer surveillance represent a much more heterogeneous population in human bone marrow than in the mouse. Our investigation has identified a number of such sub-populations that warrant further investigation.

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