Cancer Immunol Immunother (2007) 56: 101–109
DOI 10.1007/s00262-006-0141-y

SYMPOSIUM PAPER

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Lymphocyte activation in response to melanoma: interaction of NK-associated receptors and their ligands

Received: 31 December 2005 / Accepted: 22 January 2006 / Published online: 17 February 2006

Abstract In recent years, studies on the molecular and cellular mechanisms of immune responses against melanoma have contributed to a better understanding of how these tumours can be recognised by cytotoxic cells and the mechanisms they have developed to escape from innate and adaptive immunity. Lysis of melanoma cells by natural killer (NK) cells and cytolytic T cells is the result of a fine balance between signals transmitted by activating and inhibitory receptors. In addition to the T cell receptor, these were initially described as NK cell-associated receptors (NKRs) and were later also found on subsets of T lymphocytes, particularly effector-memory and terminally differentiated CD8 T cells. An increase of NKR^+CD8^+ T cells has been found in melanoma patients, correlating with the expansion of differentiated effector $CD8^+CD28^{null}$ $CD27^{null}$ T cells. NKRs can regulate the lysis of target cells expressing appropriate ligands. Activating receptors recognise ligands on tumours whereas inhibitory receptors are specific for MHC class I antigens and sense missing self. Altered expression of MHC class I antigens is frequently found on melanoma cells, preventing recognition by specific cytolytic T cells but favouring NK cell recognition. Changes in the expression of NKR-ligands in melanoma contribute in explaining the differences in the capacity of cytotoxic immune cells to control melanoma growth and dissemination.

This article is a symposium paper from the conference "Progress in Vaccination against Cancer 2005 (PIVAC 5)", held in Athens, Greece, on 20–21 September 2005.

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Cell mediated cytotoxicity against melanoma: natural killer cells and cytotoxic T lymphocytes as effectors

Major advances in cancer immunology have come from the study of melanoma. In recent years, new insights into the molecular and cellular mechanisms of immune responses against tumours have allowed the development of novel therapeutic strategies against melanoma. However, melanoma cells can evade immune recognition by innate and adaptive immunity. As tumour escape pathways have been identified, several methods have been developed to block them. However, it is significant that in most cases, the results are not as expected.

Solid tumours, such as melanoma, express tumour-specific antigens that serve as targets for immune effector T cells. Naïve cytotoxic T cells are activated exclusively in secondary lymphoid organs including the spleen and lymph nodes. In the early phases of solid tumour development, tumour cells do not usually reach lymphoid organs and cannot prime naïve T cells; in consequence, immune surveillance is inefficient. The induction of tumour-specific T cell immunity by vaccination is intended to overcome this immunological ignorance [61].

Total or partial downregulation of major histocompatibility complex (MHC) class I expression is frequently found in melanoma cells, which thereby resist killing by MHC-restricted cytotoxic T lymphocytes (CTLs). However, altered expression of MHC class I molecules can make melanoma cells more susceptible to lysis mediated by natural killer (NK) cells, a component of the innate immune system. NK cell cytotoxicity is controlled by the balance of activating and inhibitory signals mediated by different receptors, some of which bind to MHC class I molecules on target cells [12, 33, 94,

104]. Lack of MHC class I molecules renders tumour cells more susceptible to NK cell-mediated killing due to lack of ligation of MHC class I-specific inhibitory receptors. Thus, interactions of tumour cells with cytotoxic cells will produce a complex network of positive and negative signals, the integration of which will modulate immune responses [42, 56, 64, 65, 91, 93, 97].

Cytotoxic cells of the immune system play a pivotal role against tumours and viral infections. Recent advances in immunological techniques, such as the development of tetramer technology, have allowed a better characterisation of tumour-specific CTLs, concerning precursor frequency, activation mechanisms, and regulation of the immune response. The identification of new receptors expressed by cytotoxic cells, some of them initially described as NK cell-associated receptors (NKRs), has allowed new insights into the mechanisms involved in the induction of cytotoxicity [12, 41, 46, 63]. The major activating and inhibitory receptors described in NK cells and CD8 T lymphocytes are listed in Tables 1 and 2, respectively. Activation of CD8 T cells requires antigen-specific signals transmitted by the T cell receptor (TCR) and costimulatory signals mediated by several molecules including some NKRs. Thus, the expression of NKRs on different subsets of T lymphocytes, mainly effector CD8 T cells, has been correlated with terminally-differentiated effector cells [19, 56, 89, 91, 93, 97].

In this paper, results from our laboratory and others describing the significance of NK cell receptors and their ligands in the recognition of tumour targets are reviewed. A better understanding of the interactions between the immune system and tumour cells may provide a rational basis for new immunotherapy protocols [38, 93].

NK cells and melanoma

NK cells are a component of the innate immune system that contribute to the immune responses against tumours as they kill some cancer cells without prior sensitisation and without a requirement for MHC restriction [68]. Human NK cells comprise up to 15% of all peripheral blood lymphocytes and are defined phenotypically by the expression of CD56 and/or CD16 and lack of expression of CD3. Two distinct populations of human NK cells can be identified based on their cell-surface density of CD56, whereby $CD56^{dim}$ NK cells constitute the majority (90%) and express high levels of CD16 (FcRIII). They possess greater cytotoxic capacity than the minority subset of $CD56^{bright}$ NK cells, which have low expression of CD16 but produce larger amounts of cytokines. NK cell subsets differentially express other structures involved in cytotoxicity, such as chemokine receptors (e.g. CCR7, CXCR3, CXCR1) and adhesion molecules (e.g. CD2, CD44, LFA-3, or ICAM-1) and may have distinct trafficking patterns during the immune response [23, 41]. NK cells have an important role in vivo in immune defence against tumours by preventing their dissemination in experimental models and are also important in defences against viruses [20, 65, 84, 102]. Recently, emerging evidence shows that NK cells can contribute to dendritic cell maturation and in consequence help T cell-mediated immune responses as well [24, 37, 101].

Table 1 Major activating and inhibitory receptors on NK cells

Activating receptors	Inhibitory receptors
NKp46	CD94/NKG2A
NKp30	CD85j
NKp44	KIR2DL1
NKG2D	KIR2DL2/3
CD244	
CD94/NKG2C	
KIR2DS1	
KIR2DS2/3	

Table 2 Major activating and inhibitory receptors on T cells

Activating receptors	Inhibitory receptors
TcR (1st signal; specific)	CTLA4
	CD85j
Co-stimulation (2nd signal):	CD94/NKG2A
CD28	KIR2DL1
ICOS	KIR2DL2/3
NKG2D	
CD244	
CD94/NKG2C	
KIR2DS1	
KIR2DS2/3	

Role of NK cells as effector cells against melanoma

NK cells have the ability to kill a variety of tumour cells spontaneously while sparing normal cells [1, 88]. The potential role of NK cells for cancer immunotherapy is due to their capacity to recognise tumour cells without the need for preactivation and kill tumours that might evade T-cell killing by altered expression of HLA molecules [1]. It has been demonstrated that melanoma cells can be susceptible to NK-mediated lysis both in murine and human models [40, 74]. Using a melanoma cell line generated in our laboratory as a target cell, we show that both autologous and allogeneic polyclonal NK cells can effectively kill this tumour cell (Fig. 1).

The ability of NK cells to respond to cytokines, such as interleukin-2 (IL-2) and interferons (IFNs), can increase their usefulness in immunotherapy against tumours. Thus, activation of NK cells with high-dose IL-2 has been widely used and has been shown to mediate anti-tumour activity in clinical as well as experimental settings [52, 69]. In our experimental system, lymphokine-activated killer (LAK) cells were generated after NK cell activation by IL-2 ex vivo. LAK cells showed

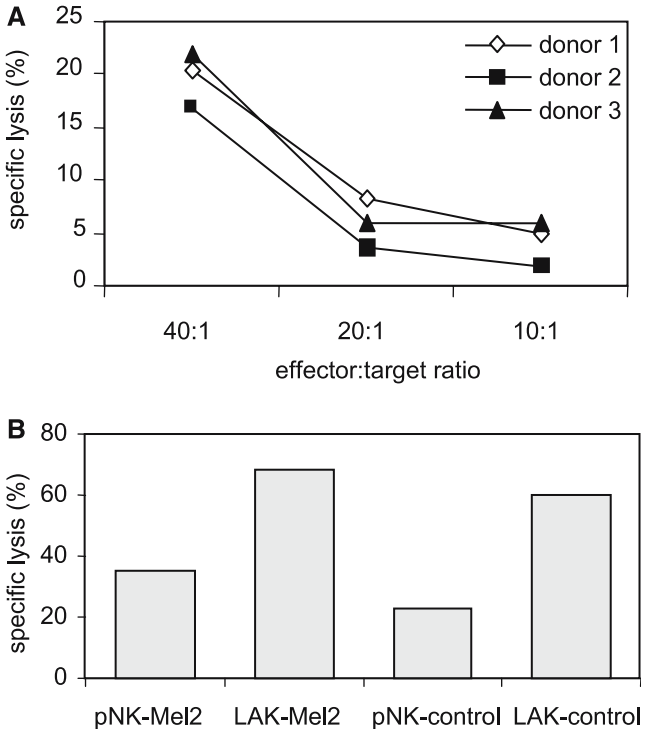

Fig. 1 Susceptibility of melanoma cells to lysis mediated by NK cells. Susceptibility of UCO-Mel2, a melanoma cell line obtained from a primary melanoma, was analysed by standard Cr51 release assay. **a** Polyclonal NK cells from three different donors were used as effectors at different ratios as indicated in the figure. UCO-Mel2 cells were used as targets (5,000 cells/well). **b** Polyclonal NK cells obtained from a melanoma patient are able to kill autologous melanoma cells. Treatment of NK cells with IL-2 increases specific lysis of melanoma cells (The effector:target ratio was 40:1). LAK cells were obtained after stimulation of polyclonal NK cells with rIL-2 (400 U/ml) during 5 days

increased cytotoxicity against the melanoma cell line UCO-Mel-2 as well as against 721.221, an NK-cell-susceptible cell line (Fig. 1b).

NK cytotoxicity: activation–inhibition balance

In the early 1980s, Kärre and colleagues proposed the "missing self" hypothesis based on observations that lymphoma cells which had lost H-2 molecules were more susceptible to lysis mediated by NK cells, suggesting that these effector cells contribute to anti-tumour immunity by detecting deleted or reduced expression of self-MHC on tumour cells [39, 44, 61, 62, 105]. MHC-class I-specific NKRs were initially described as receptors present on NK cells that regulate lysis of target cells expressing the appropriate ligand [12, 41, 46, 63]. In humans, three families of HLA class-I specific NKRs have been identified, including activating and inhibitory isoforms: killer immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILR) (also termed Immunoglobulin Like Transcripts, ILT, or CD85), and C-type lectin receptors [12, 41, 46, 59] (for review see [12]). MHC class I-specific inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains, which recruit intracellular tyrosine phosphatases that mediate inhibition.

NK-cell activation is a complex process involving multiple interactions between NK receptors and their ligands on target cells. Some of these receptors, both activating and inhibitory, show specificity for MHC class I or class I-like molecules and modulate NK-mediated cytotoxicity against targets expressing the appropriate HLA class I allele [10, 17, 47, 60]. However, lysis of MHC-deficient tumour cells emphasizes that other activating receptors, MHC independent, must be involved in natural cytotoxicity mediated by NK cells. In recent years, a novel family of receptors termed natural cytotoxicity receptors (NCRs) has been identified [57, 58]. NCRs are selectively expressed by NK cells and several members have been described. Two NCRs, NKp46 and NKp30, are expressed by all resting and activated NK cells, whereas NKp44 is selectively expressed by activated NK cells. Crosslinking of NCRs mediates NK-cell triggering, leading to target-cell lysis and cytokine production. NCRs are selectively expressed by NK cells and associate with different transmembrane-anchored polypeptides bearing immune tyrosine-based activating motifs (ITAMs) [15]. NCRs are involved in the lysis of several tumours and virus-infected cells, suggesting that the ligands for these receptors are widely distributed [13, 14]. Recently, some ligands recognised by NCRs have been defined. Thus, NKp46 and NKp44, but not NKp30, can recognise viral hemagglutinins and NKp46 recognises membrane-associated heparan sulfate proteoglycans expressed on tumour cells [5, 11, 48, 49].

Another major NK-activating receptor that has been implicated in the killing of tumours is NKG2D, which is characterised by a lectin-like extracellular domain. Its ligands are defined antigens that are frequently overexpressed on many different tumours, indicating a potential role for NKG2D in immune surveillance against cancer [21, 65, 75]. In humans, NKG2D is expressed on both NK cells and CD8 T cells. Ligation of NKG2D on NK cells directly leads to cytotoxicity, whereas on T cells it costimulates TCR signalling [28, 89]. NK cell-mediated cytotoxicity through NKG2D signalling can be inhibited by inhibitory receptors [53, 78]. A remarkable finding is the complementary role played by the NCR and the NKG2D receptors [58, 75]. Human NKG2D associates with the DAP10 transmembrane adaptor, which bears a YxxM motif and activates the phosphatidylinositol 3-kinase pathway [12, 30, 89, 103].

Other triggering receptors expressed by NK cells have recently been identified including the DNAX accessory molecule-1 (DNAM-1, CD226) that recognise the poliovirus receptor (PVR, CD155) and Nectin-2 (CD112), two closely related molecules that are expressed by several tumours such as melanomas. This receptor as well as 2B4 (C1.7, CD244) and NKp80 appear to function primarily as costimulatory molecules [13, 14].

Finally, NK-cell receptor protein 1 (NKR-P1; CD161 in humans and NK1.1 in mice), a protein that belongs to the C-type lectin superfamily has also been considered an NK triggering receptor. It is detected early during NK cell development [22]. Interestingly, an increase of $CD56^{-}$ $CD161^{+}$ NK cells and a reduction of $CD56^{+}$ $CD161^{+}$ NK cells have been described in HIV infected individuals [92]. CD161 is also expressed in a subset of $CD4^{+}$ and $CD8^{+}$ T cells, and CD161 expression is a characteristic of NKT cells [19, 26, 95]. Crosslinking of CD161 on NK cells can result in either activation or inhibition, suggesting the existence of functionally distinct isoforms [7]. Recently, the human lectin-like transcript 1 (LLT1) has been identified as a ligand for CD161. Engagement of CD161 on NK cells with LLT1 expressed on target cells inhibited NK cell-mediated cytotoxicity and IFN gamma secretion [2, 82]. By contrast, on T cells, the interaction of CD161 and LLT1 acted as a costimulatory signal, enhancing TCR mediated production of IFN gamma [2]. It has been shown that LLT1 is also an activating receptor on NK cells [51], suggesting that LLT1/CD161 signalling may be bidirectional. Thus, it is of interest to further analyse the role of LLT1/CD161 interaction on NK and T cell activation and its possible significance in the anti-tumour immune response.

Expression of ligands for NKRs on melanoma cells

Inhibitory NKRs are specific for HLA class I molecules and, therefore, the altered expression of MHC class I molecules can make melanoma cells more susceptible to NK-mediated lysis [35, 47, 73]. Total or partial loss of HLA class I antigens on tumour cells is a frequent strategy used by tumour cells to avoid T cell recognition [4, 16, 50, 79, 80, 85]. The expression of MHC class I antigens on melanoma cell lines has been analysed in a broad panel of melanoma cell lines obtained from the ESTDAB melanoma cell bank and the results show that whereas total loss of HLA class I is a rare event (< 5% of the cell lines), partial loss is frequently found (about 50% of the melanoma cell lines studied had altered HLA class I expression [81]). An example illustrating these alterations is shown in Fig. 3. UCO-MEL2, a melanoma cell line obtained in our laboratory from a primary lesion, showed downregulation of HLA-B, whereas HLA-A expression was preserved (HLA typing was A24/68 B negative). Treatment with IFNγ increased HLA class I molecules on the surface of melanoma cells (Fig. 2) and induced HLA-B expression on this cell line (HLA-B 35/50). This particular alteration in HLA class I expression corresponds to the type D downregulation defined by Garrido et al. [81], and it is found in 26% of melanoma cell lines. Thus, blocking HLA class I antigens on melanoma cells increases NK-mediated cytotoxicity (Fig. 3).

The expression of ligands for activating NKRs and costimulatory molecules on melanoma cell lines has also been analysed. In humans, the NKG2D receptor recognises MHC class I-related A and B antigens (MICA and MICB), encoded by genes within the human MHC. MIC molecules consist of three domains similar to the α chain of classical MHC class I molecules, but do not bind to $\beta 2$ microglobulin or peptide [43]. Another MHC-related family of ligands for human NKG2D, designated UL16-binding proteins (ULBPs), that bind to human cytomegalovirus glycoprotein, UL16, has been reported [25, 45]. Studies of these molecules on different tumours showed that MIC are more frequently expressed on melanoma cell lines than ULBP [75], a result confirmed by us typing a large number of melanoma lines in the ESTDAB collection that showed that 79% of the melanoma cell lines analysed expressed MICA/B (manuscript in preparation). MICA/B expression on melanoma cells can trigger NK cell-mediated cytotoxicity, supporting that MICA/B interaction with NKG2D may constitute a major effector mechanism of NK cell recognition of melanoma cells. In this sense, it has been postulated that the shedding of MICA/B by tumour cells may constitute a mechanism of tumour escape by interfering with NKG2D mediated NK and T cell activation [31, 86].

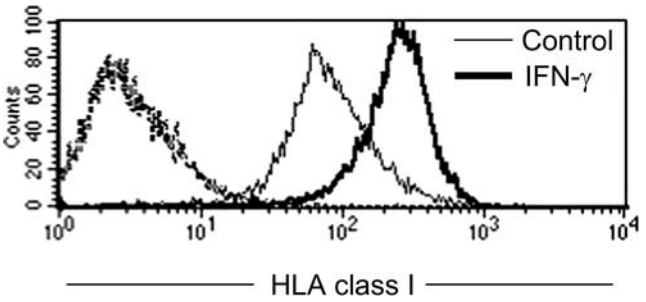

Fig. 2 Expression of HLA class I molecules on the melanoma cell line, UCO-Mel2. The treatment with 200 U/ml of gamma interferon for 48 h increased HLA class I expression as detected by flow cytometry

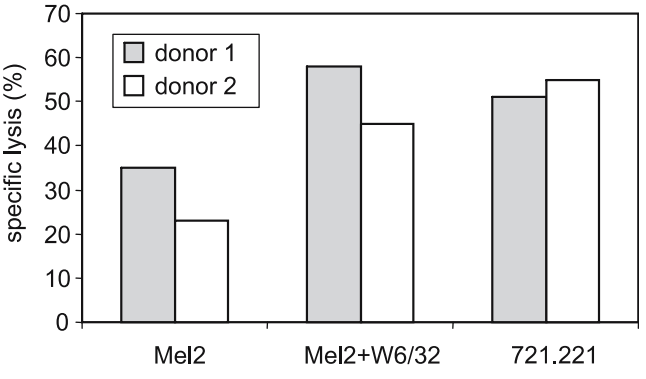

Fig. 3 Crosslinking of HLA class I-specific inhibitory receptors reduces melanoma susceptibility to NK cell cytotoxicity. Cytotoxicity was analysed by standard Cr51 release assay using polyclonal NK cells from two different donors. The lysis of UCO-Mel2 cell line by polyclonal NK cells is enhanced by the addition of anti-HLA class I mAb (W6/32) at 10 μg/ml. The effector:target ratio was 40:1

Cytotoxicity mediated by NK cells also requires an adequate interaction with the target cell mediated by adhesion molecules expressed by both effector and tumour cells. Analysis of melanoma cell lines in the ESTDAB collection (manuscript in preparation) demonstrated the expression on these cells of adhesion molecules (in particular CD54 and CD58) that may contribute to conjugate formation with the cytotoxic cells. In contrast, the expression of ligands for costimulatory molecules such as CD80, CD86, or CD48 is essentially absent. Other molecules, such as CD56 or CD57, for which biological significance has to be determined, can be also expressed in a significant percentage of melanoma cell lines.

An extensive characterisation of the melanoma cell lines of the ESTDAB cell bank, including the expression of ligands for NKRs, can be found on the on-line searchable database at http://www.ebi.ac.uk/ipd/estdab/secondary_search.html.

CD8 effector T cells and melanoma

The effector phase of the immune response against melanoma includes components from both the innate and the adaptive immune system [77]. The identification of several MHC class I-restricted melanoma-associated antigens recognised by CTL has allowed a better characterisation of T cell-mediated cytotoxicity against melanoma and the development of new strategies for immunotherapy of human melanoma. However, immune tolerance to melanoma is a frequent finding and several mechanisms of tumour escape have been proposed [29, 70].

CTLs are considered as the major effectors against cancer. Activation of T cells requires two signals, the first mediated by the TCR after specific antigen recognition, and the second one provided on engagement of costimulatory receptors, such as CD28 [3, 18]. Downregulation of HLA class I molecules on tumour cells is a well-known strategy to avoid killing by MHC restricted CTLs and is usually associated with poor prognosis. Although total loss of HLA class I molecules is rare ($<5\%$), loss of heterozygosity is an important mechanism found in melanoma to avoid T cell recognition [81].

CD8 T lymphocytes also express other NK receptors with ligands different from HLA class I molecules. Within this group of receptors, CD56, CD57, and CD244 molecules correlate with cell activation and may have functional implications in the immune response against tumour antigens [67, 76, 93, 95]. The expression of NKRs on CD8 T cells is almost entirely restricted to the effector memory and effector CD45RA+ phenotype (unpublished results).

Increase of effector T cells in melanoma patients

T-cell-mediated immune responses play a key role in the control of melanoma progression. Distinct subpopulations of circulating human CD8 T cells can be defined based on their phenotype and function. Naïve and memory subsets can be distinguished by the expression of different isoforms of the leukocyte common antigen, CD45RA or CD45RO, respectively. Four human CD8 T cell subsets, naïve ($CCR7^+CD45RA^+$), central memory ($CCR7^+CD45RA^-$), effector memory ($CCR7^-CD45RA^-$), and effector cells ($CCR7^-CD45RA^+$) are defined by the differential expression of CCR7 and CD45RA [87]. In addition, the expression of the costimulatory molecules CD28 and CD27 can also identify several stages of T cell differentiation [83]. Naïve T lymphocytes express both CD28 and CD27 molecules that become downregulated during the processes leading to T cell differentiation into effector cells. We have previously shown an expansion of $CD8^+CD28^-$ $CD27^-$ T cells in melanoma patients. This subset appears to be terminally-differentiated effector cells as defined by a $CD56^+$ and $CD244^+$ phenotype and high levels of perforin [19]. The expansion of $CD28^-$ T cells has also been reported in ageing and other situations of chronic immune stimulation [27, 71, 91, 95]. The expression of CD56 on CD8 T cells has been correlated with cytolytic effector function [76] and has been found to be decreased in HIV-infected individuals [95]. The role of CD56 on CD8 T cells from melanoma patients requires further analysis. In a previous paper, we showed that CD8 T cells from melanoma patients had a distinct phenotype regarding CD28 and CD45RA expression [19]. Most CD8 T cells from healthy young controls display a naïve phenotype ($CD28^+$ $CD45RA^+$) but in melanoma patients we found an increase of effector T cells ($CD28^-$ $CD45RA^+$). In contrast, metastatic melanoma patients had a similar phenotypic distribution as healthy individuals (Fig. 4).

NKR expression on CD8 T cells in melanoma patients

As discussed above, activating or inhibitory NKRs can be expressed on subsets of CD8 T cells. Increased expression of NKRs has been found in CD8 T cells from melanoma patients [8, 19, 54, 72, 90, 91, 99]. Furthermore, in melanoma patients, CTLs recognising melanoma epitopes can express inhibitory NKRs and signalling through these receptors interferes with CTL effector function [34, 36, 90, 100]. The characterisation of T cells expressing NKRs in melanoma patients showed that circulating NKR^+ $CD8^+$ T cells from melanoma patients display a distinct phenotype characterised by changes in the expression of costimulatory molecules [19]. Thus, the expression of NKRs is mainly restricted to the $CD8^+CD28^-$ T cell subset [19, 66, 91, 95]. We propose that the increase of perforin, CD244, and NKRs observed for CD8 T cells from melanoma patients is associated with the differentiation process, leading to the acquisition of memory and effector phenotypes. NKR expression on differentiated $CD8^+CD28^-$ T cells may constitute a regulatory

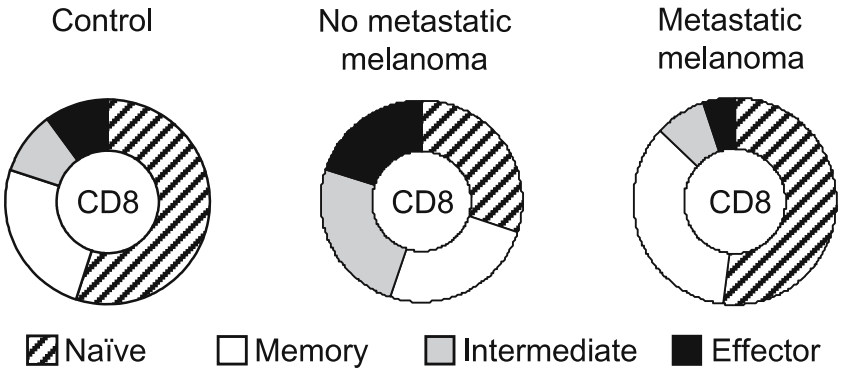

Fig. 4 $CD8^+$ T cell differentiation in healthy individuals and melanoma patients. Peripheral blood from healthy controls and melanoma patients was collected and analysed by flow cytometry to determine the percentage of naïve (CD28+ CD45RA+), memory (CD28+ CD45RA-), intermediate (CD28- CD45RA-), and effector cells (CD28- CD45RA+) based on the expression of CD28 and CD45RA. Patients were divided into metastatic ($n=5$) and no-metastatic melanoma ($n=12$)

mechanism of cytotoxicity as demonstrated for NK cells. Recently, the differential expression of HLA class-I-specific NKRs on CTLs has been correlated with transition from effector to memory T cells, suggesting a role for NKRs in the survival of CD8 memory T cells [79, 96, 106].

Escape from immunosurveillance by melanoma cells

Although tumour-specific CD8 T cells can be found infiltrating the tumour, frequently tumours escape from immunosurveillance. Hence, new immunotherapy protocols have focused on triggering or enhancing appropriate anti-tumour immunity. Different mechanisms have been implicated in melanoma escape from T cell-mediated immune responses. Some relate to decreased T cell recognition due to altered MHC class I molecules on tumour cells, changes in tumour antigen expression, or lack of costimulatory molecules and adhesion molecules required for an adequate initiation of T cell activation; all these changes can induce T cell anergy. In other instances, tumour escape occurs as a consequence of the production of a variety of immunosuppressive soluble factors by tumour cells, suppressor T cells, or both [70]. Both NK cells and CTLs possess granules containing perforin and granzymes that are released after cell activation. Perforin allows the entry of granzymes into the target cell where they induce apoptosis. In this regard, it has been postulated that certain viruses and possibly tumour cells could protect themselves from granzymes by serine protease inhibitors, and thus could escape from programmed cell death [6]. Crosslinking of inhibitory NKRs on CTLs by their ligands on melanoma cells may represent another mechanism of tumour immune escape. Thus, downregulation of HLA class I molecules on melanoma cells has been correlated with the up-regulated expression of non-classical MHC molecules such as HLA-E, the ligand for the inhibitory receptor CD94/NKG2A [4, 50]. Several cytokines such as IL-15 and TGF-β, produced by tumour cells, can induce the expression of NKRs on T cells [9, 32, 55]. In addition, the increased frequency of NKRs observed on CD8 T cells from melanoma patients might represent another mechanism of tumour escape [70, 93, 98].

Conclusions

The expression on melanoma cells of ligands for inhibitory NKRs expressed on NK cells and CTLs is likely to represent an important immune escape mechanism. In contrast, the expression of ligands for activating or co-stimulatory receptors may contribute to the efficient recognition and killing of melanoma cells. Blocking inhibitory receptor function and boosting activating receptors will enhance anti-tumour responses by both innate and adaptive immunity. Compared with T cells, NK cells can recognise tumour cells without the need for immunisation or preactivation. Furthermore, NK cells can recognise tumours that might evade T-cell killing by altered expression of HLA. These characteristics of NK cells make them good candidates for immunotherapy favouring the development of both innate and adaptive immunity. Recent advances in NK research identifying the major receptors involved in NK cell recognition and cytotoxicity of tumour cells have improved the capacity to design more effective therapies.

Acknowledgements This work was supported by grants QLRT-2001-00668 (Outcome and Impact of Specific Treatment in European Research on Melanoma, OISTER) and QLK6-CT2002-02283 (T cells in Ageing, T-CIA) from the 5th Framework Program of the European Union, FIS01/0478, FIS03/1383 (to R.S.), FIS00/0853 (to R.T.) from Ministry of Health, SAF2003-05184 (to R.T.) from Ministry of Science and Technology, 03/2 (to R.T.) from the "Consejería de Sanidad y Consumo", and 3PR05A012 (to R.T.) from the "Consejería de Infraestrucuras y Desarrollo Tecnológico" Junta de Extremadura, (Spain).

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