REVIEW

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Mechanisms of tumor escape: role of tumor microenvironment in inducing apoptosis of cytolytic effector cells

Alessandro Poggi[1] and Maria Raffaella Zocchi[2]

[1] Laboratory of Experimental Oncology D, National Institute for Cancer Research, 16132 Genoa, Italy
[2] Laboratory of Tumor Immunology, Scientific Institute San Raffaele, 20132 Milan, Italy

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Abstract

Spontaneous tumors grow and kill the host unless therapy reduces their mass to a level where the immune system, it is thought, can control their growth and diffusion. Indeed, in many instances tumors can reappear, become resistant to therapy, and escape the host immune response. Many mechanisms of tumor escape operating in the tumor microenvironment have been proposed: 1) low or absent expression of molecules on tumor cells involved in tumor target cell recognition; 2) absence of co-stimulation leading to tolerization of T cells; 3) soluble factors secreted by tumor cells inhibiting T cell response; and 4) regulatory T cells, myeloid suppressor cells, and stromal cells may impair immune-cell responses to tumors. Furthermore, tumors can release soluble molecules such as HLA-I (sHLA-I). This, in turn, reduces T cell-mediated immune response and induces apoptosis of cytolytic effector cells such as natural killer and $CD8^+$ T lymphocytes through the engagement of HLA-I receptors such as CD8 and/or activating isoforms of the inhibitory receptor superfamily. The release of soluble ligand for activating receptors, e.g. UL16 binding proteins and/or MHC class I-related proteins A and B, the natural ligands of NKG2D, may impair activation, effector cell-mediated recognition, and cytolysis of tumor cells. Furthermore, the elimination of anti-tumor effector cells may be achieved by induction of apoptosis consequent to triggering elicited via activating molecules, such as receptors responsible for natural cytotoxicity, upon their binding with ligands expressed on tumor cells.

Key words: tumor escape, soluble HLA-I, natural cytotoxicity receptor, NKG2D, apoptosis, stromal cells.

Corresponding author: Alessandro Poggi, Ph.D. M.D., Laboratory of Experimental Oncology D, National Institute for Cancer Research, c/o CBA Torre A1, Largo R. Benzi 10, 16132-Genoa, Italy, tel.: +39 0105737211, fax: +39 010354282, e-mail: alessandro.poggi@ostge.it

INTRODUCTION

Among the different therapeutic tools that a physician can utilize in fighting a tumor, immunotherapy represents undoubtedly the more fascinating one [64, 71, 73, 88]. Indeed, if it is true that the immune system can control tumor generation and growth, employing host lymphocytes appears to represent a more natural and less dangerous "drug" method to eliminate tumor cells [64, 71, 73, 88]. Several attempts have been made in trying to stimulate the reaction of host lymphocytes against tumors, stimulating both the innate or the adaptive arm of the immune system. However, as our knowledge of the mechanisms of the regulation of the anti-tumor response is currently limited, the therapeutic effect of immunotherapy is not satisfactory. It has become evident that tumor cells can escape immune system-mediated control in several ways, and an understanding of the molecular mechanisms by which this can happen is essential to plan any kind of immunotherapeutic schedule [64, 71, 73, 88].

It is generally accepted that cytotoxic $\alpha\beta$ or $\gamma\delta$ T lymphocytes (CTL) and natural killer (NK) cells play a role in limiting tumor cell expansion [36, 45, 52, 56, 57, 62, 64, 67, 71, 73, 88, 103, 113]. However, tumor cells can escape the immune system in several ways. As cancer cells are autologous cells, they do not elicit a strong reaction by the immune system. Indeed, anti-tumor response can be down-regulated by the same mechanisms which avoid immune system reaction to self-antigens. Apparently, while T lymphocytes can eliminate cancer cells by recognizing tumor-associated antigens (TAA) presented within self HLA class-I (HLA-I) antigens, NK cells can kill cancer cells which do not express

self HLA-I. Indeed, in physiological conditions it is thought that members of the inhibitory receptor superfamily (IRS) expressed by NK cells interact with self HLA-I and deliver an inhibitory signal leading to down-regulation of cytolitic activity; this mechanism is missing in the absence of HLA-I [56, 57, 62, 67, 103]. However, the large majority of tumor cells isolated from cancer patients express HLA-I but not TAA, and this might be why they are insensitive to both NK cell- and T lymphocyte-mediated killing. It was recently claimed that cancer immunotherapy using active immunization with dendritic cell (DC) vaccines for solid tumors has shown unsatisfactory results, with an objective response rate of <3% [88]. On the other hand, more encouraging results have been reported using adoptively transferred anti-tumor T cells, especially in melanoma treatment [88]. Here we will briefly analyze some of the classical mechanisms of tumor escape and focus our attention on molecular mechanisms by which tumor cells can escape innate immune-mediated control.

THE MECHANISMS OF TUMOR ESCAPE FROM LYMPHOCYTE-MEDIATED CONTROL

It is evident that many tumors must escape the host adaptive immune response as they can grow and kill the host. Indeed, tumors are composed of autologous cells and thus have a low immunogenic potential. Tumor cells can escape immune-mediated control by several molecular mechanisms (Fig. 1) that lead to subversion of anti-cancer response [5, 7, 17, 20–22, 28, 29, 32, 33, 38, 42–44, 48, 51, 86, 87]. These mechanisms include: 1) alteration of the expressions of classical and non-classical human leukocyte antigens (HLAs) and/or loss of tumor antigens, 2) loss of co-stimulatory molecules which are essential in inducing a powerful immune response, 3) the production of cytokines which are strongly immunosuppressive, and 4) induction of anergy or clonal deletion or suppressor cells.

Tumor cells can lose one or more HLA-I alleles, thus impairing the presentation of tumor-derived antigenic peptides. The loss of HLA antigens can be accomplished by several mechanisms, such as deletion of β_2-microglobulin and the loss of peptide transporter function [17, 43]. However, the loss of HLA-I molecules could promote the innate immune response, recruiting NK and γδ T cells; indeed, these lymphocyte subsets, expressing inhibitory isoforms of IRS, in absence of the corresponding ligand are able to kill tumor cells [5, 22, 32, 36, 57, 62, 67, 113]. However, it is possible that immunoselective pressure can give rise to resistant variants that have lost some HLA alleles which are involved in peptide antigen presentation, while they have up-regulated other alleles or non-classical HLA molecules (as HLA-E) which are recognized by inhibitory receptors, thus inducing the down-regulation of anti-tumor response [5, 22, 32, 36, 57, 62, 64, 67, 113].

Tumor cells can down-regulate molecules such as ICAM1 and CD40, reducing the interaction with LFA1 or CD40L on effector lymphocytes and impairing the triggering of the lytic machinery [29, 33, 42, 48]. Furthermore, the lack of co-stimulatory molecules at the tumor cell surface or on antigen-presenting cells infiltrating the neoplasia can impair the T cell-mediated immune response [21, 29, 33]. On the other hand, the expression of ligands, such as B7-H1 on different tumors, which have negative effects on anti-tumor cells should be taken into account as targets for therapy [44].

In the tumor microenvironment, tumor and/or immune cells can produce cytokines that can exert an immunosuppressive effect. Transforming growth factor (TGF)-β and interleukin (IL)-10 (Fig. 1) are the best known, although several others, such as IL-6, may be involved in inducing the T cell dysfunction [28, 33, 51, 86]. Furthermore it should be noted that, in principle, any cytokine may have different, and in may instances opposite, effects on immune response and it is thus difficult to assign a cytokine a precise role as favoring or not favoring tumor-cell escape [28, 33, 51, 86]. Indeed,

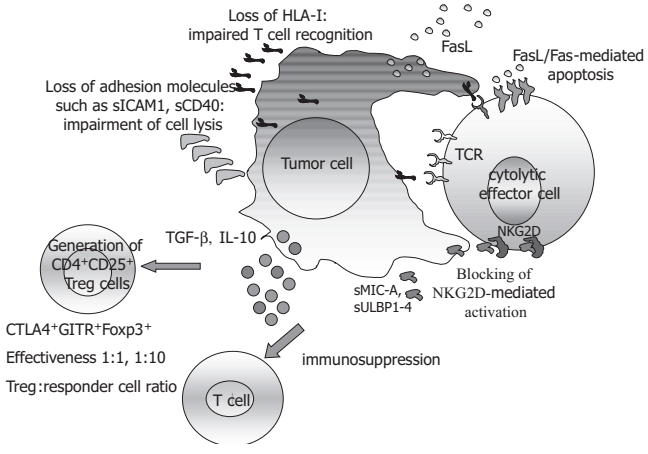

Fig. 1. Mechanisms of tumor-cell escape from immune-mediated control. Tumor cells can escape from immune system-mediated control through different mechanisms: 1) loss of expression of HLA-I molecules result in impairment of T cell-mediated recognition; 2) loss of adhesion molecules, such as ICAM1, the counter receptor of LFA1, or CD40, can render tumor cells less susceptible to cytolytic effector cells; 3) tumor cells can also release soluble factors, such as TGF-β and IL-10, that have a role in the direct immunosuppressive effect on T cell proliferation or these cytokines are important for the generation of $CD4^+CD25^+$ regulatory T cells (Treg) which are effective at 1:1-1:10 Treg:responder cell ratios and express CTLA4 and GITR besides the transcription factor Foxp3; 4) tumor cells can release MIC-A and ULBP1-4, which are the counter-ligands of NKG2D activating receptor, resulting in blocking NKG2D activation of cytolytic effector cells; 5) the release of sFasL by tumor cells by interacting with Fas expressed by anti-tumor effector cells can induce their apoptosis.

interferon (IFN)-γ can, on the one hand, reduce the immunosuppression induced by tumor-infiltrating macrophages and, on the other, reduce the expression of some tumor antigens [2, 7]. FasL released by tumor cells interacting with Fas expressed on anti-tumor effector cells can induce cell death [64, 87]; it is of note that FasL expression in melanoma is weak and rare in primary lesions, while in metastasis it is higher. It has been suggested that FasL may derive not only from tumor cells, but also from anti-tumor lymphocytes, leading to cell death of anti-tumor effector cells [4, 9, 24, 70].

Finally, the $CD4^+CD25^+$ regulatory T cells have been considered important players in immunological tolerance not only towards normal self cells, but also tumor cells. Indeed, a depletion of $CD4^+CD25^+$ regulatory T cells can prevent tumor progression [39, 40, 90, 91, 96, 106].

RELEASE OF SOLUBLE RECEPTORS AS COUNTER-LIGANDS OF THE ACTIVATING RECEPTOR NKG2D AS AN ADDITIONAL TUMOR ESCAPE MECHANISM

Besides soluble receptors that induce apoptosis, such as sHLA-I, tumor cells can release soluble counter-receptors of activating surface structures expressed by effector cells to avoid recognition by the immune system. Indeed, it has been shown that soluble MIC-A (sMIC-A) molecules are actually released by cancer cells and are present in the sera of patients affected by gastrointestinal tumors [6, 25, 46, 47, 55, 74, 81, 92, 93]. Thus it is conceivable that the NKG2D activating receptor on cytolytic effector T (either $TCR\alpha\beta^+$ or $TCR\gamma\delta^+$) and NK cells can bind to sMIC-A instead of MIC-A expressed by tumor cells. In addition, it has been reported that sMIC ligands impair NKG2D expression and consequent NKG2D-mediated cell activation [6, 25, 46, 47, 55, 74, 81, 92, 93]. These two mechanisms may allow tumor cells to evade immune system-mediated control. If this is true, it would be essential to analyze the mechanisms by which tumor cells can shed MIC-A in order to prevent or block this loss. Thus by preserving MIC-A at the tumor cell surface, effector cells could deliver the lethal hit and eliminate tumor cells. Like MIC-A, other NKG2D ligands, such as ULBPs, which are found in the sera of leukemic patients [81], might be released by tumor cells and contribute to tumor escape (Fig. 1).

TUMOR-INFILTRATING MACROPHAGES MAY LIMIT T CELL ACTIVATION AND PROLIFERATION BY INTERFERING WITH AMINO-ACID METABOLISM

Tumor-associated macrophages can inhibit T lymphocyte triggering and proliferation by affecting the metabolism of amino acids [11, 14–16, 18, 23, 26, 27, 34, 41, 49, 66, 69, 72, 89, 94, 104, 105, 109]. Indeed, myeloid suppressor cells have been identified within tumors [14, 15] These cells, in mice, express CD11b and GR1 antigens and may inhibit T and B cell activation induced by antigen or polyclonal stimuli, using an MHC-independent mechanism that needs cell-cell interaction [14, 15]. They regulate T cell activation and consequent proliferation by affecting the metabolism of L-arginine through the enzymes arginase and nitric-oxide synthase [11, 14]. The expression and function of these enzymes are regulated by cytokines produced by T helper 1 (Th1) and Th2 cells, such as IFN-γ, IL-4, and IL-13. Arginase activity causes translational blockade of the ζ-chain of CD3 (Fig. 2), while nitric-oxide synthase, by inducing cyclic GMP, interferes with the IL-2 receptor signaling pathway, reducing the phosphorylation of signal-transducing molecules that are coupled to the IL-2 receptor [11, 14, 72]. Myeloid cells can also modulate the expression of another enzyme, indoleamine 2,3-dioxigenase (IDO), which metabolizes the amino acid L-tryptofan [34, 41, 49, 89, 104]; in turn, the production of kynurenines inhibit the proliferation of T and NK cells [41]. Thus it has been suggested that L-arginine and L-tryptophan metabolism in myeloid cells are non-redundant, alternative systems that control T cell responses to antigens [14] (Fig. 2).

HUMAN NK CELLS AS ANTI-TUMOR EFFECTOR CELLS: SOLUBLE HLA-I MAY INDUCE NK CELL APOPTOSIS THROUGH THE ENGAGEMENT OF ACTIVATING IRS AND/OR CD8

It is thought that human NK cells play a role in eliminating virus-infected cells as well as in controlling tumor cell growth [56, 57, 67, 103]. This lymphocyte population originates from bone marrow, where it resides and re-circulates from the peripheral blood. NK cells are the most cytolytic effector lymphocytes which can kill target cells without prior sensitization [103]. Differently from CTL, NK cells recognize targets lacking HLA-I and peptide. Paradoxically, NK cell-mediated activities are down-regulated by interacting with HLA-I. Indeed, NK lymphocytes are characterized by the surface expression of receptors for self HLA-I molecules, including some members of the IRS, whose engagement leads to the inhibition of cytolysis and cytokine production. Thus, according to the missing self hypothesis, NK cells can kill targets that do not express (or express low levels of) HLA-I, such as tumor and virus-infected cells [56, 57, 67, 103]. The identification of isoforms of IRS that deliver an activating signal in NK cells that triggers cytolytic activity and cytokine release indicates that NK cells can be actually activated by self HLA-I [56, 57, 67, 103]. To reconcile these conflicting findings, it has been stated that the negative signal delivered via inhibiting IRS always overcomes the activating ones when these two opposing signals occur in

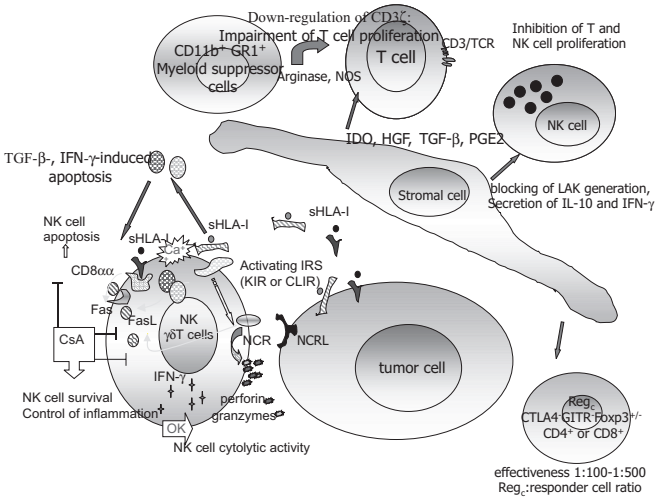

Fig. 2. Role of the microenvironment in tumor-cell escape. Natural killer or T lymphocytes (expressing either TCRγδ or αβ) may be induced to apoptosis by the interaction with soluble HLA-I (sHLA-I) derived from tumor cells (e.g. after tumor cell lysis): this HLA can interact with either CD8 (any kind of HLA) or activating isoforms of inhibitory receptors (IRS; killer Ig-like receptor: KIR; C-lectin type inhibitory receptor: CLIR) (discrete HLA alleles for a given IRS member) leading to FasL synthesis and secretion, which in turn triggers lymphocyte cell death interacting with Fas antigen. Upon the engagement of CD8 and/or activating IRS, effector cells can also secrete IFN-γ and TGF-β, which can induce apoptosis by themselves. All these effects are blocked by lymphocyte treatment with the immunosuppressive drug cyclosporin A (CsA). Indeed, CsA down-regulates FasL, IFN-γ, and TGF-β transcription and their secretion. On the other hand, the engagement of natural cytoxicity receptors (NCRs) by putative NCR ligand (NCRL) expressed on tumor cells triggers the activation of effector cell apoptosis via FasL/Fas interaction. In the tumor microenvironment are different cell types able to down-regulate effector-cell activation. Indeed, myeloid suppressor cells, expressing CD11b and GR1, can inhibit T cell proliferation by reducing CD3ζ expression and IL-2-receptor-mediated signal transduction by arginase and nitric oxide synthase (NOS), respectively. An intriguing hypothesis is that, like mesenchymal stem cells isolated from the bone marrow, tumor stromal cells, by releasing indoleamin dyoxigenase (IDO), tryptophan metabolites, hepatocyte growth factor (HGF), TGF-β, and prostaglandin E2 (PGE_2), may also inhibit T cell proliferation and survival. This idea is supported by the finding that tumor fibroblasts express these factors and can inhibit T cell proliferation to antigen and polyclonal stimuli in a dose-dependent manner. Co-culture of stromal cells and peripheral blood lymphocytes can induce the generation of $CTLA4^{-}GITR^{-}$ cells that display a strong regulatory activity (effective at 1:100–1:500 Reg_c:responder cell ratios). These cells may have a role in inhibiting anti-tumor cell response by inducing apoptosis of cytolytic effector cells. Furthermore, stromal cells may inhibit NK-cell proliferation induced by IL-2 and the generation of lymphokine-activated killer (LAK), thus reducing their anti-tumor activity and the efficacy of immunotherapy with IL-2.

close proximity. Alternatively, it has been shown that the affinity of inhibiting forms for a given HLA-I is higher than that of activating IRS [56, 57, 67, 103]. However, as both activating and inhibiting IRS are clonally distributed and each IRS can recognize one or a limited number of HLA-I alleles, it is possible that some NK cells bear an activating IRS and an inhibiting one for different HLA-I alleles; this implies that, to overcome the activating signals, inhibiting IRS are engaged together activating IRS by the corresponding HLA-I allele. Tumor cells could be eliminated only when they lack the HLA-I interacting with the inhibiting IRS or they could be better recognized if they express the HLA-I which binds the activating IRS.

Besides the allele-specific receptors, NK cells express the CD8αα homodimer [103]. This receptor can recognize any kind of HLA-I by interacting with the α3 constant region [60]. CD8 is usually expressed at lower levels on NK cells than on T cells [103], and while analyzing NK cell clones we found that they can be grouped on the basis of the percentage of $CD8^{+}$ cells and the level of expression of CD8 into $CD8^{dull}$, $CD8^{intermediate}$, and $CD8^{bright}$. Furthermore, we observed that CD8 antigen was present in the cytoplasm of all cells of $CD8^{dull}$ NK cell clones and that it was up-regulated on $CD8^{dull}$ and $CD8^{intermediate}$ cells along the culture period (45–60 days). The engagement of either activating IRS or CD8 (on $CD8^{bright}$ NK cells) by discrete alleles or any kind of HLA-I, respectively, can induce NK cell apoptosis. After the interaction of sHLA-I with either activating IRS or CD8, a strong increase in intracellular calcium concentration was detected and this calcium rise was mainly due to calcium influx from the extra-cellular milieu (Fig. 2). Calcium influx was needed for the induction of NK cell apoptosis, as either the calcium chelator EGTA or the L-type calcium-channel blockers verapamil strongly inhibited sHLA-I-induced apoptosis [99]. That actually activating IRS or CD8 are responsible for the induction of NK cell apoptosis was further reinforced by the finding that specific monoclonal antibodies to activating IRS or CD8 can block sHLA-I-mediated NK cell death. We also provided evidence that Fas/FasL interaction plays a key role in sHLA-I-mediated NK cell death. Indeed, sHLA-I-induced *de novo* transcription of mRNA coding for FasL and FasL was detectable in the culture supernatant of NK cells incubated with sHLA-I; this suggests that interaction of sHLA-I with either activating IRS or CD8 delivers an activating signal, leading to the synthesis and secretion of FasL which, in turn, binds to Fas and induces NK cell death. This interpretation of our results is further supported by blocking experiments with anti-Fas antibody and by neutralization of NK supernatant with anti-FasL monoclonal antibody [99]. The role of CD8 on NK cells is still under investigation. Interestingly, we have found that NK cell-mediated lysis of both $HLA\text{-}I^{+}$ and $HLA\text{-}I^{-}$ tumor target cells is independent of the level of CD8 expression and that the engagement of CD8 does not trigger NK cell killing. This indicates that CD8 is not essential for the activation of NK cell cytolysis. On the other hand, NK cell clones expressing a given activating IRS can lyse a tumor target cell bearing the right HLA-I alleles through the release of perforins and granzymes initiated by the engagement of activating IRS [99–101].

REGULATION OF CD8- AND ACTIVATING IRS-MEDIATED NK CELL APOPTOSIS BY THE IMMUNOSUPPRESSIVE DRUG CYCLOSPORIN A

Furthermore, we found that FasL-induced apoptosis of NK cells is strongly reduced by the immunosuppressive drug cyclosporin A (CsA). CsA treatment of NK cell clones expressing activating receptors or CD8 may therefore be a tool to maintain the killing of autologous tumor target cells without the elimination of these really potent cytolytic effector cells. In fact, CsA did not inhibit activating receptor-mediated cytolysis or the triggering of NK cells via CD16 or other activating NK cell receptors as CD69, suggesting that CsA did not affect NK cell-mediated killing [101]. It has been reported that cytolytic effector cells can lyse tumor target cells by two independent mechanisms: degranulation of perforin/granzyme and a FasL-mediated mechanism [13, 52]. In fact, several tumor target cells express Fas antigen at the cell surface and can die upon Fas engagement by FasL [52]. CsA administration could then block the second cytolytic pathway of inducing tumor target cell death. Thus, to plan a CsA treatment of patients affected by neoplasia, one should consider that a certain tumor can show different sensitivities to cytolysis mediated via the two above-mentioned mechanisms. In fact, one should first define whether a correlation exists between tumor histotypes and their sensitivity to CsA--treated NK cells in order to select which tumor is much more sensitive to cytolysis via perforin/granzymes. Secondly, CsA treatment can strongly augment the frequency of NK cells bearing activating receptor for self HLA-I antigens and thus, besides the potential and wished elimination of tumor cells, it can evoke an undesired powerful auto-immune reaction.

THE ENGAGEMENT OF CD8 OR ACTIVATING IRS BY HLA-I LEADS TO IFN-γ AND TGF-β PRODUCTION

The interaction of sHLA-I with either CD8 or activating IRS not only leads to NK cell death, but it can induce the production and secretion of detectable amounts of IFN-γ [101] and TGF-β (unpublished observation). The physiological significance of this effect is still to be defined. One can speculate that IFN-γ can, by activating antigen-presenting cells and thus by favoring optimal antigen presentation to T lymphocytes, induce a Th1-type immune response, typical of the antiviral host response. In this context, IFN-γ production which accompanies NK cell death induced by sHLA-I may be useful in switching from the innate to the adaptive immune response.

In addition, IFN-γ can also up-regulate the expression of HLA-I on target cells, possibly leading to a stronger inhibitory effect on NK cell-mediated functional activities via its interaction with the inhibitory counter-IRS. At the same time, IFN-γ can increase the shedding of HLA-I from tumor cells and thus increase the degree of NK cell apoptosis upon interaction with CD8 and/or activating IRS. This effect could take place anywhere, either far from tumor cell localization or in the close proximity of tumor cells. Thus, inactivation of NK cells with activating receptors is obtained by their own apoptosis far from tumor cells without any evident effect on tumor cells. Again, we found that IFN-γ production induced via CD8 and/or activating IRS was CsA dependent. This suggests that CsA treatment can reduce, at the same time, NK cell apoptosis and production of cytokines which may favor sHLA-I release from tumor cells or up-regulation of HLA-I at the tumor cell surface. Thus sHLA-I derived from tumor targets can interact with CD8, thus inducing NK cell death without the need of NK-target cell interaction. On the other hand, NK cells, during interaction with target cells, might receive an apoptotic signal through the direct binding of CD8 and/or activating IRS with HLA-I expressed by the target cell. These phenomena may play a key role in regulating the response of innate immunity against tumors. In this context we found NK cell apoptosis at sHLA-I concentrations as low as 1 pg/cell, and this amount is high compared with that of HLA-I present on a single lymphocyte (0.1 fg/cell), it is therefore possible that tumor targets down-regulate NK cell activity when high amounts of cancer cells are dying, such as in a necrotic portion of the tumor. In addition, tumor cells might evade the control of innate immunity simply by releasing sHLA-I, which in turn leads to NK cell apoptosis. This could explain, at least in part, how tumors with a low amount of HLA-I at the cell surface escape both NK cell-mediated killing (by releasing HLA-I) and T cell recognition (by reducing the presentation of tumor-specific antigens).

The release of TGF-β from NK cells upon interaction with sHLA-I may further down-regulate NK cell survival by inducing their apoptosis and impairment of NK cell-mediated cytotoxicity [8, 112]. In conclusion, apoptosis of NK cells through sHLA-I/CD8 or sHLA--I/activating IRS interaction could play a role in switching off NK cell-mediated cytolysis of tumor cells. The finding that CD8α is not essential for NK cytolysis suggests that blocking CD8-mediated apoptosis would be a useful tool to enhance the anti-tumor activity of NK cells.

ACTIVATION-INDUCED CELL DEATH: SUICIDE OF CYTOLYTIC EFFECTOR CELLS UPON TUMOR TARGET CELL INTERACTION

Apoptosis, or programmed cell death (PCD), represents a cellular mechanism playing a role in regulating the integrity of the immune system during the host's lifetime. PCD is a controlled process that utilizes regulating signaling cascades and leads to the organized breakdown of cellular structures [85, 107]. It is conceivable

that any time an immune response is triggered, highly regulated negative feed-back mechanisms should exist to switch down this response [85, 107]. This would also occur during anti-tumor immune response. Indeed, it has been reported that TCR$\gamma\delta^+$ T cells can undergo apoptosis upon interaction with Daudi lymphoma target cells [35, 61]. This phenomenon is called activation-induced cell death (AICD) and it should occur to avoid, after the elimination of tumor cells, an undesired reaction against autologous normal tissue. However, AICD could favor at the same time the survival of unrecognized tumor cells and facilitate tumor escape. Importantly, AICD can be down-regulated by the treatment of TCR$\gamma\delta^+$ T lymphocytes with caspase-3 inhibitors [35], thus allowing these cells to maintain their anti-tumor activity for a longer time. This indicates that blocking the executioners of cell death, such as caspases, may reinforce the immune response to cancer cells.

We have shown that that NK cells undergo apoptosis upon interaction with tumor cells through the engagement of the recently described natural cytotoxicity receptors [68]. Indeed, the cross-linking of NKp30, NKp44, or NKp46 induces the up-regulation of FasL transcription, synthesis, and release [78]. This, in turn, triggers the apoptosis of NK cells by interacting with Fas at the NK cell surface (Fig. 2). Thus it is conceivable that tumor-induced effector cell apoptosis functions as a potential mechanism of tumor escape. Indeed, any time a powerful cytolytic NK lymphocyte encounters a tumor cell, this interaction leads to the damage of the target and eventually to its death, but also to NK cell suicide. This event appears to be a consequence of the triggering of cytolytic activity; NK cell suicide might therefore also represent an intrinsic regulatory mechanism involved in switching off cytolysis in order to block dangerous NK cell-mediated effects on healthy host cells. Based on these considerations, the anti-tumor response might be down-regulated by the same molecules whereby it is triggered. This would greatly decrease the efficiency of immunotherapy of tumors, which is largely based on the ability of inducing potent cytolytic effector cells. In addition, the ratio between NK effector cells and tumor target cells should be taken into consideration. Indeed, in our experiments, NK cell apoptosis was evident at low effector-target ratios, e.g. 2:1 or 1:1, while it was negligible at 10:1. This would imply that, to perform an effective anti-tumor trial by infusing cytolytic effector cells, one should employ not only the most powerful anti-tumor cells, that is NK cells activated with high doses of IL-2 [56, 57, 62, 99, 100, 101, 103], but also an amount of NK cells tenfold higher than that of tumor cells.

In this regard, the finding that CsA can selectively inhibit the apoptosis of NK cells, but not their cytolytic function, would provide a potential tool to avoid NK cell death after interaction with tumor targets. Indeed, CsA would prolong both NK cell survival and the anti-tumor effect exerted by NK cells. This may allow the usage of low amounts of NK cells for immunotherapy, while discontinuation of CsA might render NK cells again susceptible to apoptosis after interaction with tumor cells, thus avoiding potentially harmful reactions against healthy tissues.

A WORKING HYPOTHESIS: TUMOR STROMA AS A NOVEL PLAYER TO FAVORING TUMOR CELL GROWTH AND TUMOR-CELL ESCAPE

It is evident that effector lymphocytes to kill tumor cells should egress from the bloodstream and go through the stroma matrix [12, 19, 37, 77, 82, 83, 97, 98, 110, 111]. During this journey they may interact with stromal cells. In some instances, tumor cells produce a unique kind of stroma matrix which conceivably serves as an anchorage for tumor cells. These matrix proteins can be considered ideal targets for therapy by using engineered human antibodies [12, 54]. However, the role of stromal cells in facilitating tumor escape can be another than simply synthesizing and releasing these extracellular matrix proteins which physically limit effector cells' ability to interact with tumor cells. This viewpoint comes from the finding that within the bone marrow are the so-called mesenchymal stem cells (MSCs), which can down-regulate lymphocyte cell proliferation by direct interaction with T lymphocytes [1, 3, 10, 30, 31, 50, 53, 58, 59, 63, 65, 75, 76, 83, 84, 95, 108]. This effect is thought to be mediated by different soluble factors, including TGF-β, hepatocyte growth factor, prostaglandin E2, and IDO [1, 30, 65]. In addition, MSCs can impair the maturation of DCs and thus also affect the antigen-specific T cell response [1]. These immunosuppressive effects may both favor the engraftment of bone marrow transplants and avoid the graft-versus-host disease (GVHD). Indeed, the administration of MSCs has been found to abolish GVHD in a young patient who underwent bone marrow transplantion, strongly supporting their employment in the regulation of immune responses [59]. Of course, MSCs are, by definition, able to differentiate into functional cells of mesodermal origin such as condrocytes, osteoblasts, and adypocytes, or even into cells of ectodermal origin such as neurons, although they express a surface phenotype similar to that of fibroblasts [95, 108]. For this reason, we tested the hypothesis that fibroblasts can also exert an immunosuppressive effect similar to that mediated by MSCs. We found that both the mixed lymphocyte reaction as well as CD3-driven lymphocyte proliferation can be inhibited in a dose-dependent manner by the presence of autologous foreskin fibroblasts or fibroblasts from lung tumors (unpublished observation). In these culture conditions, cells (Reg_c) lacking CTLA4 and GITR and with a strong regulatory activity were generated. Indeed, either $CD4^+$ or $CD8^+$ Reg_c inhibited T cell proliferation at 1:100–1:500 Reg_c:responder cell ratios, a 10–500 times higher effectiveness than the conventional $CD4^+CD25^+$ Treg (Fig. 2). Furthermore, co-

-culture of stromal cells with peripheral blood NK cells in the presence of IL-2 (generation of lymphokine-activated killer activity) was strongly impaired. If this is true also for stromal cells isolated from different solid tumors, an additional cell player for inducing tumor-cell escape would be present within the tumor site. The finding that autologous NK cells can kill stromal cells [79, 80, 102] may imply that NK cells can down-regulate stromal cell-mediated inhibition of lymphocyte activation.

CONCLUDING REMARKS

It is clear that immunotherapy can represent an additional tool for fighting tumor cell growth. However, too many cells and factors may play a role in facilitating tumor cell growth and tumor escape from immune-mediated control. Tumor cells are self cells which have lost the cell-growth control typical of mature cells, and for this reason they can survive in an hostile microenvironment where normal cells do not. To plan effective immunotherapy, one should consider that the survival of effector cells is the first step to getting an effective response to tumor. In addition, we should keep in mind that any kind of cell found within the tumor microenvironment may have a double-faced role. Indeed, the inhibition of T cell proliferation exerted by tumor associated macrophages or stromal cells can be the side-effect of the effort of the host of inhibiting tumor cell proliferation. The ability of tumor cells to proliferate at a higher rate than effector lymphocytes can be the mechanism by which tumor cells can escape any kind of control, thus favoring the rise of resistant tumor cell variants.

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