The Requirements for CTL-Mediated Rejection of Cancer in Humans: NKG2D and Its Role in the Immune Responsiveness of Melanoma

□□ *Commentary on Maccalli et al., p. 7459*

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The Contemporary Understanding of the Role of α/β+ CTLs in the Control of Human Cancer Growth and Its Rejection: What Are We Missing?

The contemporary understanding of the role played by CTLs that recognize tumor-associated antigens (TAA) in association with human leukocyte antigen class I molecules can be summarized as follows: (*a*) the responses of CTLs against cancers occur naturally; this is clearly exemplified by the frequent identification of TAA-specific tumor-infiltrating lymphocytes (TIL) isolated from several tumor types and, in particular, from melanoma metastases (1); (*b*) naturally occurring immune responses can also be observed at the systemic level as shown by the expansion of TAA-recognizing CTLs from peripheral blood lymphocytes of patients with metastatic melanoma (2); (*c*) these natural immune responses result from *in vivo* priming of host-circulating lymphocytes by TAA-expressing tumors (3); (*d*) these naturally observed immune responses can be enhanced by active-specific immunization with TAA-based vaccines that reproducibly increase the number of circulating (4, 5) and intratumoral (6) tumor-recognizing CTLs; and (*e*) this increase in number of TAA-recognizing CTLs is not sufficient by itself to induce cancer rejection (Fig. 1; ref 7). Thus, the recent advancement in the understanding of human tumor immunology yielded one of the most specific anticancer agents but has left us with the sobering task of better understanding the *in vivo* requirements for the complete activation of CTL function in the target organ (8), an understanding that may go beyond tumor immunology and may have to do with the understanding of the mechanisms of CTL-mediated tissue-specific destruction in the context also of acute allograft rejection, flares of autoimmunity, and response to acute infection (9).

Real-time assessment of transcriptional changes occurring during immune-mediated acute tissue destruction in various human models has suggested that together with the activation of IFN-stimulated genes, an additional requirement consists in the activation of genes associated with the effector mechanism of CTLs and natural killer (NK) cells (10–12). This finding could be explained by the understanding that antigen-specific circulating CTL display *in vivo* a quiescent phenotype deprived of most effector functions but gain an effector phenotype that overlaps that of NK cells when activated *in vitro* through antigen exposure in the presence of proinflammatory stimuli (13).

NKG2D as a Putative Biomarker of the Status of CTL Activation within the Tumor Microenvironment

NKG2D is part of the human NK complex that encodes for the leukocyte C-type lectins, CD69, the activation-induced C-type lectin AICL; the lectin-like transcript–1, CD161 and CD94; and all the NKG2 molecules. It seems that NKG2D expression is physiologically associated with the activation of lymphocytes (14, 15); NKG2D ligands comprise a diverse array of human leukocyte antigen class I–related (also called nonclassic major histocompatibility) proteins that are generally expressed in condition of stress; it is believed that their expression increases the ability of CTLs and/or NK cells to exert their effector mechanisms against target cells stressed by viral infections or other noxious conditions (15). Maccalli et al. (16) investigated, in the study concurrently published in this issue of *Clinical Cancer Research*, the expression of NKG2D by TILs, addressed its physiologic function *in vitro*, and tested whether NKG2D ligands are actually expressed by tumor tissues. This was done in the context of one of the most immune responsive of human tumors: primary and metastatic cutaneous melanoma. We have previously observed that melanomas differ at the global transcriptional level from other less immune responsive tumors because they display a unique immunologic signature that is associated with improved survival (17), and it is predominantly inclusive of transcripts associated with NK cell/activated CTL effector functions (18). In particular, several NK receptor group genes were consistently found. In this context, this study provides a first in-depth analysis of an important NK/CTL-associated gene. This analysis may yield insights about the functional requirements for activation of TILs or circulating TAA-specific CTLs; a variable beyond their simple enumeration and assessment of their ability to produce cytokines such as IFN-γ, in response to antigen exposure, which may not comprehensively represent the status of activation of CTLs. This is suggested by animal models showing that effector CTL function is short lived after antigen exposure lasting through the week-long expansion phase. During this phase, the expression of cytotoxic molecules is transient and quickly disappears during the contraction phase within 2 weeks after antigen exposure. Yet the ability of CTLs to produce IFN-γ is retained during this memory/quiescent phase (19, 20). More

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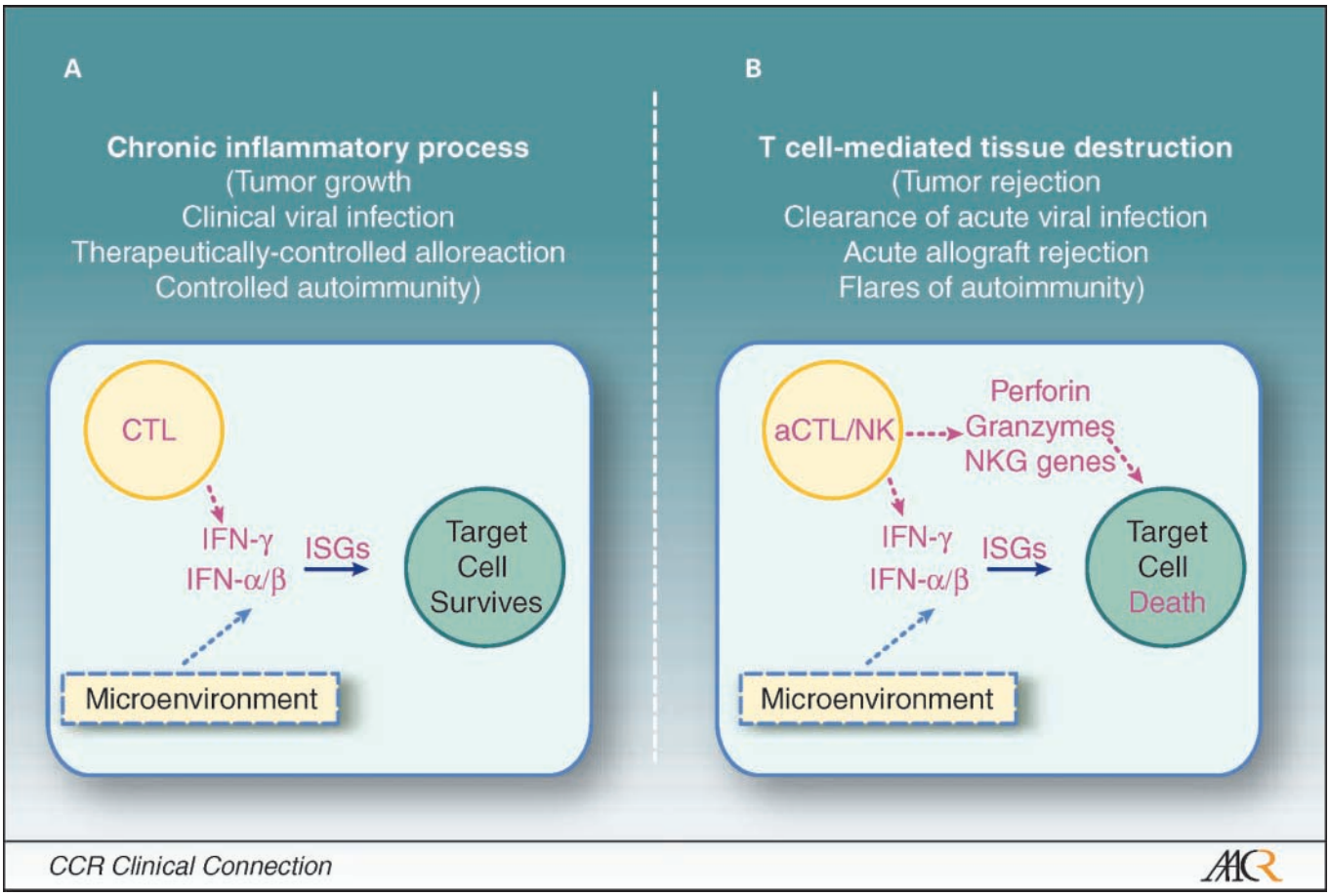

Fig. 1. Relevance of NK cell – type differentiation for tissue-specific rejection. A common pattern observed by investigators studying CTL activation/differentiation seems to point to a the expression of cytotoxic molecules as the landmark of maximally activated CTLs (10–13, 19, 21). *A*, a chronic inflammatory condition is displayed in which IFN-stimulated genes induced by the production of type-one IFNs (*IFNα/β*) by different cells in the tumor microenvironment or IFN-γ by immune cells including CTLs are often observed by genetic profiling analysis. In chronic inflammatory conditions, the expression of genes associated with NK cell/activated CTL function such as granzymes, perforin, and NK receptor group genes is never observed. *B*, the consistent addition of such effector molecules to IFN-stimulated genes signatures seems to be associated with the elimination of NK cell/CTL targets cells, resulting in either the elimination of unwanted pathogenic processes (virally infected cells and cancer cells) or the destruction of normal tissues (autoimmune phenomena) or allografts (acute rejection).

recently, Peixoto et al. (21) showed that in humans, effector/memory CTLs come in different flavors and mixed phenotypes. The expression of effector molecules by CD8 T-cells can be quite heterogeneous at different time points after primary or secondary antigen exposure, but it is characteristically cocoordinated at the single cell level with cytotoxic and NK-type molecules expressed specifically by terminally activated CTLs. Maccalli et al. (16) observed by immunohistochemistry in 10 melanoma lesions (one primary and nine metastatic lesions) that most CD3-expressing T cells expressed NKG2D in contrast with other CD3-expressing CTLs infiltrating normal tissue or nontumor cell–bearing lymph nodes. Short-term *in vitro* expansion of TILs from the same lesions as well as analysis of established TAA-specific CTL clones derived from bulk TIL cultures corroborated the expression of NKG2D by direct cell staining by fluorescence-activated cell sorting analysis. This showed, in addition, that NKG2D expression is peculiar of CD8-expressing T cells compared with CD4-expressing T cells, supporting a possible association of the expression of this marker with a development of an NK cell-type, highly activated CTL.

If NKG2D Expression by CTLs Is Relevant to Tumor Growth, How Are Tumors Infiltrated by NKG2D+ TILs Escaping Recognition?

A dynamic entity, such as a growing tumor mass, must strike a favorable balance against the effector antitumor mechanisms of the host; otherwise, it would not be there. This can be achieved by the *de novo* production of factors that may alter the physiology of immune mechanisms or by the loss of those molecules that may represent targets of immune recognition, a phenomenon called immune escape (22–24). Macalli et al. (16) investigated the expression of NKG2D ligands by immunohistochemistry in frozen section of the same melanoma lesions used for the analysis of NKG2D expression by CTLs. They observed that only the primary lesion expressed the full range of NKG2D ligands, whereas metastases were quite heterogeneous and overall scant in such expression. Importantly, it was observed that NKG2D ligands were more consistently expressed in *in vitro* established melanoma cell lines compared with melanoma tissue

sections. This finding, if not due to the higher sensitivity of the detection methods that could be used for the study of the cell lines, may suggest that a negative selection of NKG2D ligand–expressing cells may occur *in vivo* that can then be reversed in culture conditions. The investigators observed that NKG2D is important in mediating the T cell receptor–dependent recognition of TAAs by CTLs as blocking with anti-NKG2D antibodies decreased the production of IFN-γ by CTLs exposed to the correspondent target cell. Thus, it is likely that NKG2D participates in the repertoire of molecules that significantly affect the recognition of cancer cells *in vivo*, and this may be counterbalanced by loss of the correspondent ligands by the target cells. This study, however, limited the functional assessment of NKG2D-expressing CTLs to their ability to release IFN-γ in response to antigen. In the future, it will be important to assess whether NKG2D downstream signaling may affect the cytotoxic potential and NK-type differentiation of CTLs.

Remaining Questions

This important study identified a naturally occurring likely mediator/potentiator of effector immune responses against human melanomas. As knowledge of the frequency of TAA-specific CTLs in the circulation or at tumor site is not a reliable variable of their anticancer potential, studies such as the present study addressing the quality rather than the number of TAA-specific CTLs will stimulate a new generation of investigations that is necessary if the requirements for CTL-mediated tumor destruction in humans ought to be understood. Obviously, the significance of this finding will need to be further evaluated in larger cohorts and patients, including patients at various disease stages, and the information will need to be related to clinical outcome. As the immune response to tumors is a multifactorial phenomenon, it is unlikely that a perfect correlation will be identified between expression of NKG2D and its ligands on one side and clinical outcome on the other. Yet, any correlation may provide insights that will help understand the requirements for CTL-mediated tumor rejection. This work provides an outstanding example of a translational effort to study directly the tumor microenvironment where tumor cell/host interactions occur. Although limited in its experimental breath, it provides a novel road map for future studies addressing the significance of this novel putative biomarker of immune responsiveness, and it should be praised as such. It should also be emphasized that this study did not address the mechanisms responsible for the enrichment of NKG2D–expressing CTLs in melanoma. It seems, from the study, that such expression is stable as it can be observed not only *in vivo* but also in short-term and long-term TIL cultures. The *in vitro* conditions included the presence of the immunestimulatory cytokine interleukin-2, however, that could by itself induce the expression of various NK cell regulatory genes (25). Thus, it would be interesting in the future to study whether NKG2D expression by TIL *in vivo* is the result of a permanent modification of their phenotype or represents a reaction to microenvironmental factors associated with melanoma progression/regression and, in any case, a stage of their activation. In addition, future studies will need to address whether NKG2D is a biomarker of CTL differentiation or actually is primarily responsible for their coactivation through a partially T cell receptor–independent signaling cascade. This activation would be responsible for a differentiation of CTLs toward an effector, NK cell-type of fully cytotoxic cell. Moreover, it would be important to know whether the expression of NKG2D occurs independently or if it is a reflection of an activated status of CTLs that could be associated with the activation of several other genes of the NK receptor gene cluster. This analysis could be easily addressed by functional profiling of different CTLs phenotypes bearing or lacking NKG2D. Finally, the down-regulation of NKG2D ligands by melanoma metastases will need to be addressed at the physiopathologic level to test for its reversibility. Because NKG2D ligands belong to the major histocompatiblity complex family, their expression my be modulated by cytokines such as IFN-γ. In addition, epigenetic silencing of their expression may be modulated by demethylating agents. This information may provide a strategy to by pass tumor cell escape.

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