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Cancer Immunooediting of the NK Group 2D Ligand H60a

Timothy O’Sullivan,* Gavin P. Dunn,†,1 Daphne Y. Lacoursiere,‡ Robert D. Schreiber,† and Jack D. Bui*

Cancer immunooediting describes the process whereby highly immunogenic tumor cells are removed, or edited, from the primary tumor repertoire by the immune system. In immunodeficient mice, the editing process is hampered, and “unedited” tumor cells can be recovered and studied. In this study, we compared unedited and edited tumors for their expression of NK group 2D (NKG2D) ligands, a family of surface proteins expressed on tumor cells that can activate NK cell cytotoxic activity. We found that the expression of the NKG2D ligand H60a was more heterogeneous in groups of unedited 3’-methylcholanthrene sarcoma cell lines compared with that in edited 3’-methylcholanthrene sarcoma cell lines (i.e., some unedited cell lines expressed very high levels of H60a, whereas other unedited and edited cell lines expressed very low levels). We also found that some highly immunogenic cell lines displayed a bimodal distribution consisting of H60a-hi and H60a-lo cells. In one of these cell lines, the H60a-hi cells could be removed by passaging the cells through RAG2−/− mice, resulting in edited cell lines that were poor targets for NK cells and that displayed progressive tumor growth. This editing of H60a-hi cells required NK cells and NKG2D. Our studies show that the expression of H60a on tumors cells can be actively modulated by the immune system, thereby implicating this NKG2D ligand in tumor immunosurveillance. The Journal of Immunology, 2011, 187: 000–000.

Significant interactions between immune cells and tumor cells occur during tumor development and can lead to tumor promotion or tumor destruction (1). These interactions have been codified into a cancer immunooediting process that results in the generation of a tumor cell repertoire capable of surviving in immunocompetent hosts (2–4). The substrate for the editing process is thought to be a primary repertoire of nascently transformed immunogenic cells, so called “unedited” cells. In the most complex form of cancer immunooediting, the immune system acts on the unedited tumor cell repertoire by eliminating immunogenic tumor cells, leading to an equilibrium process (5) that eventually culminates in the escape and outgrowth of an edited repertoire of poorly immunogenic tumor cells.

We previously found that adaptive lymphocytes and the IFN system are required for effective tumor surveillance (2–4, 6, 7). When 3’-methylcholanthrene (MCA) is given to mice that lack adaptive immunity (RAG2−/−) or responsiveness to type I IFN (IFNAR−/−), the tumor incidence is higher compared with that of syngeneic wild-type (WT) mice. Given the requirement for RAG2 and IFNAR in tumor surveillance, we reasoned that tumors that arise in these mice would be unedited. Indeed, we found that tumors from RAG2−/− or IFNAR−/− mice displayed increased immunogenicity (3, 6). In these studies, the immunogenicity of a cell line was measured by transplanting it into WT and RAG2−/− mice and measuring its growth. Cell lines that did not grow in WT mice but grew in RAG2−/− mice were termed regressor cell lines (3, 8) and were considered highly immunogenic. Progressor cell lines grew in both WT and RAG2−/− mice and are thought to be poorly immunogenic. We found that certain unedited tumor cell lines derived from immunodeficient RAG2−/− or IFNAR−/− mice displayed a regressor growth phenotype, whereas others displayed a progressor growth phenotype (3, 6). In contrast, all of the edited tumor cell lines derived from WT mice displayed progressor growth phenotypes. It is important to note that this assay of immunogenicity, involving tumor cell transplantation, is a functional assay that does not necessarily define a molecular target for cancer immunooediting. To date, a molecular assay has not been established that can reliably identify unedited versus edited tumor cell groups.

In this study, we compared the edited and unedited tumor cell repertoire by examining MCA sarcoma cell lines generated in carcinogen-treated syngeneic WT and immunodeficient mice (3, 6). We focused on evaluating the expression of ligands for the activating receptor NK group 2D (NKG2D) (9) within the unedited tumor repertoire. NKG2D is a receptor expressed on NK cells, CD8+ T cells, γδ T cells, and NKT cells that mediates the detection of stressed cells that are infected by viruses or undergoing transformation (10–14). It binds a ligand family that is generally not expressed at functional levels in normal tissues but can be upregulated by certain stimuli, including DNA damage and virus infection (10, 15). In mice deficient in NKG2D, there is increased development of prostate tumors and lymphomas compared with that in control mice, but the development of MCA sarcomas was not changed (12). In contrast, mice treated with blocking Ab to NKG2D developed more MCA sarcomas than control-treated mice (13), although it is not clear whether NK cell function was affected throughout the course of this study.

We have described that the NKG2D ligand H60a (16–19), but not other NKG2D ligands, is downregulated by IFN-γ in MCA

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Abbreviations used in this article: MCA, 3’-methylcholanthrene; NKG2D, NK group 2D; R&K, RAG2−/− × STAT1−/−; WT, wild-type.

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Materials and Methods

All of the experiments involving mice were conducted under animal protocols approved by the Washington University Animal Studies Committee and the University of California at San Diego Institutional Animal Care and Use Committee (IACUC Protocol No. S06201) and were in accordance with their ethical guidelines.

Cell lines and mice

MCA sarcoma cell lines were generated as described in Ref. 3 by injecting 129/Sv mice with 1–400 μg MCA (Sigma-Aldrich, St. Louis, MO) dissolved in peanut oil, harvesting the tumor mass after it reached >10 mm in average diameter, dissociating the mass via collagenase treatment, and culturing the cells in vitro. In all of the experiments were done with cells passed between 4 and 12 cycles. 129/Sv, RAG2+/−, RAG2+/− × STAT1−/− (RkSk), and STAT1+/− mice used were described in Refs. 3 and 6. Cell lines were maintained in RPMI 1640 supplemented with 10% FCS, 1-glutamine, nonessential amino acids, sodium pyruvate, sodium bicarbonate, penicillin/streptomycin, and 2-ME. The IFNAR2−/− mouse was activated by culturing in media containing 0.5–2 million total cells, 0.5–1 μl Ab, and 100 μl FACS buffer (PBS, 1% FCS, and 0.09% NaNO2; Sigma-Aldrich). All of the analyses were done on live cells identified by forward and side scatter properties. Significance of variance was determined between groups by using the F test. The Levene statistic was calculated to determine homogeneity within all of the groups, Welch’s t test, which accommodates unequal variances, was used to determine differences between the means of two groups. The median fluorescence intensity was used to quantify Ab binding.

Chromium release assay

Splenocytes from RAG2−/− mice were activated by culturing in media with 1000 U/ml human IL-2 (Chiron, Emeryville, CA). Day 7 IL-2-activated NK effector cells were used in a 4-h [51Cr] release assay using tumor target cells labeled with [51Cr] as described in Ref. 19 with either control IgG or anti-NKG2D. Bars depict the SEs of triplicates. All of the experiments were done at least twice. ANOVA was used to assess statistical significance between parent and passaged cell lines.

Results

Unedited tumors display a wide range of NKG2D ligand expression

Previous carcinogenesis studies comparing primary tumor formation in WT versus NKG2D-deficient mice found no significant difference in MCA sarcoma formation (12). However, these experiments were performed using mice on a C57BL/6 background, where H60a is not expressed (18, 27). Therefore, we measured NKG2D ligand expression in MCA sarcoma cell lines generated in WT mice and mice with varying levels of immunodeficiency (3, 6), all on a 129/Sv background, which display an intact H60a gene (28). Fig. 1 shows NKG2D tetramer staining of 39 129/Sv strain MCA sarcoma cell lines. We found that the range of NKG2D ligand expression was the greatest in the MCA sarcomas that developed in the most immunodeficient mice (RkSk or STAT1−/−). MCA sarcomas from RAG2−/− mice displayed moderate heterogeneity of NKG2D ligand expression, whereas edited MCA sarcomas that developed in immunocompetent WT mice had tightly grouped levels of NKG2D ligand expression. Although the mean level of NKG2D ligand expression was similar between the groups, using the F test to compare variances, we found that there was a significant difference in the variances of NKG2D ligand expression between WT and RkSk/STAT1−/− groups (p = 0.0079) and WT and RAG2−/− groups (p = 0.043), but not between RAG2−/− and RkSk/STAT1−/− groups (p = 0.166). We also calculated the Levene statistic to test the homogeneity of variances between all of the groups and found significant heterogeneity in NKG2D ligand expression (p = 0.007).

Regressor cell lines display a higher level of heterogeneous NKG2D ligand expression than matched regressor cell lines

We previously found that ~40% of unedited MCA sarcoma cell lines tested were rejected when transplanted into syngeneic, naive WT mice, whereas 60% grew progressively (3, 6). The regressor and progressor phenotypes of these cell lines were reproducible
and were only observed in tumor cell lines derived from immunodeficient mice (either RAG2−/− or IFNAR−/−). Edited tumor cell lines from WT mice all displayed regressor phenotypes. We determined whether the tumor growth phenotype was correlated with the expression of NKG2D ligands by examining unedited MCA sarcomas from RAG2−/− or IFNAR−/− mice that had already been categorized into regressor and progressor phenotypes (3, 6). We found that the heterogeneity in NKG2D ligand levels was associated with the regressor phenotype of unedited cells (Fig. 2). This difference in heterogeneity was significant when the variances of the populations were compared in the group of tumors (F test, p = 0.0024; Levene statistic, p = 0.001) from RAG2−/− but not IFNAR−/− mice.

H60a expression correlates with the heterogeneity in NKG2D tetramer staining seen in unedited tumors

We next examined whether the heterogeneous NKG2D tetramer staining could be explained by heterogeneous expression of a specific NKG2D ligand. We found that our MCA sarcoma cell lines did not express MULTI (19), so we focused on examining the expression of H60a and RAЕ1 using mAbs. We found that H60a expression was highly variable in the groups of unedited tumors (Fig. 3A). This mirrored our results with NKG2D tetramer staining. Interestingly, RAЕ1 expression, although lower than H60a expression, also displayed heterogeneity (Fig. 3B). For both H60a and RAЕ1, there were significant differences in variance between RAG progressor and RkSk/STAT1−/− groups (p = 0.0079 for H60a; p = 0.0014 for RAЕ1) and between RAG progressor and RAG regressor groups (p = 0.0087 for H60a; p = 0.001 for RAЕ1). The Levene statistic indicated that RAЕ1 had a slightly higher level of heterogeneity than H60a (p = 0.048 for RAЕ1 variance; p = 0.075 for H60a variance). Although the heterogeneity in the expression of H60a and RAЕ1 was found in similar groups of tumors, most regressor tumors expressed H60a, whereas few expressed RAЕ1. Therefore, we focused on H60a.

H60a staining was bimodal in many cell lines

In examining the expression of H60a in our cell lines, we noticed that the staining of H60a in some MCA sarcoma cell lines did not display unimodal staining, as would be expected of a homogeneous population of cells. We have chosen nine representative regressor cell lines (Fig. 4A) to demonstrate that the pattern of H60a staining ranged from unimodal (d27m2, F510, and h31m1) and broad staining (F515 and d30m4) to bimodal staining, with equal distribution (F535 and d100) between the two cell populations or uneven, “shoulder”-type distribution (d38m2 and d42m1), suggesting that within a cell line there were populations of cells that expressed differing levels of H60a. This pattern of staining did not...
change even after 3 weeks of in vitro passaging for the bimodal d100 cell line (Supplemental Fig. 1), indicating that it was a relatively stable characteristic of this cell line. RAE1 did not display this pattern of staining, even though in some regressors (d30m4 and d27m2) it was highly expressed.

Cell lines with bimodal expression of H60a lose H60a expression after in vivo passage

We were struck by the fact that bimodal H60a but not RAE1 expression could be seen in some cell lines (F535 and d100) and wondered whether H60a-hi cells could be edited by components
of the immune system in vivo. Because d100 and F535 are both regressor cell lines that reject in WT mice, we transplanted them into immunodeficient mice to test if partial immune function could eliminate H60a-hi cells in vivo. We transplanted d100 (derived from an IFNAR\(^{-/-}\) mouse) into RAG2\(^{-/-}\) mice and F535 (derived from a RAG2\(^{-/-}\) mouse) into IFNAR\(^{-/-}\) mice. After in vivo growth for 20–30 days, we harvested the tumor mass and generated cell lines. Fig. 5 shows that the passaged cell lines indeed had lower expression of H60a compared with that of the parental cell lines. Whereas 70% of parental d100 cells had high expression of H60a (as measured by NKG2D tetramer staining), this was significantly reduced to 35% in the passaged cell lines (Fig. 5A, 5C, \(p < 0.001\)). Likewise, two of the three passaged F535 cell lines completely lost the H60a-hi population of cells and became unimodally low in H60a expression, whereas the third passaged cell line had decreased levels of H60a-hi cells (Fig. 5B, 5D). The loss of H60a-hi cells seen after in vivo passagen was not seen in the same cell line cultured in vitro for a similar period of time (Supplemental Fig. 1), and therefore it is unlikely due to inherent differences in proliferation or cell cycle between H60a-hi and H60a-lo cells.

The regressor tumor d100 shows delayed growth in RAG2\(^{-/-}\) mice and increased NK cell infiltration

We next examined the cell types that could mediate the editing process that removed H60a-hi cells. We hypothesized that NK cells would infiltrate and selectively destroy H60a-hi cells. Therefore, we transplanted d100 and a control regressor cell line F244 that expresses moderate levels of H60a (Supplemental Fig. 2) into WT and RAG2\(^{-/-}\) mice. As shown previously (3, 29), d100 was rejected, but F244 grew progressively in WT mice (Fig. 6A). Interestingly, in RAG2\(^{-/-}\) mice, the regressor F244 developed a tumor mass >5 mm (mean diameter) within 10 days of transplant (Fig. 6B), whereas the d100 tumor remained ≤3 mm for >2 weeks after tumor transplant. This delayed growth was associated with a preponderance of infiltrating NK cells in the tumor mass. When both tumors were harvested at the same size (∼13 mm), 10% of the infiltrating CD45\(^{+}\) cells in the regressor d100 tumor were NK cells (DX5\(^{+}\)CD122\(^{+}\)) compared with 3% in the regressor F244 tumor (\(p < 0.001\)) (Fig. 6C).

Passage of the d100 regressor tumor through RAG2\(^{-/-}\) mice leads to reduced NK cell recognition

We next examined whether the NK cell infiltration and delayed growth of d100 in RAG2\(^{-/-}\) mice led to a sculpted tumor repertoire that was resistant to NK cell cytotoxicity. We performed a standard chromium release assay using IL-2–activated NK cells as effectors and the parental or RAG2\(^{-/-}\)-passaged cell lines as targets. As predicted, we found that the passaged cell lines were 2-fold less susceptible to NK cell killing compared with the parental cell line (Fig. 7, \(p = 0.0216\)).

Passage of the d100 regressor tumor through RAG2\(^{-/-}\) mice converts it into a regressor tumor through NK cell NKG2D editing of H60a-hi cells

Having shown that NK recognition was decreased in vitro in RAG2\(^{-/-}\)-passaged cell lines, we next determined whether RAG2\(^{-/-}\)passaging of d100 in the presence or absence of NK cells or NKG2D activity had functional consequences on tumor growth in vivo. We transplanted d100 into RAG2\(^{-/-}\) mice that had received i.p. injections of either PBS, anti-ASGM1 (to deplete NK cells), or a blocking anti-NKG2D mAb and monitored tumor growth in vivo. Depletion of NK cells with anti-ASGM1 showed a slight increase in tumor growth in RAG2\(^{-/-}\) mice compared with control PBS treatment, whereas there was no change in growth when NKG2D function was blocked with anti-NKG2D (Fig. 8A, upper panel). When cell lines were generated from the tumors, we found that cells passaged in the presence of functional NK cells (PBS) showed a progressive growth phenotype in WT mice, whereas those passaged without NKG2D function or NK
cells still retained their regressor phenotype (Fig. 8A, lower panel). Moreover, daughter cell lines generated in mice treated with anti-ASGM1 or anti-NKG2D, and tumor size was measured over time (upper panel). Tumor masses growing in RAG2−/− mice were harvested at day 25 and disaggregated, and daughter cell lines were generated and injected into WT mice (lower panel). B, H60a expression of daughter cell lines was detected by flow cytometry, and H60a-hi populations were quantified for each cell line. C, An edited, passaged, progressor d100 cell line was transduced with retrovirus expressing either GFP or both GFP and H60a and injected into WT mice. *p < 0.05, **p < 0.01.

**FIGURE 8.** H60a is functionally edited by NK cell recognition through NKG2D. A, The IFNAR−/−-derived regressor tumor d100 was injected into RAG2−/− mice that received i.p injections of either PBS, anti-ASGM1, or anti-NKG2D, and tumor size was measured over time (upper panel). Tumor masses growing in RAG2−/− mice were harvested at day 25 and disaggregated, and daughter cell lines were generated and injected into WT mice (lower panel). B, H60a expression of daughter cell lines was detected by flow cytometry, and H60a-hi populations were quantified for each cell line. C, An edited, passaged, progressor d100 cell line was transduced with retrovirus expressing either GFP or both GFP and H60a and injected into WT mice. *p < 0.05, **p < 0.01.

Discussion
Tumor heterogeneity has been appreciated as an inherent characteristic of genetically unstable neoplasms (30, 31). Various studies have shown that among the cancerous cells in a tumor mass, or even within a tumor cell line, there is heterogeneity in the phenotype (32, 33), capacity to metastasize (34, 35), response to chemotherapy (36), ability to initiate new tumors (32, 35, 37), and signaling through surface receptors (33, 38). Indeed, the cancer stem cell hypothesis (32, 37) is supported by the observation that a tumor mass is heterogeneous and therefore made of cells that have different capacities to initiate new tumors after transplantation. In this study, we have applied the idea of tumor heterogeneity to the measurement of immunogenicity. We have hypothesized that the tumor cell repertoire is immunologically heterogeneous (i.e., it consists of cells that have varying levels of immunogenicity due to diversity in Ags or innate ligands). We further hypothesized that cancer immunoediting acts by limiting the immunologic heterogeneity of the tumor cell population by removing cells of increased immunogenicity. In support of our hypotheses, we have found that the expression of H60a, as a potential surrogate marker of immunogenicity, is more heterogeneous in unedited tumor cells compared with that in edited tumor cells. This heterogeneity in H60a expression is found in groups of cell lines (unedited versus edited) and even within single unedited cell lines. These data suggest that tumor heterogeneity may be detected and eliminated by the immune system via a cancer immunoediting process and that NKG2D ligands may serve as markers for this process.

We conclude that H60a is an edited molecule, and this editing process requires NK cells and NKG2D activity. A previous study
(12) using NKG2D-deficient mice found that NKG2D is not involved in the surveillance of MCA sarcomas, whereas a study from another group showed increased MCA tumor formation in mice treated with blocking Abs to NKG2D (13). Both of these studies were performed in C57BL/6 mice, which lack H60a, and therefore do not shed light on the mechanism of H60a editing in our model system. The finding that some unedited cell lines from mice deficient in STAT1 or common γ chain display even higher levels of H60a points to NK cells as an effector of the editing process, because they are functionally defective in the absence of STAT1 (39) and are completely absent in mice lacking common γ chain (40). We found increased levels of activated NK cells in the d100 regressor tumor as it is undergoing editing when transplanted into RAG2−/− mice compared with those in a progressor tumor control. In addition, daughter cell lines generated in the presence of functional NK cells showed a progressive growth phenotype in WT mice, whereas those generated without NKG2D function or NK cells still retained their rejection phenotype, suggesting that NK cell killing through NKG2D is required for the editing of these tumors in vivo. Overexpression of H60a into edited tumors causes their rejection in vivo, showing that H60a can be a functional marker of immunoediting for certain tumors.

We found that H60a can be expressed at higher levels in progressor tumor cell lines than regressor tumor cell lines. These results suggest that H60a is not sufficient or necessary for tumor rejection. We envision that NKG2D ligands are only one component of the immunologic landscape of tumor cells and that tumors with very high antigenicity or innate ligands might not need NKG2D ligand expression to be rejected. However, tumor cells with moderate NKG2D ligand expression can escape immune detection if they decrease their antigenicity or increase inhibitory ligands or cytokines. In our model system using d100, the decrease in H60a expression is required for the conversion to a progressor. In contrast, although H60a is edited in F535 after passage through IFNAR−/− mice, the IFNAR−/−-passaged F535 daughter cell lines remain regressors, indicating that other immune targets can be sufficient to mediate rejection. It will be interesting to examine the expression of inhibitory molecules, such as CD47 (41) and the H60a-hi and H60a-lo cell clones. Because it is known that NK cell activation and killing can lead to T cell priming (23, 44), we envision that NK/NKG2D-dependent killing of H60a-hi cells releases Ags that are targeted in an adaptive immune response to H60a-lo cells, leading to complete tumor rejection. Future studies will address whether the H60a-hi cells can immunize against edited H60a-lo cells.

It is not known what regulatory mechanism allows for very high and very low NKG2D ligand levels in similarly treated cells. The fact that H60a expression is also heterogeneous within cell lines indicates that H60a expression could be controlled at the level of a single cell via intrinsic mechanisms. We envision that during carcinogenesis some tumor cells suffer lesions that induce H60a expression, whereas others do not. These lesions could be quantitative in nature and may loosely correlate with H60a expression. We believe that these lesions could be in the cis elements that regulate H60a and also throughout the genome, to be sensed by trans factors that regulate H60a. Although DNA damage has been shown to regulate Rae1, Mult1, and MICA, we have not found similar regulation of H60a (J.D. Bui, unpublished observations).

We have found that the transcript levels for H60a also displayed heterogeneity and are not always correlated with H60a protein levels, implying that posttranscriptional mechanisms of H60a regulation may exist. Indeed, we and others have found that microRNAs can regulate the human NKG2D ligand MICA (47, 48) and H60a (28).

Our cell lines are not clonal, and therefore, the heterogeneous expression of H60a is likely due to multiple cell clones expressing different levels of H60a. Interestingly, when we sorted H60a-lo cells from the bimodal parental d100 cell line, the H60a expression was unstable, and the sorted H60a-lo cells gave rise to H60a-hi cells after several days of culture (data not shown). The sorted H60a-lo cells, when transplanted into WT mice as a bimodal population due to H60a instability, were still regressor cells. We could not obtain cell clones that stably expressed H60a at low levels, even after multiple limiting dilution cloning procedures. Thus, we still do not know whether the parent d100 is a mixture of cell lines or is a single “unstable” cell line that then gives rise to different daughter cells. Nevertheless, our observations are consistent with the concept that the substrate for editing is a mixture of various cell clones, each with differing immunogeneticities. Tumors that are not edited therefore would be more heterogeneous in their expression of immunogenic molecules such as NKG2D ligands.

An alternative scenario could be that H60a expression is associated with intrinsic cellular physiology and could be related to the proliferation state of the cell, as has been shown for MICA (49). Although cell starvation is not sufficient to affect H60a levels (19), cell cycle signals could collaborate with other signals to regulate H60a. It should be noted that the Rae1 levels in our cell lines are fairly homogeneous (i.e., display a unimodal, narrow peak in flow cytometric analysis), and thus, the mechanisms that underlie the observed heterogeneous expression of H60a must be specific for individual NKG2D ligands.

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**Disclosures**

The authors have no financial conflicts of interest.
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