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Studies on the antigenicity of the NKG2D ligand H60a in tumour cells

Deepak Yadav,* Jennifer Ngolab,* Natalie Dang and Jack D. Bui

Department of Pathology, University of California, San Diego, La Jolla, CA, USA

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*These authors contributed equally to the paper.
Correspondence: Dr J. D. Bui, Department of Pathology, University of California, San Diego, La Jolla, 9500 Gilman Drive, CA 92093-0612, USA. Email: jbui@ucsd.edu
Senior author: Jack D. Bui

Summary

H60a is a minor histocompatibility antigen expressed in BALB and 129/Sv but not C57BL/6 mouse strains. The majority of $CD8^+$ T cells in C57BL/6 mice responding to BALB.B splenocytes are specific for H60a. Interestingly, H60a is expressed constitutively on tumour cells, but its nature as a tumour rejection antigen, as a parallel to its function as a transplant rejection antigen, has not been studied. In this report, we show that tumour cells that constitutively express H60a at the cell surface can be recognized by H60a-specific T cells. Furthermore, when H60a-expressing sarcoma cell lines are transplanted into C57BL/6 mice, H60a-specific T cells can be found at high percentages among the tumour-infiltrating $CD8^+$ T cells. These findings were seen in C57BL/6 but not F1 (C57BL/6 × 129) mice (which express H60a), suggesting that endogenous tolerance mechanisms suppress the antigenic properties of H60a. Our findings have implications for the generation of tumour vaccines against human natural killer group 2D ligands, such as MHC class I chain-like gene A, that are also transplantation antigens.

Keywords: cancer; minor histocompatibility antigens; natural killer cell receptors; tumour antigens

Introduction

H60a is a minor histocompatibility (H) antigen expressed in BALB.B but not C57BL/6 mice.[1] It is known to be expressed by haematopoietic cells and in certain tumour cell lines from various strains, including BALB/C and 129/Sv,[1–4] but is a pseudogene in C57BL/6 mice.[5] The highly immunogenic minor H peptide of H60a (H60p) was identified to be an eight amino acid peptide (LYL8) that is presented on $H\text{-}2K^b$.[1] LYL8-$H\text{-}2K^b$ tetramers can be used to detect and monitor H60p-specific $CD8^+$ T cells during minor histocompatibility immune responses in C57BL/6 mice challenged with BALB.B splenocytes.[6] Studies of H60p-specific responses have been performed in C57BL/6 mice, where H60a is not expressed. It is not known whether responses to H60a can be detected in 129/Sv strains of mice, where it is expressed by endogenous cells. In addition, whether H60p-specific T cells can mount effective anti-tumour responses is not known.

H60a also binds natural killer group 2D (NKG2D) and can participate in tumour surveillance and cancer immunoediting.[7–9] NKG2D is an activating receptor on NK cells[10,11] and recognizes a ligand family (including H60a) that is up-regulated by certain stimuli including DNA damage and virus infection.[12,13] The importance of interactions between NKG2D and NKG2D ligand in tumour immunosurveillance is supported by several findings. For example, mice deficient in NKG2D activity are more susceptible to primary tumour formation.[14,15] In addition, enforced expression of NKG2D ligands in tumour cells leads to their rejection via a mechanism that requires NK cells.[16,17] In humans, soluble inhibitory forms of NKG2D ligands can be detected in the serum of certain patients with cancer and can lead to defective immune responses.[18] These studies highlight

Abbreviations: FACS, fluorescence activated cell sorting; GFP, green fluorescent protein; H, histocompatibility; MCA, methylcholanthrene; MICA, MHC class I chain-like gene A; NK, natural killer; NKG2D, natural killer group 2D; ULBP, UL16-binding protein.

the potential significance of NKG2D ligands as targets for tumour immune therapy.

It is interesting to note that among mouse and human NKG2D ligands, H60a and the human NKG2D ligand MHC class I chain-like gene A (MICA) have both been implicated in histocompatibility rejection responses.[19,20] As mentioned before, H60a is the dominant minor antigen in MHC-compatible transplants. MICA also seems to be an important transplantation antigen, because antibodies to MICA can be found in patients after renal and heart transplantation and are associated with poor outcomes.[21,22] Notably, antibodies to MICA are most predictive of poor outcome in patients who are well-matched at the HLA loci, suggesting that similar to H60a, MICA is an important minor antigen.

Antibodies to MICA are also found in patients with cancer and correlate with better rather than worse prognosis.[23] In this scenario, it is thought that the progression of cancer is associated with a break in tolerance, allowing for antibodies to develop against MICA, which could be considered a tumour antigen. These results are consistent with the paradigm that NKG2D ligands, such as MICA and H60a, could represent a novel category of non-mutated tumour antigens similar to cancer-testis antigens, whose expression is normally restricted to certain tissues but is up-regulated in tumour tissue.[24] Importantly, T-cell reactivity to peptides derived from NKG2D ligands during anti-tumour responses has not been documented. In this study, we show 'proof-of-principle' by reporting that tumour-expressed H60a can induce anti-H60p T-cell responses *in vitro* and *in vivo*.

Materials and methods

Cell lines

Methylcholanthrene (MCA)-induced sarcomas were isolated and passaged *in vitro* as described elsewhere.[25,26] The 129/Sv MCA sarcoma cells with varying levels of H60a expression have been described previously[4] and the C57BL/6 MCA sarcoma cell lines were generated in wild-type and immune-deficient mice (T. O'Sullivan, R. Saddawi-Konefka, W. Vermi, R. Uppaluri, C. D. Arthur, J. M. White, M. J. Smyth, R. D. Schreiber and J. D. Bui, manuscript in preparation) in a manner similar to the 129/Sv MCA sarcoma cells.[26] Highly immunogenic tumour cell lines are derived from tumours that developed in immune-deficient mice, whereas poorly immunogenic cell lines are derived from wild-type mice.[26] Cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, L-glutamine, non-essential amino acids, sodium pyruvate, sodium bicarbonate, penicillin/streptomycin and β-mercaptoethanol. H60a-specific T-cell clones were maintained as described previously.[27] These T-cell clones displayed specificity for the H60a-derived peptide LYL8 complexed with K^b. The C57BL/6 MCA sarcoma cell line 9609 was transduced with a retrovirus-expressing green fluorescent protein (GFP) and H60a.[4] This construct has GFP under the control of an internal ribosomal entry site downstream of H60a. This allows for the use of GFP as a suitable reporter for H60a.

T-cell cytokine production assay

H60a-specific T-cell lines (kindly provided by Derry Roopenian) were cultured with tumour cells at a 1 : 1 ratio overnight in the presence of brefeldin A. The next day, T cells were harvested and stained for the expression of interferon-γ (IFN-γ). In some experiments, tumour cells were pulsed with LYL8 peptide for 30 min at 37° before co-culture with T cells. The phorbol ester PMA and ionomycin (Sigma, St. Louis, MO) were used at 10 μg/ml and 1 μM, respectively.

Mice

129/SvEv mice (H-2^b haplotype) were purchased from Taconic Farms (Germantown, NY) and C57BL/6 mice (H-2^b haplotype) were purchased from Charles Rivers (Wilmington, MA). These mice were interbred to generate F_1 (C57BL/6 × 129) mice. All animal procedures were approved by the University of California, San Diego Institutional Animal Care and Use Committee (UCSD IACUC) under protocol #S06201.

Tumour transplantation and processing

Tumour cell lines were transplanted into recipient naive mice as described previously.[25] The mice were monitored for tumour growth and on various days post-transplant, tumour was excised from mice, minced and treated with 1 mg/ml type Ia collagenase (Sigma) in complete RPMI-1640 medium for 30 min at 37°. The ipsilateral inguinal (tumour-draining) or contralateral (non-draining) lymph nodes were also harvested, and single-cell suspensions were made by crushing the lymph nodes between two glass slides. All cell suspensions were vigorously resuspended, washed in FACS staining buffer (PBS + 1% fetal bovine serum + 0·1% sodium azide), and filtered before staining. For cell lines generated from tumours (Fig. 5), the cell suspension was cultured in complete RPMI-1640 medium for 3–5 days before analysis by flow cytometry. All cell lines were split at least once during this period.

Antibodies and FACS analysis

Monoclonal antibodies to CD3, CD8 and CD45 were obtained from eBiosciences (San Diego, CA). Monoclonal antibody to H60a was obtained from R&D (Minneapolis, MN). Anti-IFN-γ-phycoerythrin was from BD Biosciences

(San Jose, CA); Fc block was purchased from eBiosciences; and K^b-LYL8 tetramers were obtained from the National Institute of Allergy and Infectious Diseases tetramer facility and used at 1 : 1000. To analyse tumour-infiltrating cells (Fig. 2), a 7-AAD$^-$ CD45$^+$ gate was used as described elsewhere[25] and also as shown in Fig. 4. For experiments using GFP-expressing tumours, the plots are shown in bi-exponential display. The gates shown are identical across the different samples/conditions but appear different because of the bi-exponential display.

Results

Recognition of H60a-expressing tumour cell lines by H60p-specific T cells

Tumours possess qualities that may prevent antigen presentation or otherwise reduce immunogenicity so we tested whether tumour-expressed H60a could serve as an effective target for T-cell recognition. We examined the production of IFN-γ by H60a-specific CD8$^+$ T-cell lines.[6,27,28] When stimulated with PMA in combination with ionomycin, the H60p-specific T-cell line B6/H60 could be induced to express IFN-γ as a marker of activation (Fig. 1a). Likewise, B6/H60 could also be stimulated by C57BL/6 or 129/Sv MCA sarcoma cell lines that were loaded with exogenous LYL8 peptide, confirming that MCA sarcomas of the H-2^b haplotype could present peptide and maximally stimulate these T cells (Fig. 1b,c, right panels). Notably, a 129/Sv-strain cell line expressing high levels of endogenous H60a could stimulate B6/H60 without the addition of exogenous peptide (Fig. 1c left panel). This was not seen with a C57BL/6 MCA sarcoma that did not express H60a (Fig. 1b left panel).

We then expanded this analysis to examine whether the levels of endogenous H60a that tumour cell lines express influence their recognition by H60p-specific T cells. We have previously identified a panel of MCA sarcoma cell lines expressing low and high levels of surface H60a as detected by flow cytometry using anti-H60a monoclonal antibodies.[4] When the two different H60p-specific T-cell lines (B6/H60 and SP/H60) were cultured with the sarcoma cell lines the percentage of IFN-γ-secreting T cells correlated with the amount of H60a surface protein expressed by the MCA sarcoma cell lines (Fig. 1d,e, open bars). To confirm that the different cell

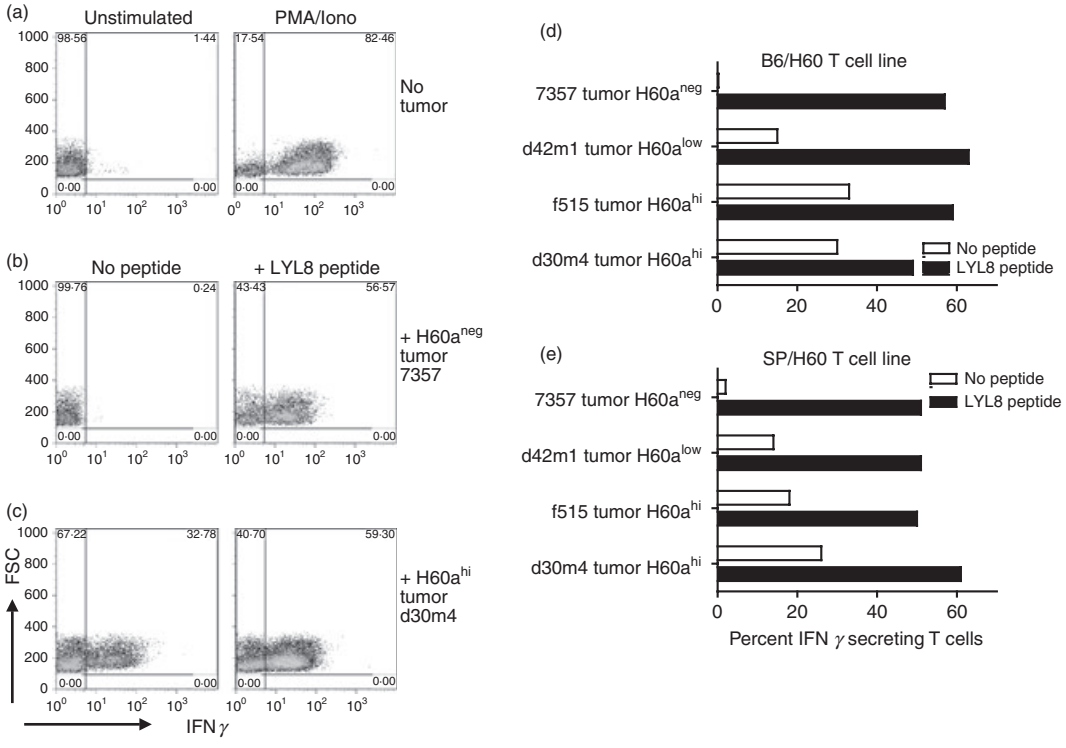

Figure 1. Recognition of H60a-expressing tumour cell lines by H60a-specific T cells in the presence and absence of exogenous LYL8 H60a peptide. Methylcholanthrene (MCA) sarcoma cell lines expressing varying levels of H60a were co-cultured with LYL8-specific T-cell clones, and interferon-γ (IFN-γ) production was measured by intracellular cytokine staining. Representative primary FACS data are shown for the LYL8-specific T-cell clone B6/H60 cultured (a) without (left panel) or with (right panel) PMA/Ionomycin (b) with the H60a^{neg} tumour 7357 not pulsed (left panel) or pulsed (right panel) with LYL8 peptide, or (c) with the H60a^{hi} tumour d30m4 not pulsed (left panel) or pulsed (right panel) with LYL8 peptide. The LYL8-specific T-cell clones (d) B6/H60 or (e) SP/H60 were stimulated with the indicated tumour cell lines pulsed with LYL8 H60a peptide (black bars) or unpulsed (white bars). The percentage of IFN-γ-secreting T cells is shown. The data are representative of two independent experiments.

lines had equivalent antigen presentation capabilities, exogenous LYL8 peptide was added to the cultures. Figure 1(d,e; closed bars) shows that all of the MCA sarcoma cell lines induced 50–60% of the T cells to express IFN-γ in the presence of exogenous LYL8 peptide. Together, the results show that differences in levels of endogenous H60a protein expression correlate generally with their H60p display to antigen-specific $CD8^+$ cells. The IFN-γ production seen here is probably the result of the H60a presented as a peptide and not the result of cognate NKG2D-H60a interactions because NKG2D ligation on T cells does not lead to IFN-γ production.[29]

H60-expressing tumours induce an anti-H60 T-cell response in allogeneic C57BL/6 mice

We next determined whether an H60p-specific immune response could be induced by H60a-expressing tumours in mice. We first examined the anti-H60p response in C57BL/6 mice where H60a is one of many minor H antigens. We transplanted 129/Sv-strain MCA sarcoma cell lines into C57BL/6 mice and tracked H60p-specific T cells using K^b-LYL8 tetramers. Endogenous H60p-specific T cells could be found at high levels within the tumour and at measurable but minimal levels in tumour-draining lymph nodes of C57BL/6 mice bearing the $H60a^{hi}$ tumour d30m4 (Fig. 2a). When this experiment was repeated with a panel of 129/Sv-strain MCA sarcoma cell lines expressing low and high levels of H60a,[4] we found high levels of H60p-specific T cells within the tumour but not draining lymph node (Fig. 2b).

H60-expressing tumours fail to induce a detectable anti-H60 T-cell response in syngeneic transplantation models

In 129/Sv-strain mice, H60a would be considered a self-antigen, and it is expected that adaptive immune responses to H60p would be censored because of tolerance mechanisms. Nevertheless, as H60a expression is inducible and furthermore restricted to certain tissues,[5,30] anti-H60a responses could presumably occur, especially in the context of NKG2D-mediated activation of the innate immune system[31] and in the context of a tumour, where responses to tissue-specific self antigens have been described.[24] To test this possibility, we examined the anti-H60p immune response in C57BL/6, F_1 (C57BL/6 × 129), and 129/Sv-strain mice. The F_1 (C57BL/6 × 129) mice have one copy of H60a and presumably would have reduced expression of the H60a gene, potentially lowering the threshold for T-cell tolerance. We transplanted a 129/Sv-strain MCA sarcoma cell line (f515) with high levels of surface H60a into C57BL/6, F_1 (C57BL/6 × 129), and 129/Sv mouse hosts and analysed the tumour-draining lymph nodes and tumour mass for the presence of H60a-specific T cells. As shown in Fig. 3(a,b), we found high levels of LYL8-K^b-tetramer-positive T cells within the tumours of C57BL/6 mice, but negligible numbers in F_1 (C57BL/6 × 129) and 129/Sv-strain mice, demonstrating that anti-H60a responses are poorly detected in mice that possess the intact H60a gene. These results indicate that a syngeneic response to H60a does not occur under these circumstances. Since f515 is a highly immunogenic cell

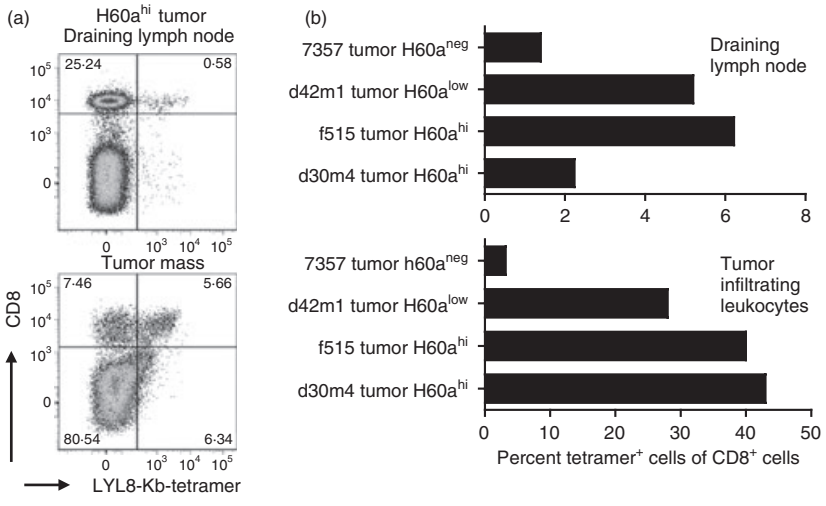

Figure 2. Tumours with high levels of H60a display high levels of tumour-infiltrating H60a-specific T cells in an allogeneic transplantation model. C57BL/6 mice were challenged with 129/Sv-strain methylcholanthrene (MCA) sarcomas (d42m1, f515, d30m4) expressing varying levels of H60a or an $H60a^{neg}$ C57BL/6-strain tumour (7357). At day 7 after tumour transplant, the (top panels) tumour draining lymph node or (bottom panels) tumour mass were harvested, and H60a-specific T cells were detected using LYL8-K^b tetramers. (a) Primary FACS plots of a representative $H60a^{hi}$ tumour or (b) the percentage of $tetramer^{pos}$ T cells within the $CD8^+$ population calculated by the following formula: $tetramer^{pos}/(tetramer^{pos} + tetramer^{neg})$. The data are representative of three experiments.

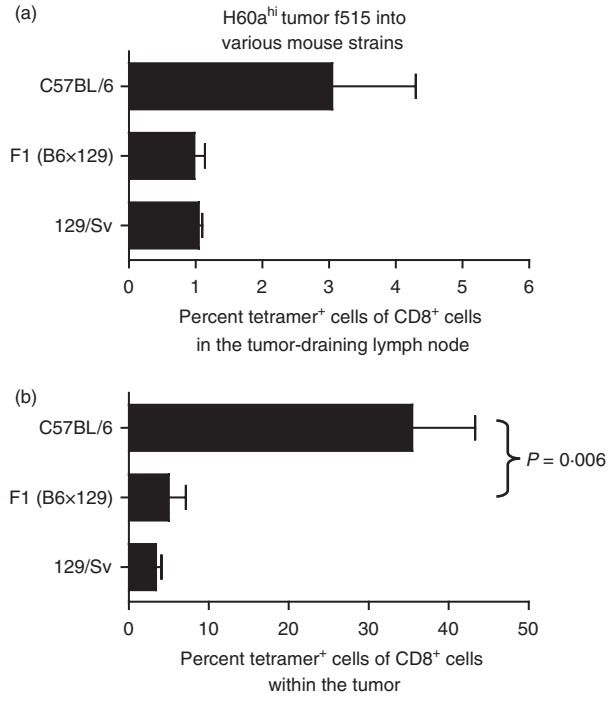

Figure 3. Tumours with high levels of H60a do not display high levels of tumour-infiltrating H60a-specific T cells in syngeneic transplantation models. The highly immunogenic H60a^{hi} tumour f515 was transplanted into various mouse strains, and at day 7 after tumour transplant, (a) the tumour draining lymph node or (b) tumour masses were harvested, and H60a-specific T cells were detected using LYL8-K^b tetramers. The tumour cell line f515 (derived from 129/Sv-strain mice) is rejected in C57BL/6, 129/Sv, and F_1 (C57BL/6 × 129/Sv) wild-type mice because it is immunogenic (data not shown).[25,26] The data are representative of two experiments.

line that is rejected by T cells when transplanted in syngeneic hosts,[26] the lack of an anti-H60a response is not the result of immune-suppressive properties of this tumour.

Ectopic expression of H60a in C57BL/6 tumour cells is sufficient to induce anti-H60a responses

Previous studies have shown that the presence of other minor H antigens are important for productive responses to H60a,[6] and therefore, the inability of F_1 (C57BL/6 × 129) and 129/Sv strain mice to mount anti-H60p responses (as shown in Fig. 3 above) could be the result of a lack of other minor antigens and tolerance mechanisms. To determine whether immune responses to H60a could be detected in the absence of all other minor histocompatibility antigens, we transduced a C57BL/6 MCA sarcoma cell line (MCA-9609) to express GFP and H60a[4] and transplanted it into C57BL/6 or F_1 (C57BL/6 × 129) recipients. Next, we harvested the tumour when the tumour mass was approximately 15 mm in mean diameter and examined the specificity of CD8$^+$ tumour-infiltrating T cells using LYL8-K^b-tetramers. Using a CD45$^+$7AAD$^-$ gate to examine tumour-infiltrating leucocytes, we found that the majority of CD8$^+$ T cells in the tumour were stained by LYL8-K^b-tetramers in C57BL/6 recipient mice, whereas LYL8-specific T cells were largely undetectable in F_1 (C57BL/6 × 129) mice (Fig. 4a,b, tumour-infiltrating leucocyte gate). These results were seen in a majority of mice and were statistically significant ($P = 0{\cdot}0002$), suggesting that H60a-specific T cells can be induced to infiltrate syngeneic tumours (with H60a as a transgene) in the absence of other minor H antigens.

Tumour-expressed H60a is edited by the immune system to generate escape variants expressing low levels of H60a

Having shown that H60p-specific T cells infiltrated H60a-expressing tumours (Fig. 4), we next examined whether these T cells imposed any functional effect. We hypothesized that the tumour was able to grow because the cancer cells decreased H60a expression to escape T-cell recognition. We therefore measured the expression of H60a on tumour cells growing in C57BL/6 mice (that had anti-H60p T-cell responses) versus F_1 (C57BL/6 × 129) mice (that did not generate detectable anti-H60p T-cell responses). As freshly harvested tumours were treated with collagenase, which could destroy H60a epitopes, we used GFP expression as a marker of H60a levels (GFP is expressed under the control of an internal ribosomal entry site downstream of the H60a gene in these cells). Figure 4(a,b,d; tumour gate) shows that the GFP mean fluorescence intensity of tumour cells growing in C57BL/6 mice was on average 40% lower ($P = 0{\cdot}0049$) than similar cells growing in F_1 (C57BL/6 × 129) mice, suggesting that H60a was edited by tumour-infiltrating H60p-specific T cells present in C57BL/6 tumours but not F_1 (C57BL/6 × 129) tumours. Direct measurement of H60a on tumour cells (Fig. 5a,b) cultured for several days *in vitro* (to allow for re-expression of H60a) showed a slight decrease in H60a expression in the tumour cells that had been isolated from C57BL/6 versus F_1 (C57BL/6 × 129) mice. We also stained the parental cell line and found that the F_1-passaged cells had higher expression, while the C57BL/6-passaged cells had lower expression, of GFP (Fig. 4) and H60a (Fig. 5) than the parental cells, suggesting that *in vivo* passage can influence the level of H60a expression on tumours in both directions.

Discussion

Tumour antigens can be derived from mutated proteins, tissue-specific proteins, or oncofetal proteins.[32] Tumour antigens are typically not broadly expressed so vaccine

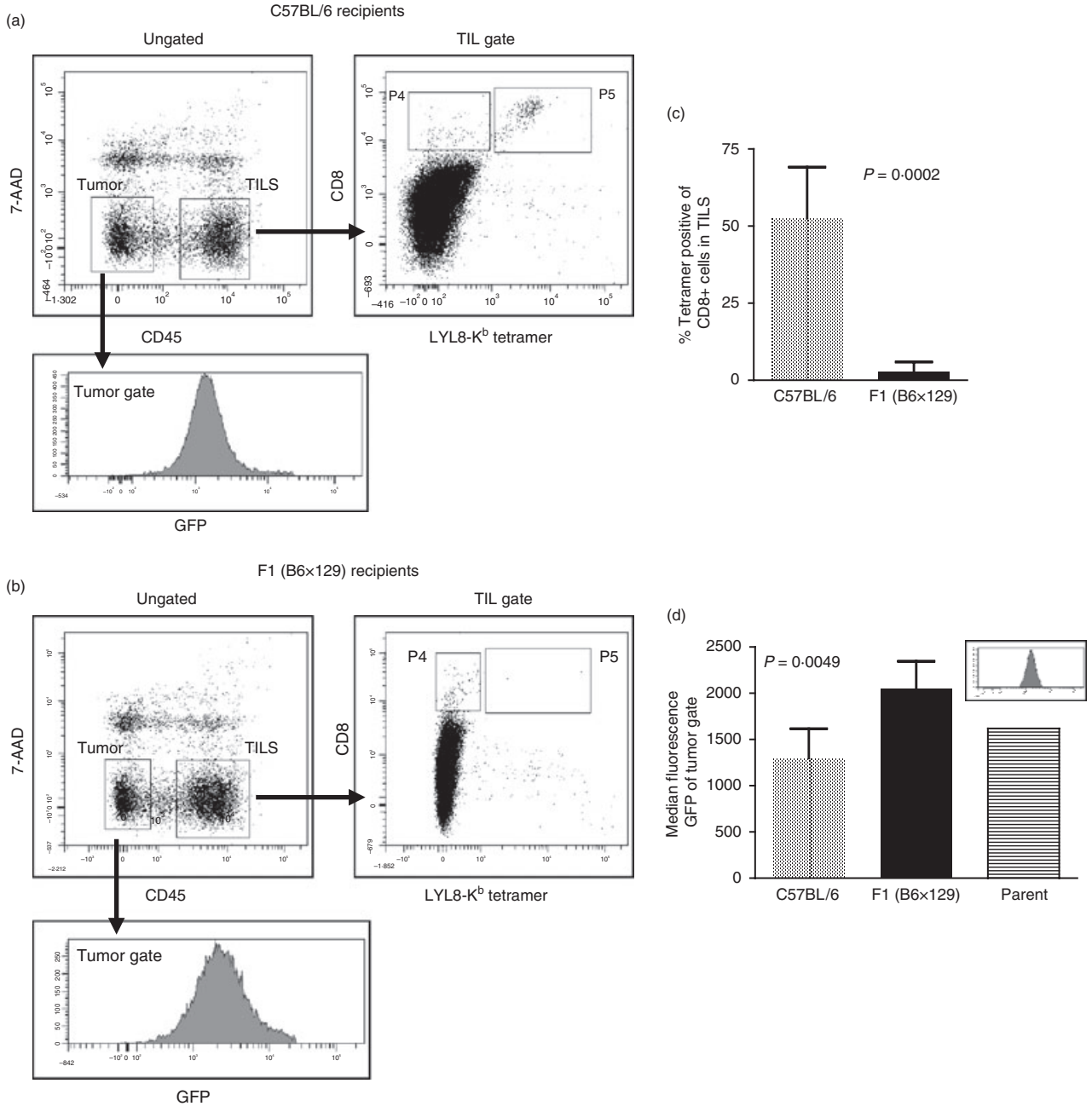

Figure 4. Expression of H60a in C57BL/6 tumours is sufficient to recruit H60a-specific T cells leading to tumour editing in C57BL/6 mice. H60a was transduced into a C57BL/6 sarcoma cell line and transplanted into (a) C57BL/6 or (b) F_1 (C57BL/6 × 129) mice. At days 18–20 after tumour transplant, the tumour masses were harvested, and tumour infiltrating leucocytes (TILs, $CD45^+7AAD^-$) or tumour cells ($CD45^-7AAD^-$) were analysed. Shown are (a,b) representative dots plots or (c,d) an experiment with five mice per group. (c) The percentage of H60-specific T cells is shown in tumours in C57BL/6 or F_1 (C57BL/6 × 129) mice. (d) The median fluorescence intensity of green fluorescence protein (GFP), as a surrogate measure of H60a expression, is shown in tumours harvested from C57BL/6 or F_1 (C57BL/6 × 129) mice. The data are representative of two experiments. (inset) A histogram showing GFP expression of the parental cell line cultured *in vitro* is shown.

strategies targeting known tumour antigens cannot be applied to all patients. The NKG2D ligand family[2,3] is expressed on a wide range of tumour tissue, including breast, melanoma, prostate, sarcoma, glioma, colorectal, lymphoma and lung. In this manuscript, we study the possibility that the NKG2D ligand H60a, which is also a transplantation antigen, can serve as a broadly expressed tumour antigen. We document that H60a can activate H60p-specific T cells *in vitro*. This also occurs *in vivo*, but only when H60a is not expressed endogenously. The

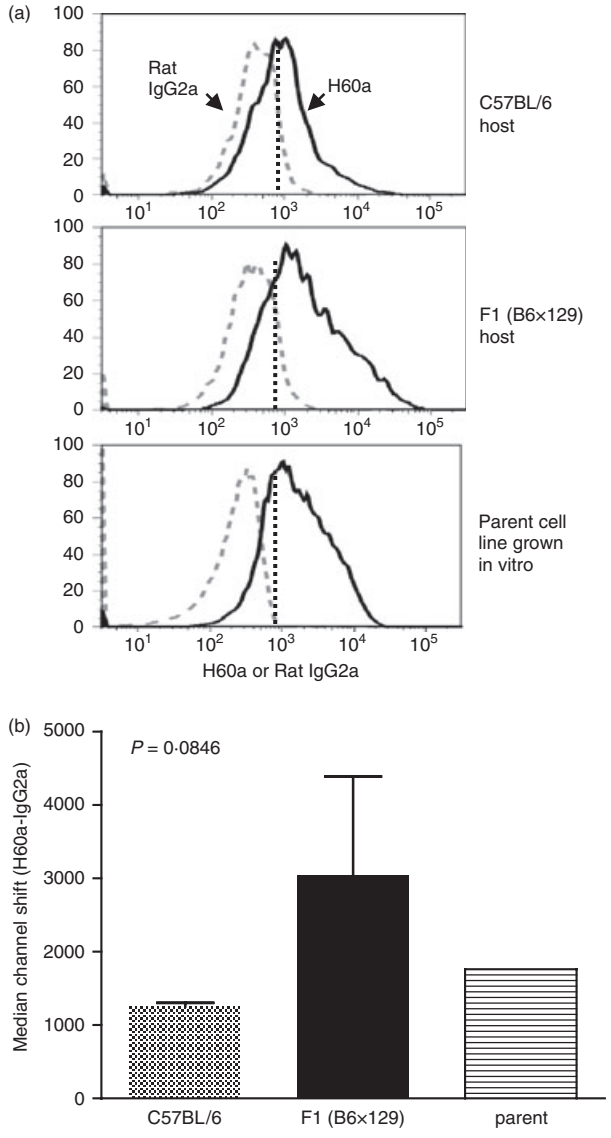

Figure 5. H60a is edited to a greater extent in C57BL/6 versus F_1 (C57BL/6 × 129) mice. H60a was transduced into a C57BL/6 sarcoma cell line and transplanted into C57BL/6 or F_1 (C57BL/6 × 129) mice. Cell lines were generated from the tumour mass, and H60a expression was measured on the cell lines. (a) A representative histogram of H60a (solid) or IgG2a (dashed) staining of the cell lines and the parental cell line cultured *in vitro*. The dashed line is at the same spot on all graphs for comparison purposes and is meant to represent the midpoint of the H60a histogram in the top graph. (b) The median channel shift of H60a staining is shown for three independent cell lines generated from three mice and for the parental cell line.

infiltration of H60p-specific T cells (as detected by LYL8-K^b tetramers) into H60a-expressing tumours is correlated with the emergence of tumour escape variants that have slightly decreased H60a expression. These results suggest that H60a is an immunogenic molecule that is edited by anti-H60a immune responses under conditions where H60a is a foreign antigen. Based on our findings, we suggest that tumour immune therapy targeting H60a or other NKG2D ligands as antigens is not feasible unless tolerance mechanisms can be broken and antigen escape variants can be dealt with.

We found that the majority of $CD8^+$ cells infiltrating H60a-expressing tumours in C57BL/6 mice are specific for LYL8, indicating that H60p is an immunodominant epitope in solid tumours. This immunodominance is consistent with previous findings that H60p is the dominant minor H antigen in C57BL/6-anti-BALB.B responses and C57BL/6-anti-129/Sv response in solid and haematopoietic allograft transplants.[6,27,28] In human organ transplants, the NKG2D ligand MICA is also known to be a transplantation antigen,[33] and antibodies to MICA are predictive of kidney transplant rejection in recipients who are well-matched at the HLA loci.[22] In this setting, antibodies to MICA recognize polymorphisms in the MICA gene,[34] but such serologically detected polymorphisms have not been described for H60a.[11] Both H60a and MICA are regulated by IFN-γ[4,35] and by microRNAs,[35,36] so we believe that our findings regarding H60a in the mouse model may have relevance to vaccines targeting MICA in human patients with cancer.

Although we readily elicited anti-H60p T cells in C57BL/6 mice, we did not detect these T cells in mice that express endogenous H60a, [129/Sv and F_1 (C57BL/6 × 129) mice] wherein H60a would be an auto-antigen. Previous studies have supported a role for NKG2D ligands in autoimmune disease, but these studies did not examine the possibility that NKG2D ligands could be auto-antigens. For example, the mouse NKG2D ligand RAE1 is expressed in the pancreas of diabetic NOD mice, but the autoreactive T cells found were not tested for their ability to recognize RAE1 as an antigen.[37] In patients with rheumatoid arthritis, the NKG2D ligands MICA/B are expressed in the synovium of patients with rheumatoid arthritis, leading to stimulation of autoreactive T cells, but the specificity of these T cells is not known.[38] Recently, expression of the human NKG2D ligand UL-16 binding protein (ULBP3) was found in the hair follicles of patients with alopecia areata, an autoimmune disease causing hair loss.[39] To our knowledge, our study is the first to use a mouse model to determine whether auto-T-cell responses to an NKG2D ligand can occur. The results suggest that in the setting of tumour transplantation, allogeneic T-cell responses to H60p are detectable, but syngeneic $CD8^+$ T-cell responses do not occur, probably as the result of tolerance mechanisms.

Syngeneic responses to the NKG2D ligand MICA are detected in patients with multiple myeloma not only during therapy[23] but also as part of normal tumour progression.[40] This finding is consistent with the observation that the progression of human cancer is associated with a break in tolerance to certain tumour antigens, even if they

are expressed endogenously.[24] In our experiments, we were not able to break tolerance to H60a (Fig. 3), even when we used highly immunogenic tumour cells. Furthermore, in preliminary studies, tolerance to H60a could not be broken by multiple immunizations of F_1 (C57BL/6 × 129) mice with LYL8 peptide + adjuvant (D. Yadav and J. D. Bui, unpublished observations). It is likely that tolerance to H60a in F_1 (C57BL/6 × 129) mice is mediated by central deletion of H60a-specific T cells, because H60a is highly expressed in the thymus.[30] This mechanism of tolerance would not be abrogated by simple immunizations. For MICA, it is possible that central tolerance does not operate, and therefore, anti-MICA adaptive immune responses may be detectable during normal tumour progression.

When we compared tumour cells that had been passaged through F_1 (C57BL/6 × 129) or C57BL/6 mice with the parental cells, we found that the parental cells expressed intermediate levels of H60a. In examining the histograms, there was a high degree of overlap in the passaged cells and the parental cells. It is not clear why the median fluorescence intensity of H60a (or the reporter GFP) is increased in tumours passaged through F_1 (C57BL/6 × 129) mice compared with the parental cells. We speculate that H60a might actually promote tumour cell proliferation. This effect is counterbalanced by immune cells that recognize H60a, either as an NKG2D ligand or a peptide. Hence, when there is high immune pressure (in C57BL/6 mice), H60a is decreased, whereas in conditions of tolerance to H60a (in C57BL/6 × 129 mice), the tumour cells with high levels of H60a are enriched. In addition, our findings show small differences in H60a expression in the passaged cell lines that do not reach statistical significance, indicating that the editing of H60a in this system is probably weak. One explanation for this is that the cells were cultured *in vitro* for several days in the absence of immune pressure. Another possibility is that the ectopic expression of H60a under the control of an exogenous strong promoter precludes mechanisms of editing that may act on the endogenous H60a promoter or 3′UTR.

In this report, we tested the antigenic properties of the minor histocompatibility antigen and NKG2D ligand H60a. Based on multiple previous observations, we hypothesized that anti-H60a T-cell responses might be used to effectively reject tumours expressing H60a. Although we were able to show that H60a can function as a tumour antigen, leading to expansion of H60a-specific T cells and tumour editing, this finding could not be extended to conditions wherein H60a was expressed as a self antigen. Future studies will focus on the antigenic properties of other NKG2D ligands, especially MICA, to determine whether NKG2D ligands can be targeted during tumour immunotherapy. We believe that their potent ability to activate innate immune cells and their high levels of expression in tumours make them attractive targets for tumour vaccines. In this context, our studies on the antigenic properties of H60a suggest that blocking tolerance mechanisms would be paramount in the successful targeting of NKG2D ligands as tumour antigens.

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Disclosure

The authors have no conflicts of interest to disclose.

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