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Strong spontaneous tumor neoantigen responses induced by a natural human carcinogen

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Abbreviations: dLN, lymph node draining the tumor; HA, hemagglutinin; MCA, methylcholanthrene; NGS, next-generation sequencing; SNV, single nucleotide variant.

A key to improving cancer immunotherapy will be the identification of tumor-specific "neoantigens" that arise from mutations and augment the resultant host immune response. In this study we identified single nucleotide variants (SNVs) by RNA sequencing of asbestos-induced murine mesothelioma cell lines AB1 and AB1-HA. Using the NetMHCpan 2.8 algorithm, the theoretical binding affinity of predicted peptides arising from high-confidence, exonic, non-synonymous SNVs was determined for the BALB/c strain. The immunoreactivity to 20 candidate mutation-carrying peptides of increased affinity and the corresponding wild-type peptides was determined using interferon-γ ELISPOT assays and lymphoid organs of non-manipulated tumor-bearing mice. A strong endogenous immune response was demonstrated to one of the candidate neoantigens, Uqcrc2; this response was detected in the draining lymph node and spleen. Antigen reactive cells were not detected in non-tumor bearing mice. The magnitude of the response to the Uqcrc2 neoantigen was similar to that of the strong influenza hemagglutinin antigen, a model tumor neoantigen. This work confirms that the approach of RNAseq plus peptide prediction and ELISPOT testing is sufficient to identify natural tumor neoantigens.

Introduction

Although it has been known for many years that tumors express a large number of mutated proteins that could potentially elicit strong immune responses, the difficulty of detecting these antigens and quantifying their specific antitumor reactivity has meant that few such neoantigens have been identified.[1] This situation has changed recently with the development of high-throughput next-generation sequencing (NGS), which has enabled rapid delineation of the mutational profile of expressed cancer proteins, prediction of altered MHC binding of these mutated peptides, and testing of the peptides to identify those that induce specific $CD8^+$ T-cell responses.

NGS of tumors, identification of non-synonymous mutations, and *in silico* epitope prediction have successfully been used to identify tumor neoantigens in several mouse cancers, including spontaneous melanoma[2] and methylcholanthrene (MCA)-induced sarcoma,[3,4] and in some human cancers, including melanoma,[5] cholangiocarcinoma,[6] ovarian cancer,[7] and chronic lymphocytic leukemia[8] in a variety of treatment settings.

Despite this progress, it will not be possible to answer in humans some of the key outstanding questions addressing how best to use such reactivities to track responses and/or treat patients. Questions such as "What is the main anatomical compartment that the immune response occurs in?" cannot be answered in patients because of limited access to lymph nodes and tumors. Similarly, questions relating to the mechanisms of action and optimization of new checkpoint blockade-based treatments and the capacity of neoantigen vaccines to alter antitumor responses, especially when used in combination with other chemo- or immunotherapies, need to be addressed in relevant animal models.

Most immunocompetent animal models do not mimic human cancer in terms of how the cancer is initiated; most use

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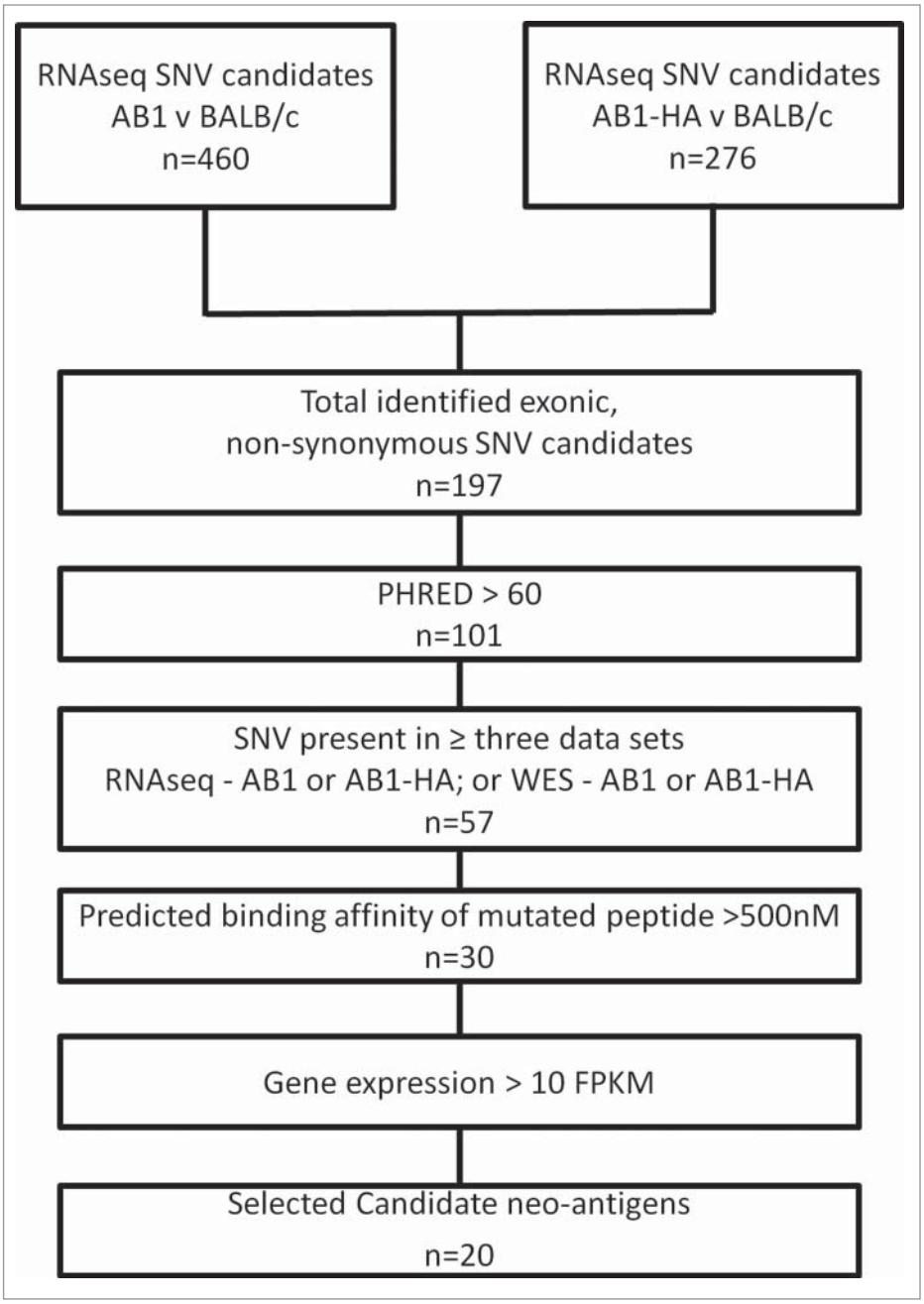

Figure 1. Flow diagram showing the selection process to identify candidate neoantigens for testing.

artificial carcinogens, occur spontaneously, or are gene-targeted by transgenesis. One of the rare animal tumor models that is actually induced by a known human carcinogen and also faithfully mimics the human tumor counterpart is the asbestos-induced tumor mesothelioma.[9] We therefore conducted this study in mouse asbestos-induced mesothelioma to examine whether such a natural carcinogen induces tumor neoantigenic immune responses. As most published studies examined blood or spleen despite the finding that neoantigenic responses are preferentially localized to the lymph node draining the tumor (dLN),[10] we then compared neoantigen reactivities in dLN and the spleen. Finally, to investigate the strength of neoantigen reactivity, we compared endogenous neoantigen responses to those observed against a transduced strong tumor neoantigen, influenza hemagglutinin HA, in the same tumor cells, examining in the process any potential competition between HA and the "natural" neoantigen.[11]

Results

Identification of non-synonymous mutations in expressed genes and predicted neoantigens in murine mesothelioma

The transcriptomes of the BALB/c mesothelioma cell lines AB1 and AB1-HA and the background BALB/c wild-type liver were sequenced. To increase the probability of identifying mutations that were expressed by the cancer, we used RNAseq. For each sample, more than 50 million sequenced reads with a mean length of 90 base pairs were generated. The concordant pair alignment was greater than 80%, and the average coverage depth was 133× in BALB/c liver, 77× in AB1, and 70× in AB1-HA. To improve confidence in the identification of the mutations called, the total exome of the cell lines and normal tissue was also sequenced such that a median of 40 million 150-nucleotide reads were generated with an average coverage depth of 70×.

We identified 460 and 276 somatic single nucleotide variants (SNV) in the transcriptomes of AB1 and AB1-HA, respectively. A total of 197 SNVs that were predicted to result in non-synonymous protein changes were identified in exomic regions. Considering only high-quality data, a total of 57 mutations were detected in 3 of the 4 available sequence datasets; i.e., either RNAseq or exome sequencing of the 2 cell lines. *In silico* epitope prediction using netMHC pan 2.8 determined that 30 mutated peptides had the potential to bind to MHC class I of BALB/c mice: 21 with high affinity and 9 with weak affinity. Of these, 20 were selected for further study. PCR-based Sanger sequencing validated 18 of the 20 candidates; 2 candidates were not validated because of a failure to generate a clean amplicon by PCR **Fig. 1**, **Table 1**. Thus, mouse mesothelioma has a similar mutation rate to human mesothelioma (manuscript in preparation).

Table 1. Sequence and predicted binding characteristics of the 20 candidate peptide neoantigens

Gene name	Mutation site	Validated by Sanger sequencing	Peptide	Length	Position of mutation	BLOSUM score	NetMHC predicted binding[a]		
							D^d	K_d	L^d
Anapc2	C2338G	NA	AYIQAMLTNL/v	10	10	−2		SB	
Ap3d1	G3142C	Y	VSNEA/pQFVF	9	5	−2	WB		
Arhgap5	G3994T	Y	VA/sGALKAFF	9	2	−1	WB		
Dpp9	C1207A	Y	RAVP/tKNVQPF	10	3	1	WB		
Exosc5	G418C	Y	LNAACMA/pL	8	7	−2	WB		
Ganab	G2087A	Y	QPFFR/qAHAHL	10	5	0			SB
Glud1	C356T	NA	KPCNHVLSLS/fF	11	10	0			SB
Hjurp	A437G	Y	LPQDEE/gYF	8	6	6			WB
Lmf1	T878G	Y	RMRILHGV/gLQI	11	8	−2	WB		
Mrpl23	C284G	Y	A/gYVQLAHGQTF	11	1	−2		WB	
Mrpl28	G763C	Y	AYLEA/gECVEWL	11	5	−2		SB	
Pop7	T77G	Y	YTLRKRL/rPHRL	11	7	−5	WB		
Rdx	G237C	Y	NPLQFK/nFRAKF	11	6	−3			SB
Rpn1	C1383A	Y	IYVRLD/eFSI	9	4	−4		SB	
Sf3b2	C2087T	Y	TPWGELEP/I	8	8	−1			SB
Tmem161a	C1150T	Y	TPLIL/fTLHCTL	11	5	−3			SB
Ube2b	C292G	Y	RWSP/aTYDVSSI	11	4	−2		SB	
Unc45a	C2206T	Y	IYEVVRP/sL	8	7	4		SB	
Uqcrc2	C1222G	Y	SYMP/aPSTVL	9	4	−2		SB	WB
Zfp524	A770C	Y	APHF/vCPVCL	9	4	−2			SB

[a]SB, strong binder; WB, weak binder.

In vivo testing of predicted neoantigens

To determine whether an endogenous T-cell response was induced to any of these putative neoantigens we inoculated wild-type mice subcutaneously with the AB1 mesothelioma cell line. Tumors were grown to approximately 70 mm^2 without intervention and then the dLNs were harvested. Twenty hours after recovery, dLN cells were tested for specific $CD8^+$ T-cell reactivity using peptide-induced interferon γ production in an ELISPOT assay. We focused on $CD8^+$ T-cell responses using short peptides and tested the peptides first in pools of 5 peptides/pool and then as separate peptides. All mice generated a $CD8^+$ T-cell response that was restricted to the mutant peptides in pool B only (**Fig. 2A**); no response was detected against peptides of pools A and D. Reactivity was demonstrated against the wild-type and mutant peptide C pool. Deconvolution of pool B revealed that the immune response was directed against only one of the 5 peptides, mutated Uqcrc2, with a median of 0.17 (interquartile range [IQR], 0–1.42) spot-forming units (SFU)/10^5 cells against the wild-type peptide detected in 9 mice, which was significantly less than the 14.38 (IQR 9.32–29.42) SFU/10^5 cells demonstrated against the mutant peptide ($P < 0.01$) **Fig. 2B**. This finding demonstrates that spontaneous antigenic reactivity induced by this natural human carcinogen to at least one mutant peptide could readily be detected.

Comparison of responses in spleen *versus* tumor-draining lymph node

Because our previous studies using model antigens demonstrated that the immune response may be restricted to the tumor dLN, and because most human studies are not able to measure responses in this anatomical compartment, we were concerned that specific natural neoantigen reactivities might be missed when spleen and/or blood was studied. We therefore compared the response in spleen with that in dLN. Typically, responses were higher in the dLN but not restricted to that location. Although the total number of SFU/10^5 cells was lower in the spleen than in the dLN, there was still a significantly greater response against the mutant Uqcrc2 peptide (4.5 [IQR 0.34–7.5] SFU/10^5 cells) compared to the wild-type peptide (0.34 [IQR 0–1.12] SFU/10^5 cells; $P < 0.05$) in this compartment **Fig. 3A**. Thus both dLN and spleen reveal detectable neoantigenic responses, at least to this mutant peptide.

Comparison of the immune response to natural and model neoantigens

To determine whether the response to the natural neoantigen Uqcrc2 was similar to that of the strong transduced neoantigen HA, we compared the responses to these two peptides. Mice were inoculated with the AB1-HA mesothelioma cell line that expresses both the Uqcrc2 neoantigen and the HA antigen. There was no significant difference in the immune response in the dLN to the 2 neoantigens. The median response to the mutant Uqcrc2 peptide was 15.38 (IQR 4.90–58.50) SFU/10^5 cells, which is in the range of that seen with the HA CL4 peptide (5.04 [IQR 0.40–63.17] SFU/10^5 cells) **Fig. 3B**. There was considerable intermouse variability in the response to the natural and artificial neoantigens in the same experiments with all conditions being identical; for example, one of the 12 mice tested (mouse 4) had a dramatic response to both peptides **Fig. 3C**, whereas a second animal (mouse 1) had a significantly stronger response to the mutant Uqcrc2 peptide than to the HA peptide **Fig. 3C**. Therefore, the response to Uqcrc2 is clearly not a weak response.

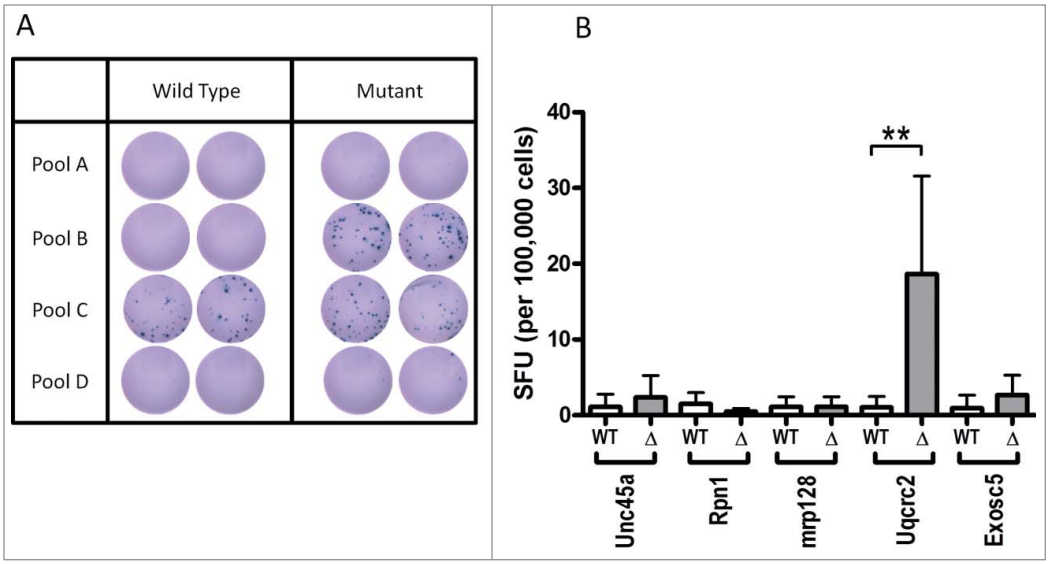

Figure 2. Endogenous immune responses to candidate neoantigens in mesothelioma tumor-bearing mice. (**A**) Representative duplicate wells from interferon-γ ELISPOT analysis of total dLN cell preparation from non-treated AB1 tumor-bearing mice against pools of peptides. (**B**) Summary of ELISPOT data showing mean ± SD for the deconvolution of peptide pool B. ELISPOT assays were performed in triplicate; $^{**}P < 0.01$.

Discussion

The notion that the immune system is not ignorant of the presence of a cancer and mounts a measurable $CD8^+$ T-cell response to the tumor has received a lot of attention in the past decade and is supported by studies showing that cross-presentation of tumor antigens is actually highly efficient[12] and cross-primes, albeit weakly, tumor-specific $CD8^+$ T cells in the dLN.[13] Such observations using artificial model tumor antigens have reinvigorated the search for "natural" tumor-specific antigens that arise through the process of carcinogenesis. Most studies of tumor antigens have focused on "self" antigens such as cancer-testis antigens and overexpressed or cellular differentiation proteins.[14] Antigenic mutated tumor proteins have been much harder to study and characterize, although over the past 20 years a number have been described.[1] It has been assumed that such neoantigens would be stronger than self antigens because they would have escaped central tolerance, and such a notion has recently received support from human studies.[15,16]

As neoantigens are almost always different in each individual patient, specific responses against them have been hard to study.[1] NGS has changed this situation by enabling the identification of the mutational profile of cancer proteins, which can then be coupled with peptide binding algorithms and *in vitro* studies. Identifying neoantigenic responses is potentially an important step in advancing cancer research and treatment. First, the immune response of the host to individual neoantigens can be tracked over time, enabling identification of the steps in the immunologic response to mutated proteins that are either effective or blocked during anticancer therapies. Second, immunoreactive neoepitopes could be exploited as vaccine candidates.

Although some neoantigenic reactivities have been described in patients,[7,15-17] many of the key outstanding questions relating to the use of such reactivities to track responses and/or treat patients cannot be answered in humans and will require relevant animal models. It is essential to study models that use a natural human carcinogen to determine whether such a carcinogen can induce any neoantigenic reactivities. Our data from this study of an asbestos-induced tumor, mesothelioma, in mice suggest that the number of mutations is similar to that seen in human mesothelioma (unpublished data). Although it is hard to compare DNA sequencing and analysis methodologies between studies, we found approximately 10-fold fewer non-synonymous transcribed exomic mutations in the murine AB1 mesothelioma cell line compared to the B16F10 melanoma cell line[2] and at least 50-fold fewer than in the MCA-induced sarcoma model.[3]

Importantly, when neoantigenic reactivities were examined, one strong neoantigen was detected among 20 candidates. Uqcrc2 is a component of the respiratory chain protein ubiquinol cytochrome c reductase that plays an important role in oxidative phosphorylation.[19] Although this molecule is involved in cellular respiration and its mutation might affect the altered glycolysis that is common in tumor biology, we do not yet know whether this mutation has any non-immunologic relevance in this model. The immune response in our study was not as broad as that seen to B16F10 tumors, in which 11 of 50 tested peptides elicited a mutant peptide-specific response,[2] but was similar to the oligoreactivity seen in the MCA model.[4]

One of the main reasons for studying neoantigenic responses in mice is to gain access to different cellular compartments, which is hard to do in humans. For example, clinical priorities mean that at presentation only limited, if any, access to the dLN is possible. Furthermore, even if access to compartments such as dLN and tumors is possible at presentation, it is rarely possible afterwards. Such an issue becomes crucial when we consider that most human studies involve examination of blood and/or tumors[7,15-17] yet mouse studies show that strong neoantigenic reactivities can be largely restricted to the dLN,[10,13,20,21] meaning that relevant reactivities might be missed if only blood or tumor is examined. Fortunately, we found that although the dLN has a higher level of response, neoantigenic responses were also readily detectable in the spleen. Thus, systemic studies in humans using blood as a source of responding T cells should be sufficient,

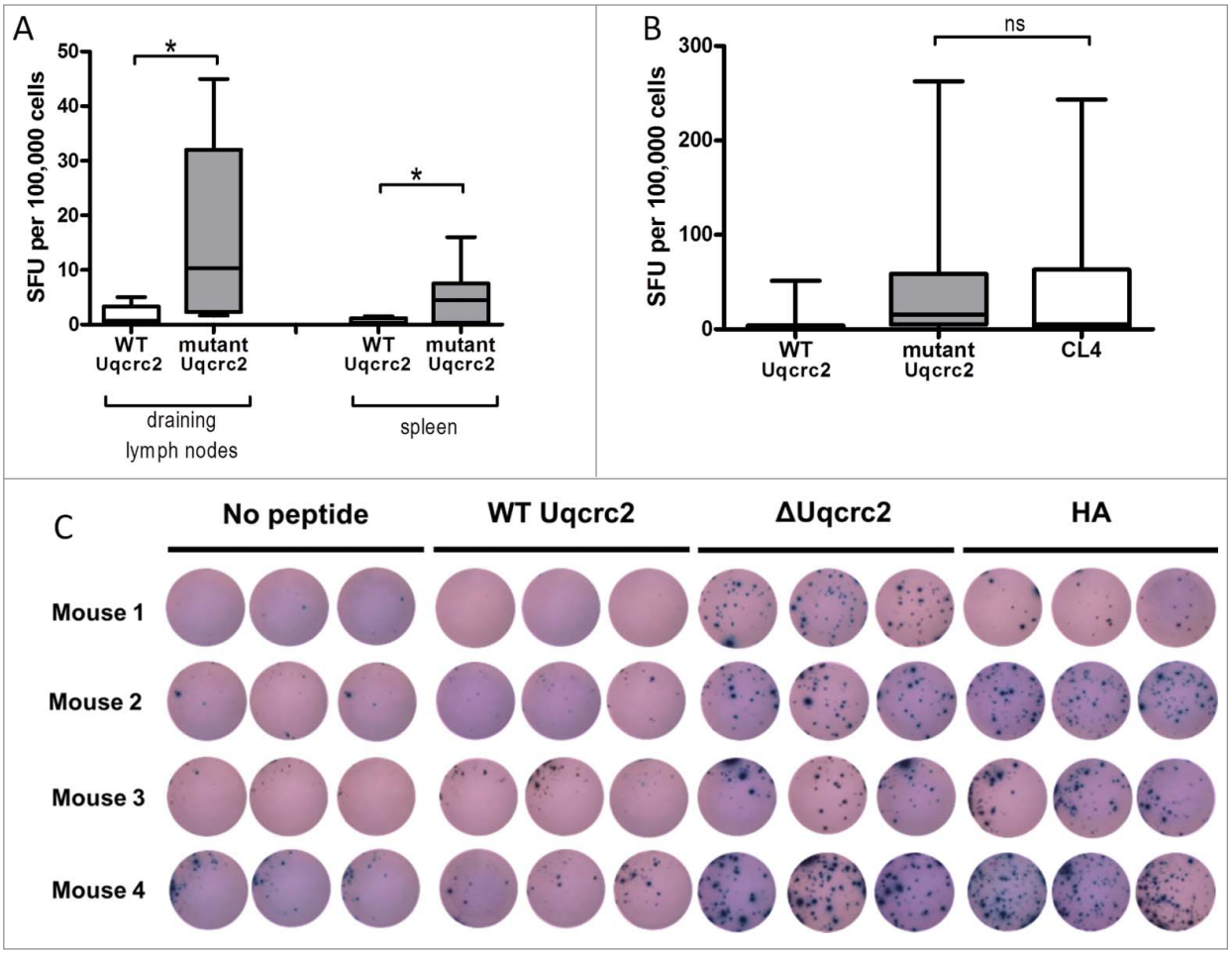

Figure 3. (**A**) Endogenous immune responses to wild-type and mutant Urcqc2 in the dLN and spleen of 7 mesothelioma-bearing mice. ELISPOT assays were performed in triplicate; * $P < 0.05$. (**B**) Comparison of endogenous immune responses to wild-type and mutant Urcqc2, and the hemagglutinin peptide CL4 in the dLN of 15 AB1-HA tumor-bearing mice. ELISPOT assays were performed in triplicate; ns, not significant. (**C**) Representative triplicate wells from interferon-γ ELISPOT analysis of total dLN cell preparation from 4 non-treated AB1-HA tumor-bearing mice.

rather than having to rely on obtaining dLNs to identify relevant neoantigenic reactivities.

The natural neoantigen reactivity discovered in this study was strong, as the level of response was similar to that to the artificial viral neoantigen HA used in this model. The notion that Uqcrc2 induced a strong rather than weak response is further supported by the fact that these responses were observed after 20 hours *ex vivo* rather than requiring prolonged *ex vivo* culture and T-cell amplification with peptide.

Importantly, although some strong antigens can compete with each other in viral systems,[11] there was no evidence of such competition between the natural neoantigen Uqcrc2 and the artificial neoantigen HA in this system. This suggests that oligoreactivity in response to multiple tumor mutations may not be hampered by competition, as has been seen in studies of tumors bearing strong poly-epitopes,[22] and raises the possibility that poly-epitope vaccines might be effective. Nevertheless, the response to the 2 strong neo-antigens Uqcrc2 and HA did not eradicate the tumor in tumor-bearing mice. This is consistent with other studies showing the presence of circulating antitumor $CD8^+$ T cells with no discernible antitumor activity.[23] This implies a failure of some other essential component, such as $CD4^+$ help,[20] inadequate lytic activity within the tumor,[24] or local suppression. These functions are all the subject of intense current study and are important to understand in the context of the current checkpoint blockade therapies, which broaden the $CD8^+$ repertoire but may not be the only factor leading to tumor eradication.

In summary, studies of neoantigen-specific T-cell responses in this relevant animal model reveal a strong reactivity to a single mutated neoantigen, Uqcrc2, a protein important in oxidative phosphorylation. This response was greater in the dLN compared to spleen but detectable in both locations, and was not out-competed by the presence of a second strong neoantigen in the same tumor. This model allows examination of many aspects of the specific host antitumor responses to antigens induced by a natural human carcinogen, with direct applicability to the equivalent human disease. It also provides an ideal opportunity to study the

effects of conventional cancer therapies such as chemotherapy, surgery, and radiation therapy on the specific antitumor response, to undertake preclinical testing of new immunotherapies such as new checkpoint blockade molecules, and to study the capacity of neoantigen vaccines to alter antitumor responses, especially in synergy with other chemo- or immunotherapies

Materials and Methods

Mice

Female BALB/c mice aged between 6 and 8 weeks were purchased from the Animal Resources Center Murdoch, Australia and maintained under standard specific pathogen-free housing conditions. Animal experiments were carried out in accordance with protocols approved by the University of Western Australia Animal Ethics Committee.

Cell lines

The murine mesothelioma cell line AB1 was previously generated by injecting crocidolite asbestos intraperitoneally into BALB/c mice;[9] the cell line was subsequently transfected with the PR8 influenza virus HA gene to produce AB1-HA.[25] Cell lines were maintained from original stocks in RPMI 1640 (Invitrogen, Mulgrave, Australia) supplemented with 20 mM HEPES, 0.05 mM 2-mercaptoethanol, 100 units/mL penicillin (CSL, Melbourne, Australia), 50 μg/mL gentamicin (David Bull Labs, Kewdale, Australia), and 10% FCS (Invitrogen). AB1-HA cells were maintained in media containing the neomycin analog geneticin (Invitrogen) at a final concentration of 400 μg/mL. Cell lines were used at below 20 passages for experiments and were regularly confirmed to be negative for *Mycoplasma spp*.

Next-generation sequencing

Total RNA was extracted from the mesothelioma cell lines AB1 and AB1-HA and from BALB/c liver tissue. RNA was sequenced using the Hiseq2000 sequencer (Illumina, CA) at Beijing Genomics Institute Hong Kong, China. Data were aligned to the murine reference genome mm9 (UCSC mm9 /NCBI build 37), derived from mice of the C57BL/6 strain using Bowtie 2.0[26] with quality analysis performed with Qualimap 2.0 software.[27]

DNA was extracted from AB1 and AB1-HA cells and from BALB/c tail tissue. Library creation and exome capture were carried out with the SureSelectXT Mouse All Exon kit (Agilent, Mulgrave, Australia) following the manufacturer's instructions. Exomes were sequenced on the Hiseq2000 by the Australian Genome Research Facility (Parkville, Australia) and data were aligned to mm9 using the Burrows-Wheeler Alignment tool.[28] All exomes were sorted using SAMtools[29] and duplicate reads were marked using Picard. Local realignment and base quality score recalibration were performed using the Genome Analysis Toolkit.[30]

Mutation selection and epitope prediction

From RNAseq data, somatic SNVs were identified using Varscan v2.3[31] and high-confidence calls were selected that had a minimum phred score of 60 or greater. Data were annotated using Annovar.[32] Homozygous SNVs were assumed to be germline mutations and excluded. For each high-confidence somatic, non-synonymous, exonic SNV identified in RNAseq data the corresponding data from exome data were examined using the Integrative Genomics Viewer.[33] Mutations that were present in 3 of the 4 sequenced data sets with a reads per kilobase of exon per million reads mapped (RPKM) expression score greater than 10 were selected. For validation, variants were amplified from cell lines and BALB/c DNA and subjected to Sanger sequencing (AGRF, Nedlands, Australia) and the results were visually examined. Protein sequences were retrieved from Genbank and for each of the mutations a set of peptides spanning the amino acid change and ranging from 8 to 11 amino acid in length was created *in silico*. For each peptide sequence, 2 sets of sequences were generated, one with the reference amino acid and one with the mutant variant. Matched sequences were analyzed using NetMHCpan 2.8 algorithm[34] and the potential class I binding affinity to murine MHC class I haplotype of H-2 D^d, H-2 K_d and H-2 L^d were determined. Strong binders, with a predicted binding affinity IC_{50} values less than 50 nM, and weak binders, with values of 50–500 nM, were selected as potential neoantigens.

Mouse mesothelioma model

Mice were inoculated subcutaneously with 5×10^5 AB1 or AB1-HA cells in a total volume of 100 μl PBS on the right flank. Mice were euthanized when tumors reached approximately 70 mm^2 and the spleens and tumor-draining ipsilateral axillary and inguinal nodes lymph nodes (dLN) were harvested. Non-tumor bearing naïve mice were used as controls. Single-cell suspensions of spleen and dLN cells were prepared as previously described[23] and used in ELISPOT assays.

ELISPOT

ELISPOT assays were performed as previously described.[35] Briefly, either 1×10^5 or 2×10^5 freshly isolated splenocytes or dLN cells were incubated in a microtiter plate pre-coated with 1 μg/mL anti-interferon-γ antibody (clone AN18; Mabtech, Preston, Australia) with 1 μg/mL test peptide (Mimtopes, Notting Hill, Australia), 1 μg/mL CL4 peptide from hemagglutinin of the Mt-Sinai strain of PR8 influenza virus (JPT, Berlin, Germany), 1 μg/mL anti-mouse CD3 monoclonal antibody (Raybiotech, GA), or media alone. After 20 h at 37°C, cytokine secretion was detected with 1 μg/mL biotinylated anti-interferon-γ antibody clone (R4–6A2; Mabtech), 1 μg/mL streptavidin horseradish peroxidase (Mabtech), and TMB substrate (Mabtech). Spots were visualized and enumerated using the Autoimmun Diagnostika AID ELISPOT reader system running count algorithm v.3.2.x. For each mouse the mean background number of SFU/10^5 cells was subtracted from the value for test wells.

Statistical analysis

Data were analyzed using Graph Pad Prism Software (Graph Pad Software, CA, USA). The paired Student's *t* test was used to compare responses of individual mice to different peptides. A P value < 0.05 using a 2-tailed test was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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