High frequency of T cells specific for cryptic epitopes in melanoma patients

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A number of cytotoxic T-cell epitopes are cryptic epitopes generated from non-conventional sources. These include epitopes that are encoded by alternative open reading frames or in generally non-coding genomic regions, such as introns. We have previously observed a frequent recognition of cryptic epitopes by tumor infiltrating lymphocytes isolated from melanoma patients. Here, we show that such cryptic epitopes are more frequently recognized than antigens of the same class encoded by canonical reading frames. Furthermore, we report the presence of T cells specific for three cryptic epitopes encoded in intronic sequences, as a result of incomplete splicing, in the circulation of melanoma patients. One of these epitopes derives from antigen isolated from immunoselected melanoma 2 (AIM2), while the two others are encoded in an alternative open reading frame of an incompletely spliced form of N-acetylglucosaminyl-transferase V (GNT-V) known as NA17-A. We have detected frequent T-cell responses against AIM2 and NA17-A epitopes in the blood of melanoma patients, both prior and after one round of in vitro peptide stimulation, but not in the circulation of healthy individuals and patients with breast or renal carcinoma. In summary, our findings indicate that the T-cell reactivity against AIM2 and NA17-A in the blood of melanoma patients is extensive, suggesting that—similar to melan A (also known as MART1)—these antigens might be used for immunomonitoring or as model antigens in several clinical and preclinical settings.

Introduction

When dissecting the antigen specificity of tumor-infiltrating lymphocytes (TILs) in melanoma patients, we detected T-cell responses against 18 different epitopes, predominantly from differentiation antigens such as melan A (also known as MART1) and gp100. Surprisingly, we also observed frequent responses against tumor-associated antigens (TAAs) that contain cryptic T-cell epitopes. Two of these epitopes were encoded by antigen isolated from immunoselected melanoma 2 (AIM2), while the two others are encoded in an alternative open reading frame of an incompletely spliced form of N-acetylglucosaminyl-transferase V (GNT-V) known as NA17-A. AIM2 was originally identified when T-cell clones established from a mixed lymphocyte-tumor cell culture were found to recognize a previously undescribed antigen. An autologous cDNA library was screened in the presence of a reactive T-cell clone to precisely identify the target of reactivity, leading to the discovery of AIM2. Two of these epitopes were encoded by antigen isolated from immunoselected melanoma 2 (AIM2) and by an alternative open reading frame of an incompletely spliced form of N-acetylglucosaminyl-transferase V (GNT-V) known as NA17-A.

AIM2 was originally identified when T-cell clones established from a mixed lymphocyte-tumor cell culture were found to recognize a previously undescribed antigen. An autologous cDNA library was screened in the presence of a reactive T-cell clone to precisely identify the target of reactivity, leading to the discovery of AIM2. AIM2 seems to be composed of two short open reading frames (ORFs) and a retained intronic sequence. ORF2, which is overlapping with the intron, encodes an HLA-A1-restricted T-cell epitope, namely the decapeptide RSDSGQQARY. AIM2 is expressed by the vast majority of melanomas and glioblastomas, and—at comparatively lower levels—by several other neoplasms, including breast carcinomas, ovarian carcinomas, colon carcinomas, and neuroectodermal tumors. Only low expression levels of AIM2 have been detected in (a few) normal tissues.

NA17-A was identified in a similar manner as AIM2, but the primordial NA17-A-reactive T-cell clone was established from TILs. The cDNA clone encoding the target for T-cell reactivity was found to contain two exons in common with GNT-V. However, these exons were separated by an unspliced intron, which encodes two T-cell epitopes. A putative cryptic promoter region was identified in the intronic sequence, driving the transcription of an alternative ORF. This promoter is active in melanoma, but not in normal cells, causing the melanoma-specific expression of NA17-A in 50% of patients. NA17-A encodes two HLA-A2-restricted T-cell epitopes, the nonapeptide VLPDVFIRC, and the overlapping decapeptide VLPDVFIRCV.

Based on the notion that these AIM2- and NA17-A-derived epitopes are frequently recognized by TILs isolated from melanoma lesions, we decided to study the corresponding T-cell reactivity in the peripheral blood of healthy individuals and cancer patients, including patients with melanoma, breast carcinoma, and renal cell carcinoma. Furthermore, we decided to compare the T-cell recognition of cryptic epitopes to that of epitopes encoded by canonical reading frames, to better understand the impact of these antigens in antitumor immune responses.
Our findings indicate that there is an extensive T-cell reactivity against AIM2 and NA17-A in the circulation of melanoma patients, nearly as abundant as that against MART1.1 Thus, AIM2- and NA17-A-derived peptides stand out as ideal candidates for immunomonitoring and as model antigens in multiple clinical and preclinical settings.

Results and Discussion

In a previous study, we screened the reactivity of TILs isolated from melanoma lesions against all known melanoma-associated T-cell epitopes (n = 175, selected for HLA-A1, -A2, -A3, -A11, and -B7) (Fig. 1A). For the most part, TILs recognized and reacted against differentiation antigens (Fig. 1B), but—surprisingly—we also detected frequent T-cell reactivity against various cryptic epitopes encoded in alternative ORFs or in generally non-coding genomic regions, such as introns.1 These cryptic epitopes were sorted according to the corresponding canonical ORF and therefore inaccurately classified as cancer-testis or overexpressed antigens. We have examined a peptide library including all known melanoma-associated T-cell epitopes for the occurrence of cryptic epitopes, and found that 14 of 175 peptides corresponded indeed to cryptic epitopes (Fig. 1C). We detected responses in melanoma-infiltrating lymphocytes against five of these epitopes, suggesting that this group of antigens significantly contributes to antigen recognition in this setting. In absolute terms, cryptic epitopes were the second most frequently recognized group of antigens, only outcompeted by differentiation antigens (Fig. 1D). However, when the number of cryptic (n = 14) vs. differentiation (n = 32) epitopes tested was taken into account, the fraction of responses against the former (36%) and the latter (35%) was comparable. Furthermore, we observed a tendency for cryptic epitopes to promote more T-cell responses (7 of 37) than what expected based on their relative abundance within the epitope library (14 of 175) (P = 0.06, Fisher’s exact test), as this was clearly the case for differentiation antigens (24 of 37 responses, 32 of 175 epitopes; P < 0.0001, Fisher’s exact test). The prominent contribution of cryptic epitopes to TIL reactivity against overexpressed TAAs was evident by the comparison of Figure 1B and D. Indeed, the majority of T-cell responses against overexpressed TAAs detected among melanoma-infiltrating lymphocytes was in reality specific for cryptic epitopes.

These results not only indicate that cryptic epitopes are frequently recognized by T cells in melanoma patients, but also suggest that the identification and correct classification of cryptic epitopes may be relevant for understanding the differences in the immune recognition of different classes of antigens. It may be speculated, but remains to be experimentally verified, that the high response rate of TILs isolated from melanoma lesions, demonstrating that these epitopes may be relevant for both tumor targeting and monitoring in the course of immunotherapy.

To elucidate the occurrence of T-cell reactivity against AIM2 and NA17-A, we screened the peripheral blood of cancer patients and healthy individuals using MHC multimers and an interferon γ (IFNγ)-specific ELISPOT assay. Since the expression of both these antigens had previously been shown to be predominantly expressed by malignant cells,2,3,5,6 Furthermore, frequent T-cell reactivity against AIM2 and NA17-A was indeed detected among TILs isolated from melanoma lesions, demonstrating that these epitopes may be relevant for both tumor targeting and monitoring in the course of immunotherapy.

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Contribution of tumor-associated antigen classes to T-cell reactivity in melanoma-infiltrating lymphocytes. (A and B) Pie charts show the classification of 175 previously described melanoma-associated T-cell epitopes (A) and the distribution of 37 T-cell responses against these tumor-associated antigens (TAAs) previously detected among tumor-infiltrating lymphocytes (TILs) (B) into classes. Please note that each response is only counted once in each patient. (C and D) Pie charts as in (A and B), respectively, with cryptic epitopes considered as a standalone class of TAAs.

**Figure 1.** Contribution of tumor-associated antigen classes to T-cell reactivity in melanoma-infiltrating lymphocytes. (A and B) Pie charts show the classification of 175 previously described melanoma-associated T-cell epitopes (A) and the distribution of 37 T-cell responses against these tumor-associated antigens (TAAs) previously detected among tumor-infiltrating lymphocytes (TILs) (B) into classes. Please note that each response is only counted once in each patient. (C and D) Pie charts as in (A and B), respectively, with cryptic epitopes considered as a standalone class of TAAs.
to TCRs specific for our NA17-A-derived decapeptide. To get further insights into this issue, we tested freshly thawed PBLs for their reactivity against the NA17-A-derived decapeptide by IFNγ-specific ELISPOT assays, but no responses were detected (data not shown).

After investigating AIM2 and NA17-A T-cell reactivity in melanoma patients, we focused on subjects affected by two other types of cancer as well as on healthy individuals. To this aim, we screened the PBLs from 7 (NA17-A) or 9 (AIM2) breast carcinoma patients and from 4 patients with renal cell carcinoma, as well as the peripheral blood mononuclear cells (PBMCs) of 15 (NA17-A) or 13 (AIM-2) healthy subjects, by the exact same approach that we had used earlier for melanoma patients. We detected no reactivity against AIM2 and NA17-A in healthy donors as well as in patients bearing neoplasms other than melanoma (Table 1). Statistical analyses revealed a significant difference in the T-reactivity of melanoma patients and healthy donors against AIM2- and NA17-A-derived epitopes, as well as between AIM2-specific responses in melanoma patients and subjects bearing breast carcinoma (Table 1). There was no statistically significant difference between melanoma patients and any other

Two epitopes from NA17-A were screened, a nonapeptide and a decapeptide. We detected responses against the nonapeptide in freshly thawed PBLs from 3/16 melanoma patients tested. The PBLs from the same patients were reactive against the NA17-A-derived nonapeptide also upon culture in the presence of peptide stimulation (Fig. 2C). In this setting, we observed T-cell responses in 6/16 (38%) patients, although not all of them were detectable with both methods (Fig. 2C and D). A reactivity against the decapeptide was observed in the PBLs of 6/16 (38%) melanoma patients tested, upon culture in the presence of peptide stimulation. Surprisingly, these were more readily detected by IFNγ-specific ELISPOT than with MHC multimers (Fig. 2E and F). Furthermore, we were unable to document any of these responses on freshly thawed PBLs. It may therefore be speculated, but remains to be formally demonstrated, that T-cell responses that are only detectable by ELISPOT may involve T-cell receptors (TCRs) that exhibit a low avidity for peptide-MHC complexes, making them more difficult to be detected with MHC multimers than with ELISPOT assays. It has been reported that the half-life of some TCR-MHC multimers might be excessively short for an efficient staining at some conditions,7 perhaps also applying to TCRs specific for our NA17-A-derived decapeptide. To get further insights into this issue, we tested freshly thawed PBLs for their reactivity against the NA17-A-derived decapeptide by IFNγ-specific ELISPOT assays, but no responses were detected (data not shown).

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AIM2- and NA17-A-specific T cells are frequently detected in the circulation of melanoma patients, and thus far resembling MART1. Nonetheless, neither of these antigens has widely been used in experimental and clinical settings. AIM2 is poorly described, and after its discovery in 2001 only a few groups have worked with this antigen, and mostly in the context of glioma. A limited number of publications describe the use of NA17-A-derived epitopes as model antigens, but the majority of these publications come from the same research groups. NA17-A-specific T cells have been studied in melanoma-bearing lymph nodes as well as in ascites from a melanoma patient, two settings in which a T-cell reactivity against NA17-A was reported. In a vaccination study in mice, strong and frequent T-cell responses against NA17-A (measured as reactivity against the nonapeptide VLPDVFIRCV) were achieved. This nonapeptide has also been included in one clinical trial, reporting an increase in the frequency of NA17-A-specific T cells after the administration of peptide-loaded dendritic cells in 2/9 patients and the presence of NA17-A-specific TILs upon treatment in a third patient. No objective responses were observed in this study, but two patients achieved stable disease. Notably, both the subjects exhibited T-cell reactivity against NA17-A. Together with these results, our findings indicate that NA17-A may represent a highly relevant melanoma-associated antigen.

In conclusion, both AIM2 and NA17-A are frequently detected by T cells in melanoma patients, and hence may serve as relevant targets for immunotherapy or might be used for the immunomonitoring of clinical trials testing unspecific immunotherapies, such as the CTLA4-targeting antibody ipilimumab or the adoptive transfer of ex vivo expanded TILs.

Materials and Methods

Peptides. Peptides were purchased from Pepscan (Pepscan Presto BV) or KJ Ross-Petersen ApS (Denmark) and dissolved in DMSO or H2O to stock concentrations of 10 or 2 mM, respectively. Sequences: AIM2-derived decapetide, RSDSGQQARY; NA17-A-derived nonapeptide, VLPDVFIRCV; NA17-A-derived decapetide VLPDVFIRCV; HIV-1-derived negative control peptide HIV-A1, GSEEKLRSLY; and HIV-1-derived negative control peptide HIV-A2 (ILKEPVHGV).

Samples from cancer patients and healthy subjects. PBLs were obtained from leukapheresis products of late-stage melanoma, breast and renal cell carcinoma patients enrolled in immunotherapeutic protocols at the University Hospital Herlev, depleted of monocytes (by adherence) and cryopreserved at -150°C in fetal bovine serum (FBS) containing 10% DMSO. Alternatively, PBLs were obtained from peripheral blood samples of breast cancer patients at primary diagnosis, purified by density centrifugation on Lymphoprep™ (Axis-Shield PoC) and cryopreserved at -150°C in FBS containing 10% DMSO. No patients received therapy at the time of PBL collection, but late-stage patients had been subjected to several rounds of previous therapy. Blood from healthy individuals was obtained from the blood bank of the Copenhagen University Hospital. PBMCs were isolated by density centrifugation on Lymphoprep™ (Axis-Shield PoC) and

Table 1. Overview of T-cell responses against three cryptic epitopes in healthy subjects and melanoma, breast carcinoma, and renal cell carcinoma patients

|                | AIM2            | NA17-A nonamer | NA17-A decamer |
|----------------|-----------------|----------------|----------------|
| Melanoma       | 6/13 (46%)      | 6/16 (38%)     | 6/16 (38%)     |
| Breast cancer  | 0/9 (0%)*       | 0/7 (0%)       | 0/7 (0%)       |
| Renal cell carcinoma | 0/4 (0%) | 0/4 (0%) | 0/4 (0%) |
| Healthy donors | 0/13 (0%)*      | 0/15 (0%)*     | 0/15 (0%)*     |

*P < 0.05 (Fisher’s exact test), as compared with melanoma patients.
cryopreserved at −150°C in FBS and 10% DMSO. All the procedures were approved by the Scientific Ethics Committee for the Capital Region of Denmark. Written informed consent was obtained, according to the Declaration of Helsinki.

T-cell staining. MHC multimers were produced as described in Supplemental Materials and Methods (final concentration: 10 µg/mL). No more than 2 × 10^6 freshly thawed or cultured PBLs/PBMCs were stained with 2.5 µL phycoerythrin (PE)-conjugated MHC multimers and 2.5 µL allophycocyanin (APC) or brilliant violet 421 (BV421)-conjugated MHC multimers in a final volume of 50 µL PBS supplemented with 2% (for most stainings) or 50% (for some AIM2 stainings, due to high background) FBS. Cells were incubated at 37°C for 15 min and then placed on ice. An antibody mix was prepared with anti-CD3, anti-CD8, anti-CD4 antibodies (all from BD), and the dead cell marker NIR-ViD (LIVE/DEAD Fixable Near-IR; Invitrogen) in PBS supplemented with 2% FBS (for most stainings) or FBS-free PBS (for AIM2 stainings with a high background). Cells were stained with 50 µL of this antibody mix and incubated for 30 min on ice. Next, cells

Figure 3. Functionality of AIM2-specific T-cell cultures. Four FACS-sorted, expanded AIM2-specific T-cell cultures were established from patients MM5, MM7, MM8, and MM9, (A) Three of these cultures were stimulated with BM36.1 cells pulsed with the AIM2 decapptide or HIV-A1 (control peptide) at a 10:1 effector:target ratio, and cytokine production was measured by intracellular cytokine staining. Background was < 0.4% and was subtracted from positive samples. The frequency of AIM2-specific multimer-CD8^+ T lymphocytes (among total CD8^+ T cells) in each culture was: 19.6%, 14%, and 61.6% for MM7, MM8, and MM9, respectively. (B) T-cell cultures from patients MM7, MM8, and MM9 were stimulated with BM36.1 cells pulsed with the AIM2 decapptide or HIV-A1 (control peptide) at the indicated effector:target ratio and cytotoxicity was measured by ^51Cr-release assays. The frequency of AIM-2 specific multimer-^T cells (among total live cells) was: 17.2%, 2.7%, and 31.6% for MM7, MM8, and MM9, respectively. (C) The T-cell culture established from patient MM5 was stimulated with the indicated melanoma cell lines at the indicated effector:target ratio and cytotoxicity was measured by ^51Cr-release assays. FM28 cells are HLA-A1^+ while FM48 and FM74 cells are HLA-A1^− yet all express AIM2 (as determined by PCR; data not shown). To test peptide-specificity and HLA-restriction, FM28 cells were pulsed with the AIM2-derived decapptide or incubated with an anti-MHC Class I antibody (W6/32). Furthermore, cold target inhibition was performed using unlabeled BM36.1 cells pulsed with either HIV-A1 or the AIM2-derived decapptide at an inhibitor:target ratio of 20:1. There were 68% AIM2 specific T cells in the culture. Data are reported as mean ± SD of 2 replicate measurements.
were washed twice with PBS supplemented with 2% FBS. Data acquisition was performed on LSR-II, FacsAria or FacsCanto flow cytometers (BD), and first line analyses were performed by means of the FacsDiva software (BD). We gated on single (FSC-A, FSC-W), live CD3+CD4−CD8+multimer+ lymphocytes. We always included HIV-specific multimers as negative controls. The detection limit was set at ≥ 10 MHC multimer+ events.

**IFNγ-specific ELISPOT assays.** ELISPOT assays to quantify the amount of peptide-specific IFNγ-releasing effector cells were performed as described previously and in Supplemental Materials and Methods. Before analysis, PBLs/PBMCs were stimulated once with 20 μM peptide and 40 U/ml IL-2 (PeproTech), to extend the sensitivity of the assay. Effector cells were added to the wells in duplicate instances, at different IL-2 (PeproTech), to extend the sensitivity of the assay. Effector M peptide and 40 U/ml IL-2 was added. After three weeks, MM7, MM8, and MM9 patients) IL-2 was added. After three weeks, MM7, MM8, and MM9 cultures were restimulated with irradiated, and MM5 patients) IL-2 was added. After three weeks, MM7, MM8, and MM9 patients) or 3000 U/mL (for MM3 and MM5 patients) or 1000 U/mL (for MM3 and MM5 patients) IL-2 was added. After three weeks, MM7, MM8, and MM9 cultures were restimulated with irradiated, allogeneic feeder cells (in a 1:1 ratio), 15 ng/mL anti-CD3 antibodies, and 120 U/mL IL-2. Cultures from MM3 and MM5 patients were tested before re-stimulation. An MHC multimer staining was always performed in parallel with intracellular cytokine stainings and 51Cr-release assays to test the purity of the culture at the time of functional tests.

**Chromium release assays.** Conventional 4 h 51Cr-release assays to assess T-cell cytotoxicity were performed as described elsewhere.22 In brief, target cells were labeled with Na251CrO4 (Perkin Elmer) for 1 h, washed, and co-cultured with effector cells for 4 h. Next, the level of 51Cr in the supernatant was measured using a Perkin Elmer Wallac Wizard 1470 automatic γ counter. BM36.1 cells, FM48 cells (HLA-A1+ melanoma cells, generated in house), FM74 (HLA-A1+ melanoma cells, ESTDAB-022), and FM28 (HLA-A1+ melanoma cells, ESTDAB-006) were used as target cells. In one case, lysis was inhibited with an anti-MHC class I antibody (W6/32; Dako). Alternatively, cold target inhibition was performed by means of unlabeled BM36.1 cells pulsed with 10 μM HIV-A1 or AIM2-derived decapeptide.

**Intracellular cytokine staining.** Intracellular cytokine staining was performed as previously described.1 Briefly, target cells (BM36.1) were pulsed with HIV-A1 or the AIM2-derived decapeptide for 1 h, washed, and then used to stimulate T-cell cultures at 37°C in X-vivo medium supplemented with 5% human serum (effector to target ratio = 10:1). After 1 h, 1 μL/mL GolgiPlug reagent (BD) was added, and incubation was continued for additional 4 h. Cells were harvested, stained with anti-CD3 and anti-CD8 antibodies, fixed, permeabilized, and stained with anti-IFNγ, anti-TNFα, and anti-IL-2 antibodies (all from BD). Data acquisition was performed on an LSR-II flow cytometer.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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**Supplemental Material**
Supplemental materials may be found here: http://www.landesbioscience.com/journals/oncoimmunology/article/25374
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