Human $\mathrm{T}_{\mathrm{h}}17$ Immune Cells Specific for the Tumor Antigen MAGE-A3 Convert to IFN-$\gamma$–Secreting Cells as They Differentiate into Effector T Cells In Vivo

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Abstract

The role of $\mathrm{T}_{\mathrm{h}}17$ cells in cancer is being investigated, but the existence of tumor antigen–specific $\mathrm{T}_{\mathrm{h}}17$ cells has yet to be ascertained. Here, we report the first description of a spontaneous $\mathrm{T}_{\mathrm{h}}17$ (IL-$\gamma$+) response to the important tumor antigen MAGE-A3, which occurred concurrently with a $\mathrm{T}_{\mathrm{h}}1$ (IFN-$\gamma$+) response in a lung cancer patient. MAGE-A3–specific interleukin (IL)-17+ T cells were mainly CCR7$^+$ central memory T cells, whereas IFN-$\gamma$+ cells were enriched for CCR7$^-$ effector memory T cells. An assessment of the fine specificity of antigen recognition by these T cells indicated that the CCR6$^+$CCR4$^+$ and CCR6$^+$CXCR3$^+$ fractions contained the same $\mathrm{T}_{\mathrm{h}}117$/$\mathrm{T}_{\mathrm{h}}1$ population at early and late differentiation stages, respectively, whereas the CCR6$^+$CXCR3$^+$ fraction contained a distinct $\mathrm{T}_{\mathrm{h}}1$ population. These findings are important because they suggest a differentiation model in which tumor antigen–specific CD4$^+$ T cells that are primed under $\mathrm{T}_{\mathrm{h}}17$ polarizing conditions will progressively convert into IFN-$\gamma$–secreting cells in vivo as they differentiate into effector T cells that can effectively attack tumors. Cancer Res; 72(5); 1059–63. ©2012 AACR.

Introduction

$\mathrm{T}_{\mathrm{h}}17$ cells have been recently defined as a distinct subset involved in the pathogenesis of inflammatory autoimmune diseases, but also in host protection against extracellular bacteria, fungi, and protozoa (1). Consistent with a physiologic role of $\mathrm{T}_{\mathrm{h}}17$ in protecting mucosal surfaces such as the gut, lungs, and skin, the subset has been shown to be prevalent at these locations (2). In humans, $\mathrm{T}_{\mathrm{h}}17$ cells are found among memory populations, suggesting that they differentiate in response to antigen in vivo, but little direct evidence of their antigen specificity has been reported (3). In this context, a recent report has suggested that generation of intestinal $\mathrm{T}_{\mathrm{h}}17$ requires microbiota but not microbial derived antigens (4). In addition, it has been recently proposed that interleukin (IL)-17–secrating T cells represent a transient phenotype of populations that tend to convert to IFN-$\gamma$–producing cells (5).

The role of $\mathrm{T}_{\mathrm{h}}17$ in cancer is being investigated. Several recent findings indicate a beneficial role for $\mathrm{T}_{\mathrm{h}}17$ in antitumor immunity. Among them are the positive association between intratumoral $\mathrm{T}_{\mathrm{h}}17$ and IFN-$\gamma$ effector cells, CTL and natural killer cells, reported for some human tumor types (6) along with evidence that murine transgenic T cells polarized in vitro to $\mathrm{T}_{\mathrm{h}}17$ induce tumor regression following in vivo transfer (7). Other data, however, depict a more complex picture and suggest instead a negative effect. These include the reported proangiogenic and protumoral activity of the $\mathrm{T}_{\mathrm{h}}17$ signature cytokine IL-$\gamma$, documented by early studies in immune-deficient mice (8), reports of association between the prevalence of tumor-associated $\mathrm{T}_{\mathrm{h}}17$ and bad clinical outcomes in some tumor types (9), and the close relationship between $\mathrm{T}_{\mathrm{h}}17$ and FOXP3$^+$ Treg, that play opposite immune functions (10–12).

As the role of $\mathrm{T}_{\mathrm{h}}17$ in cancer is still to be ascertained, no evidence for the existence of $\mathrm{T}_{\mathrm{h}}17$ specific for human tumor antigens has been yet provided. In this study, we report the first description of $\mathrm{T}_{\mathrm{h}}17$ specific for the tumor antigen MAGE-A3 in a patient affected by lung cancer. Together, our results support a differentiation model in which MAGE-A3–specific CD4$^+$ T cells primed under $\mathrm{T}_{\mathrm{h}}17$ polarizing conditions progressively convert into IFN-$\gamma$–secreting as they differentiate into effector cells in vivo.

Materials and Methods

Patients’ samples and cell sorting

Peripheral blood samples were collected from lung cancer patients seen at the CLCC René Gauducheau upon written informed consent and approval by the Institutional Review Board (Comité de Protection des Personnes Ouest 2–Angers).

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

doi: 10.1158/0008-5472.CAN-11-3432
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CD4\(^+\) T cells were enriched by positive selection from peripheral blood mononuclear cells by magnetic cell sorting (Miltenyi Biotec). For ex vivo flow cytometry cell sorting, enriched CD4\(^+\) T cells were stained with fluorochrome-labeled mAb (from BD Biosciences unless indicated otherwise) specific for CD45RA, CCR7, CD25 (Beckman Coulter), CD127 (eBioscience), CCR4, CCR6, and CXCR3 and sorted into the indicated populations to high purity (>97%; FACSARia; BD Biosciences).

**In vitro stimulation and functional assessment of MAGE-A3–specific CD4\(^+\) T cells**

Total CD4\(^+\) T cells or ex vivo sorted subpopulations were stimulated in vitro with a pool of 20 to 24 amino acid long peptides overlapping by 10 amino acids and covering the MAGE-A3 sequence (Supplementary Table S1), in the presence of irradiated autologous antigen-presenting cells (APC), and cultured in the presence of recombinant human IL-2 (Chiron). Days 10 to 14 cultures were assessed in a 4-hour intracellular cytokine staining assay using mAb specific for IFN-\(\gamma\) (BD Biosciences) and IL-17 (eBioscience) following stimulation with the MAGE-A3 peptide pool and analyzed by flow cytometry (FACSARia; BD Biosciences). In some experiments, cells were stained with cytokine-specific mAb together with mAb specific for ROR\(\gamma\)/\(\gamma\)t and T-bet (eBioscience; according to the manufacturer’s instructions). To determine the fine specificity of antigen recognition, aliquots of CD4\(^+\) T-cell cultures were stimulated in the absence or presence of the peptide pool or of individual MAGE-A3 peptides and IL-17, and IFN-\(\gamma\) were assessed in 24-hour culture supernatants by ELISA (R&D Systems and Invitrogen, respectively).

**Results and Discussion**

The human tumor antigen MAGE-A3, of the cancer/testis antigen group (13), is frequently expressed in lung cancers (14) and a MAGE-A3–based anticancer vaccine is currently being evaluated in lung cancer patients (15). We assessed circulating lymphocytes from 38 lung cancer patients (Supplementary Table S2) for spontaneous CD4\(^+\) T-cell responses to MAGE-A3. To this end, we stimulated isolated circulating CD4\(^+\) T cells from the patients with a pool of long overlapping peptides spanning the entire MAGE-A3 protein (Supplementary Table S1) and assessed the cultures 12 days later for IFN-\(\gamma\) and IL-17 production in response to the Ag. We detected significant proportions of specific CD4\(^+\) T cells secreting IFN-\(\gamma\) in 3 patients (Fig. 1A). In one of them, patient NA171, a significant IL-17 response was also detected. Simultaneous assessment of IFN-\(\gamma\)- and IL-17-secretion revealed 3 distinct subpopulations, 2 major ones secreting IL-17 or IFN-\(\gamma\) alone and one cosecreting IL-17 and IFN-\(\gamma\) (Fig. 1B).

CD4\(^+\) memory T cells expressing the chemokine receptor CCR7, called central memory (CM), represent a reservoir at an early differentiation stage that recirculate in lymphoid organs, whereas CCR7\(^-\) populations, called effector memory (EM), are at a more advanced differentiation stage and can localize in peripheral tissues (16). To address the in vivo differentiation stage of MAGE-A3–specific T\(_{IL17}\), we assessed them in conventional (CD25\(^-\)) CM and EM CD4\(^+\) T cells isolated ex vivo by flow cytometry cell sorting (Fig. 2A). Because of the reported relationship between T\(_{IL17}\) and Treg (CD25\(^+\)CD127\(^+\); ref. 12), we also assessed them in memory Treg (MTreg). MAGE-A3–specific cells secreting IL-17 alone were mostly found in CM, whereas those secreting IFN-\(\gamma\) alone or with IL-17 were enriched in EM (Fig. 2B). We did not detect MAGE-A3–specific cells in MTreg.

Expression of other chemokine receptors distinguishes CD4\(^+\) T cell subsets with different migratory ability and effector functions. Whereas expression of CXCR3 characterizes T\(_{h1}\) and CCR4 T\(_{h2}\), CCR6 has been reported to characterize T\(_{IL17}\) and Treg (17). To further characterize MAGE-A3–specific T\(_{IL17}\), we assessed them in CD4\(^+\) T cell populations sorted ex vivo based on the expression of CCR6, CXCR3, and CCR4 (Fig. 3A). Cells secreting IL-17 in response to MAGE-A3 were almost exclusively found in the CCR6\(^+\)CCR4\(^+\) fraction, whereas IFN-\(\gamma\)-secreting cells were found in the CCR6\(^-\)CCR4\(^+\) and CCR6\(^-\)CXCR3\(^+\) fractions (Fig. 3B). To support the identification of MAGE-A3–specific T\(_{IL17}\) and T\(_{IL1}\) cells, we assessed the expression of the lineage-specific transcription factors ROR\(\gamma\)t and T-bet, associated respectively with T\(_{IL1}\) and T\(_{IL1}\), in MAGE-A3–specific cells, by combined staining of Ag-stimulated subpopulations with antibodies against cytokines and transcription factors. As expected, we detected higher expression levels of ROR\(\gamma\)t in the CCR6\(^+\)CCR4\(^+\) fraction than in the

![Image](1060_CancerRes_72(5)March12_Fig1.png)

**Figure 1.** CD4\(^+\) T-cell responses to MAGE-A3 in lung cancer patients. Circulating CD4\(^+\) T cells were stimulated in vitro with a pool of long, overlapping peptides spanning the entire MAGE-A3 protein. Day 12 cultures were assessed following stimulation in the absence or presence of the peptide pool by intracellular cytokine staining. A, the proportions of CD4\(^+\) T cells producing IFN-\(\gamma\) and IL-17 in response to MAGE-A3 are shown for all patients (n = 38, symbols correspond to individual patients). Responses greater than 0.2 were considered significant. B, dot plots for patient NA171 are shown.
other populations (Fig. 3C and D). Expression of T-bet was inversely correlated with that of RORγt and was instead higher in the CXCR3⁺ fractions.

To further clarify the relationship between the identified MAGE-A3–specific populations, we assessed their fine specificity. We initially assessed total CD4⁺ T cells with MAGE-A3 peptides and detected reactivity against 3 peptides, 141–160, 241–260, and 271–290 (Fig. 4A). We then assessed the populations isolated according to chemokine receptors expression with the active peptides. We detected reactivity to peptide

Figure 3. MAGE-A3–specific Th17 and Th1 cells are detected in CD4⁺ T-cell populations with distinct chemokine receptors expression profiles. A, CD4⁺ T cells from patient NA171 were stained with mAb specific for CD45RA, CCR7, CD25, CD127, CCR4, CCR6, and CXCR3, and conventional memory (Mconv; CD25⁺CD45RA⁻) cells were sorted into CCR6⁺CCR4⁺, CCR6⁺CXCR3⁺, CCR6⁻CCR4⁺, and CCR6⁻CXCR3⁺ populations. B, sorted populations were stimulated with the MAGE-A3 peptide pool and cultures were assessed as in Fig. 2B. C and D, cultures were assessed, following stimulation with the MAGE-A3 peptide pool, for RORγt and T-bet expression and for IL-17 and IFN-γ production with specific mAb in an intracellular staining assay. Examples of dot plots for CCR6⁺CCR4⁺ and CCR6⁻CXCR3⁺ cultures are shown in C and the mean fluorescence intensity (MFI) of RORγt and T-bet staining is summarized in D for MAGE-A3–specific IL-17⁺, IL-17⁺/IFN-γ⁺, and IFN-γ⁺ cells, defined as in B, in the indicated responder cultures.
141–160 in the CCR6^+CCR4^+ fraction and found the same reactivity in the CCR6^+CXCR3^+ fraction (Fig. 4B). In contrast, in the CCR6^+/CXCR3^+ fraction the reactivity was distinct and directed against peptides 241–260 and 271–290. Together, these results indicated that the CCR6^+CCR4^+ and CCR6^+CXCR3^+ fractions contained the same MAGE-A3–specific T_H^17/T_H^1 population at early (CM) and late (EM) differentiation stages, respectively, whereas the CCR6^+CXCR3^+ fraction contained a distinct T_H^1 population. To further support the conclusion that MAGE-A3 141–160–specific CD4^+ T cells in this patient represented a single population, we isolated them based on CD154 upregulation following antigen stimulation and expanded them in vitro under clonal conditions. We obtained several MAGE-A3 141–160–specific CD4^+ T cells from patient NA171 and performed TCR analysis of the clones using anti-TCR V_b mAb revealed that they all used V_b2 (data not shown) and molecular analysis of the TCR beta variable gene (TRBV) 20-1, showed that they all used a unique TCR beta joining gene (TRBJ) and displayed an identical CDR3_b (Supplementary Fig. S1D).

Together, the findings reported here show for the first time that T_H^17 specific for a common tumor antigen can be found in cancer patients as part of their spontaneous immune response to the autologous tumor. In addition, they support a recently proposed differentiation model in which CD4^+ T cells primed in vivo under T_H^17 conditions progressively convert into IFN-γ–secreting as they differentiate into effector cells (5).

The significance and potential impact of tumor antigen–specific T_H^17 responses in lung cancer warrant further investigation, as both positive and negative correlations between the...
presence of tumor-associated IL-17-secreting cells and survival have been reported (18, 19), a discrepancy that may be explained by the involvement of cells other than those of adaptive antitumor immunity (e.g., IL-17–secreting γ/δ T cells) as recently suggested (20). In favor of the antitumor potential of adaptive T_{H17} immunity, it has been recently shown that, in a B16 melanoma model, transfer of in vitro polarized antitumor T_{H17} lines controlled tumor growth better than T_{H1} lines, an effect that was dependent on IFN-γ and independent of IL-17 (7). The existence of spontaneously arising tumor antigen–specific T_{H17} cells in patients with lung cancer, along with their penchant to convert into IFN-γ-secreting cells as they differentiate into effectors, therefore encourages the development of immunotherapeutic approaches aimed at their amplification.

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

Acknowledgments
The authors are grateful to Dr. Gerd Ritter for providing the recombinant MAGE-A3 and Melan-A proteins.

Grant Support
The study was supported by the Cancer Research Institute, the Ludwig Institute for Cancer Research, the Institut National du Cancer (France), and the Cancéropôle Ile de France (France).

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Received October 14, 2011; revised December 1, 2011; accepted December 29, 2011; published OnlineFirst January 17, 2012.

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