Priority Report

**Human T_{H}17 immune cells specific for the tumor antigen MAGE-A3 convert to IFN-γ secreting cells as they differentiate into effector T cells in vivo**

Ahmed Hamai\(^1\), Pascale Pignon\(^1\), Isabelle Raimbaud\(^1\), Karine Duperrier-Amouriaux\(^1\), Hélène Senellart\(^2\), Sandrine Hiret\(^2\), Jean-Yves Douillard\(^2\), Jaafar Bennouna\(^2\), Maha Ayyoub\(^1,4\) and Danila Valmori\(^1,3,4\)

\(^1\) Institut National de la Santé et de la Recherche Médicale, Unité 1102 and \(^2\) Department of Medical Oncology, Institut de Cancérologie de l'Ouest, 44800 Saint Herblain, France and \(^3\) Faculty of Medicine, University of Nantes, 44093 Nantes, France

\(^4\) Maha Ayyoub and Danila Valmori share senior authorship

**Running title**: Spontaneous T_{H}17 responses to MAGE-A3 in lung cancer

**Keywords**: CD4\(^+\) T cell responses; T_{H}17; tumor antigens; MAGE-A3; lung cancer

**Financial support**: The study was supported by the Cancer Research Institute (USA), the Ludwig Institute for Cancer Research (USA), the Institut National du Cancer (France) and the Cancéropôle Île de France (France)

**Corresponding authors**: Maha Ayyoub and Danila Valmori, INSERM U1102, Institut de Cancérologie de l'Ouest, 44800 Saint Herblain, France; Phone: 33 (0)2 40 67 97 26; fax: 33 (0)2 40 67 97 63; e-mail: Danila.Valmori@univ-nantes.fr (Danila Valmori) and Maha.Ayyoub@univ-nantes.fr (Maha Ayyoub)

The authors declare no conflict of interest.

2500 text words and 4 figures
Abstract

The role of T<sub>H</sub>17 cells in cancer is being investigated but the existence of tumor antigen-specific T<sub>H</sub>17 cells has yet to be ascertained. Here we report the first description of a spontaneous T<sub>H</sub>17 (IL-17<sup>+</sup>) response to the important tumor antigen MAGE-A3, which occurred concurrently with a T<sub>H</sub>1 (IFN-γ<sup>+</sup>) response in a lung cancer patient. MAGE-A3-specific IL-17<sup>+</sup> T cells were mainly CCR7<sup>+</sup> central memory T cells, whereas IFN-γ<sup>+</sup> cells were enriched for CCR7<sup>−</sup> effector memory T cells. An assessment of the fine specificity of antigen recognition by these T cells indicated that the CCR6<sup>−</sup>CCR4<sup>+</sup> and CCR6<sup>−</sup>CXCR3<sup>+</sup> fractions contained the same T<sub>H</sub>17/T<sub>H</sub>1 population at early and late differentiation stages, respectively, whereas the CCR6<sup>−</sup>CXCR3<sup>+</sup> fraction contained a distinct T<sub>H</sub>1 population. These findings are important, because they suggest a differentiation model in which tumor antigen-specific CD4<sup>+</sup> T cells that are primed under T<sub>H</sub>17 polarizing conditions will progressively convert into IFN-γ-secreting cells in vivo as they differentiate into effector T cells that can effectively attack tumors.
Introduction

TH17 cells have been recently defined as a distinct subset involved in the pathogenesis of inflammatory autoimmune diseases, but also in host protection against extra-cellular bacteria, fungi and protozoa (1). Consistent with a physiologic role of TH17 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, TH17 cells are found among memory populations, suggesting that they differentiate in response to antigen \textit{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 TH17 requires microbiota but not microbial derived antigens (4). In addition, it has been recently proposed that IL-17-secreting T cells represent a transient phenotype of populations that tend to convert to IFN-\(\gamma\)-producing cells (5).

The role of TH17 in cancer is being investigated. Several recent findings indicate a beneficial role for TH17 in anti-tumor immunity. Among them are the positive association between intra-tumoral TH17 and IFN-\(\gamma\) effector cells, CTL and NK cells, reported for some human tumor types (6) along with evidence that murine transgenic T cells polarized \textit{in vitro} to TH17 induce tumor regression following \textit{in vivo} transfer (7). Other data, however, depict a more complex picture and suggest instead a negative effect. These include the reported pro-angiogenic and pro-tumoral activity of the TH17 signature cytokine IL-17, documented by early studies in immune-deficient mice (8), reports of association between the prevalence of tumor-associated TH17 and bad clinical outcomes in some tumor types (9) and the close relationship between TH17 and FOXP3\(^{+}\) Treg, that play opposite immune functions (10-12).

As the role of TH17 in cancer is still to be ascertained, no evidence for the existence of TH17 specific for human tumor antigens has been yet provided. In this
study, we report the first description of TH17 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 TH17 polarizing conditions progressively convert into IFN-γ-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). 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 20-24 amino acid long peptides overlapping by 10 amino acids and covering the MAGE-A3 sequence (Supplementary Table S1), in the presence of irradiated autologous APC, and cultured in the presence of recombinant human IL-2 (Chiron). Day 10 to 14 cultures were assessed in a 4 h intracellular cytokine staining assay using mAb specific for IFN-γ (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 RORγ/γ 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 the presence of the peptide pool or of individual MAGE-A3 peptides and IL-17 and IFN-\(\gamma\) were assessed in 24 h 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 anti-cancer 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-γ and IL-17 production in response to the Ag. We detected significant proportions of specific CD4+ T cells secreting IFN-γ in 3 patients (Fig. 1A). In one of them, patient NA171, a significant IL-17 response was also detected. Simultaneous assessment of IFN-γ and IL-17 secretion revealed 3 distinct subpopulations, two major ones secreting IL-17 or IFN-γ alone and one co-secreting IL-17 and IFN-γ (Fig. 1B).

CD4+ memory T cells expressing the chemokine receptor CCR7, called central memory (CM), represent a reservoir at an early differentiation stage that re-circulate 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 Th17, 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 Th17 and Treg (CD25+CD127+) (12), we also assessed them in memory Treg. MAGE-A3-specific cells secreting IL-17 alone were mostly found in CM, whereas those secreting IFN-γ alone or with IL-17 were enriched in EM (Fig. 2B). We did not detect MAGE-A3-specific cells in Treg.
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_h17 and Treg (17). To further characterize MAGE-A3-specific T_h17, 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-γ-secreting cells were found in the CCR6⁺CXCR3⁺ and CCR6⁻CXCR3⁺ fractions (Fig. 3B). To support the identification of MAGE-A3-specific T_h17 and T_h1 cells, we assessed the expression of the lineage specific transcription factors RORγt and T-bet, associated respectively with T_h17 and T_h1, 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 RORγt in the CCR6⁺CCR4⁺ fraction than in the 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 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 Th17/Th1 population at early (CM) and late (EM) differentiation stages, respectively, whereas the CCR6’CXCR3+ fraction contained a distinct Th1 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 clones that secreted IL-17 and/or IFN-γ (Supplementary Fig. S1A and B). In addition to recognizing peptide MAGE-A3 141-160, the clones recognized autologous DC incubated with a recombinant MAGE-A3 protein but not with a control protein (Supplementary Fig. S1C). TCR analysis of the clones using anti-TCR Vβ mAb revealed that they all used Vβ2 (data not shown) and molecular analysis of the TCR β chain mRNA from 7 clones using specific primers further confirmed that they used the T-cell receptor beta variable gene (TRBV) 20-1, showed that they all used a unique T-cell receptor beta joining gene (TRBJ) and displayed an identical CDR3β (Supplementary Fig. S1D).

Together, the findings reported here demonstrate for the first time that Th17 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 Th17 conditions progressively convert into IFN-γ-secreting as they differentiate into effector cells (5).

The significance and potential impact of tumor antigen-specific Th17 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 anti-tumor immunity (e.g. IL-17-secreting γ/δ T cells) as recently suggested (20). In favor of the anti-tumor potential of adaptive TH17 immunity, it has been recently shown that, in a B16 melanoma model, transfer of in vitro polarized anti-tumor TH17 lines controlled tumor growth better than TH1 lines, an effect that was dependent on IFN-γ and independent of IL-17 (7). The existence of spontaneously arising tumor antigen-specific TH17 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.

**Acknowledgments:** We 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 (USA), the Ludwig Institute for Cancer Research (USA), the Institut National du Cancer (France) and the Cancéropôle Île de France (France).
References


Figure legends

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-γ and IL-17 in response to MAGE-A3 are shown for all patients (n=38, symbols correspond to individual patients). Responses > 0.2 were considered significant. B, Dot plots for patient NA171 are shown.

Figure 2. MAGE-A3-specific CD4⁺ T cells are detected in conventional central and effector memory CD4⁺ T cells but not in memory Treg. A, CD4⁺ T cells from patient NA171 were stained with mAb specific for CD45RA, CCR7, CD25 and CD127 and memory (CD45RA⁻) cells were sorted into MTreg (CD25⁺CD127low), conventional CM (CD25⁺CCR7⁺) and conventional EM (CD25⁺CCR7⁻) populations. B, Sorted populations were stimulated *in vitro* with the MAGE-A3 peptide pool and assessed 12 days later for IFN-γ and IL-17 production in response to stimulation with the Ag by intracellular cytokine staining.

Figure 3. MAGE-A3-specific T_H17 and T_H1 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 (M_conv, 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 Figure 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 using 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.

Figure 4. Fine specificity of MAGE-A3-specific IL-17⁺ and IFN-γ⁺ CD4⁺ T cells. A, CD4⁺ T cells from patient NA171 were stimulated in vitro with the MAGE-A3 peptide pool. Aliquots of day 14 cultures were stimulated in the absence or presence of the peptide pool or of individual MAGE-A3 peptides, as indicated, and IFN-γ and IL-17 were measured in 24 h culture supernatants by ELISA. Responses to single peptides were considered significant when the cytokine level was > 3 folds the background cytokine level detected in the absence of peptide (indicated by the dotted line for each cytokine). B, CD4⁺ T cells from patient NA171 were sorted into the indicated subpopulations according to chemokine receptors expression as in Figure 3A and stimulated in vitro with the MAGE-A3 peptide pool. Aliquots of the cultures were stimulated in the absence or in the presence of the indicated MAGE-A3 peptides and IFN-γ and IL-17 were assessed in 24 h culture supernatants by ELISA.
Figure 1

A

B

No peptide

MAGE-A3 peptide pool
Figure 3

A

B

C

D
Figure 4

A

Total CD4+ T cells

IFN-γ (pg/ml)

IL-17 (pg/ml)

MAGE-A3

B

CCR6+CCR4+

CCR6+CXCR3+

CCR6+CCR4+

CCR6+CXCR3+

MAGE-A3

IFN-γ (pg/ml)

IL-17 (pg/ml)