

Matrix Metalloproteinase-2 Conditions Human Dendritic Cells to Prime Inflammatory T_H2 Cells via an IL-12- and OX40L-Dependent Pathway

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SUMMARY

Matrix metalloproteinase-2 (MMP-2) is a proteolytic enzyme degrading the extracellular matrix and overexpressed by many tumors. Here, we documented the presence of MMP-2-specific CD4⁺ T cells in tumor-infiltrating lymphocytes (TILs) from melanoma patients. Strikingly, MMP-2-specific CD4⁺ T cells displayed an inflammatory T_H2 profile, i.e., mainly secreting TNF- α , IL-4, and IL-13 and expressing GATA-3. Furthermore, MMP-2-conditioned dendritic cells (DCs) primed naïve CD4⁺ T cells to differentiate into an inflammatory T_H2 phenotype through OX40L expression and inhibition of IL-12p70 production. MMP-2 degrades the type I IFN receptor, thereby preventing STAT1 phosphorylation, which is necessary for IL-12p35 production. Active MMP-2, therefore, acts as an endogenous type 2 “conditioner” and may play a role in the observed prevalence of detrimental type 2 responses in melanoma.

INTRODUCTION

A large array of human melanoma-associated antigens (MAA) has been identified and used in various immunization strategies to treat cancer patients. However, despite significant induction of tumor-specific T cells (Coulie and van der Bruggen, 2003; Rosenberg, 2004), the therapeutic efficacy of these approaches has been suboptimal indicating a need for improving current strategies. Possible explanations for failure (Loose and Van de Wiele, 2009) include malignant cells producing immunosuppressive cytokines (IL-10, TGF- β , IL-6, and M-CSF), prostaglandins, and vascular endothelial growth factor thereby skewing the immune response toward type 2 or regulatory T cells and deleteriously modulating the differentiation, maturation, and function of antigen-presenting cells (APCs). Furthermore, malignant cells that chronically stimulate infiltrating T cells can actively exhaust

and eliminate T cells through expression of molecules such as FasL, PDL-1, or RCAS1. Finally, due to immune pressure, immunoresistant tumor cell variants emerge through selection of mutants with reduced antigenicity. This can affect the expression/function of molecules implicated in antigen processing and presentation or the expression of tumor antigens themselves (Hirohashi et al., 2009; Yee et al., 2000).

A way to circumvent this latter limitation would be to vaccinate against immunogenic proteins whose expression is critical for tumor growth and/or invasiveness. The matrix metalloproteinase-2 (MMP-2), overexpressed in many tumors including melanoma, may be such an antigen. MMP-2 is a proteolytic enzyme that degrades numerous components of extracellular matrix such as collagens, laminin, or fibronectin and contributes to cell migration by clearing the surrounding extracellular matrix and basement membrane barriers. MMP-2 overexpression has

Significance

Several melanoma-associated antigens (MAA) have been targeted in immunization strategies to treat melanoma patients. However, the therapeutic efficacy of these approaches remains limited, indicating an urgent need for improvement. Because MMP-2 activity is critical for melanoma progression, it represents an interesting target for vaccine therapy. We show that MMP-2 is an immunogenic tumor antigen. However, MMP-2-specific CD4⁺ T lymphocytes display a suboptimal inflammatory T_H2 profile. MMP-2-conditioned DCs prime T_H2 responses against several MAA, suggesting that MMP-2 can create a T_H2 skewing microenvironment in a bystander fashion. Elucidation of the underlying mechanisms opens the way to improving immune responses toward a more effective T_H1 response, and highlights the potential of MMP-2 as a target antigen in melanoma.

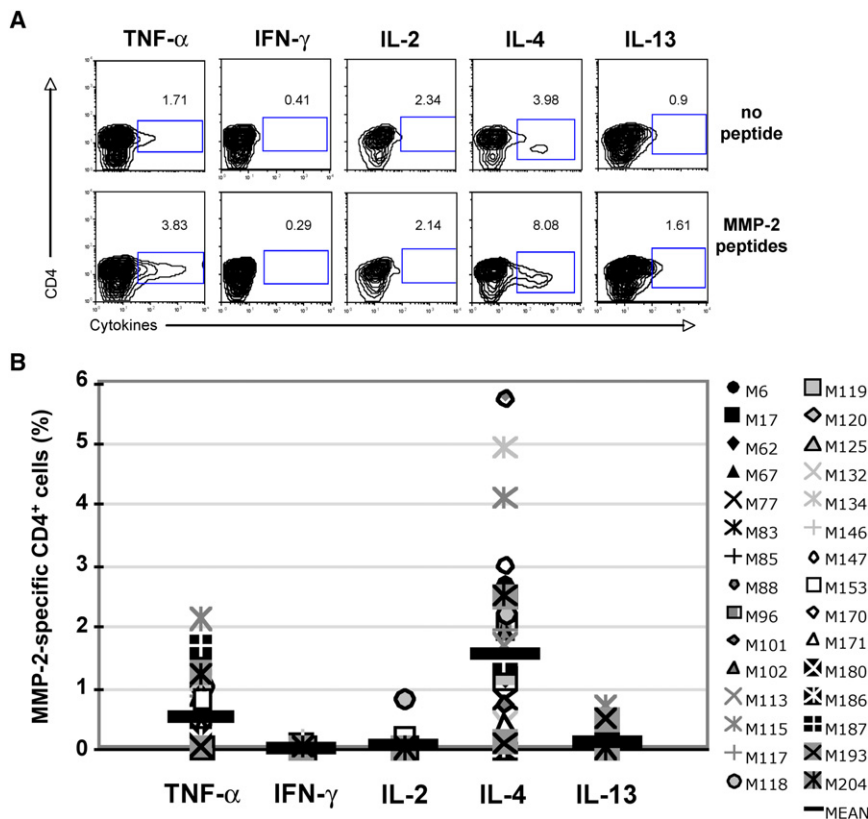


Figure 1. MMP-2-Specific CD4⁺ T Cell Responses in Melanoma Patients

TILs from representative melanoma patient M186 (A) or from 31 unselected patients (B) were stimulated with the MMP-2 peptide pool (2 μ M) for 6 hr and assessed for cytokine production by intracellular staining. Percentage of secreting cells upon peptide stimulation was considered specific/positive when it exceeded by more than 2-fold the background (cytokine-secreting cells in the absence of stimulation) and had more than 0.5% of responding cells (after background subtraction) for at least one cytokine. See also Figure S1 for MMP-2-derived peptides and patient survival analysis.

CD8⁺ T cells infiltrating their tumors and whose tumor cells produced MMP-2 protein (Godefroy et al., 2005). Strikingly, MMP-2-specific CD4⁺ T cells were found in 13 out of 31 unselected TIL populations (Figures 1A and 1B). Among these 13 responders, the percentage of cells secreting TNF- α upon stimulation ranged from 0.04% to 2.12% (mean = 0.78% \pm 0.53) and IL-4 from 1.17% to 4.91% (mean = 2.44% \pm 1.21). Very few cells produced IFN- γ and IL-2 (Figures 1A and 1B). Therefore, more than 40% of

tested TILs contained MMP-2-specific CD4⁺ T cells preferentially secreting IL-4 and TNF- α .

been associated with tumor progression. Indeed, MMP-2 modulates various oncogenic processes such as angiogenesis (Brooks et al., 1998; Itoh et al., 1998) and tumor dissemination (Kessenbrock et al., 2010; Liotta et al., 1980; Westermarck and Kahari, 1999).

We previously identified MMP-2 as a melanoma-associated antigen (MAA) recognized by HLA-A*0201-restricted CD8⁺ tumor-infiltrating lymphocytes (TILs) (Godefroy et al., 2005). Because MMP-2 activity is critical for melanoma progression, MMP-2 is a promising tumor antigen to target in immunotherapy against malignant melanoma. Accordingly, several patients administered CD8⁺ T cells that recognize this epitope among others have remained tumor-free up to 15 years after treatment (Godefroy et al., 2005; Khammari et al., 2007).

As CD4 help is essential for generating effective antitumor immunity we evaluated whether MMP-2 could also be recognized by CD4⁺ T cells. Here, we characterize these cells and identify mechanisms by which they are generated.

RESULTS

MMP-2-Specific CD4⁺ T Cell Responses in Melanoma Patients

Whether CD4⁺ T cells recognize MMP-2-derived epitopes has not previously been established. We used a pool of 20 amino acid long, partially overlapping peptides spanning the entire sequence of MMP-2 (see Figure S1A available online) to evaluate specific responses in TILs derived from melanoma patients, in some of whom we had previously detected MMP-2-specific

To try and evaluate whether these TILs played any therapeutic role, we assessed the clinical outcome of patients who had CD4⁺ TILs targeting MMP-2. Survival status was available for 21 of these patients. Twelve patients displayed MMP-2-specific CD4⁺ T cell responses and demonstrated a trend toward a poorer clinical outcome compared with patients with no detectable MMP-2 responses (Figure S1B). However, the observed difference in patient's survival was not statistically significant ($p = 0.121$).

MMP-2-Specific CD4⁺ T Cell Responses in Healthy Donors

To further study and characterize MMP-2-specific CD4⁺ T cells, we stimulated circulating isolated CD4⁺ cells from 16 healthy donors with the same pool of MMP-2 peptides. Twelve days later, cultures were assessed for MMP-2-specific CD4⁺ T cells by monitoring intracellular cytokine staining upon stimulation with the peptide pool. We derived 14 MMP-2-reactive polyclonal populations (Figures 2A and 2B). Among these 14 responders and upon peptide restimulation, the percentage of cells secreting TNF- α ranged from 0 to 2.8% (mean = 0.46% \pm 0.69), IFN- γ from 0 to 0.44% (mean = 0.07% \pm 0.11), IL-2 from 0 to 0.24% (mean = 0.06% \pm 0.06), IL-4 from 0.55 to 4.93% (mean = 1.69% \pm 1.3) and IL-13 from 0 to 1.21% (mean = 0.15% \pm 0.31) (Figure 2B). No MMP-2-specific CD4⁺ T cells secreted IL-10 and very few cells secreted type 1 cytokines (Figures 2A and 2B). To determine whether MMP-2 responses

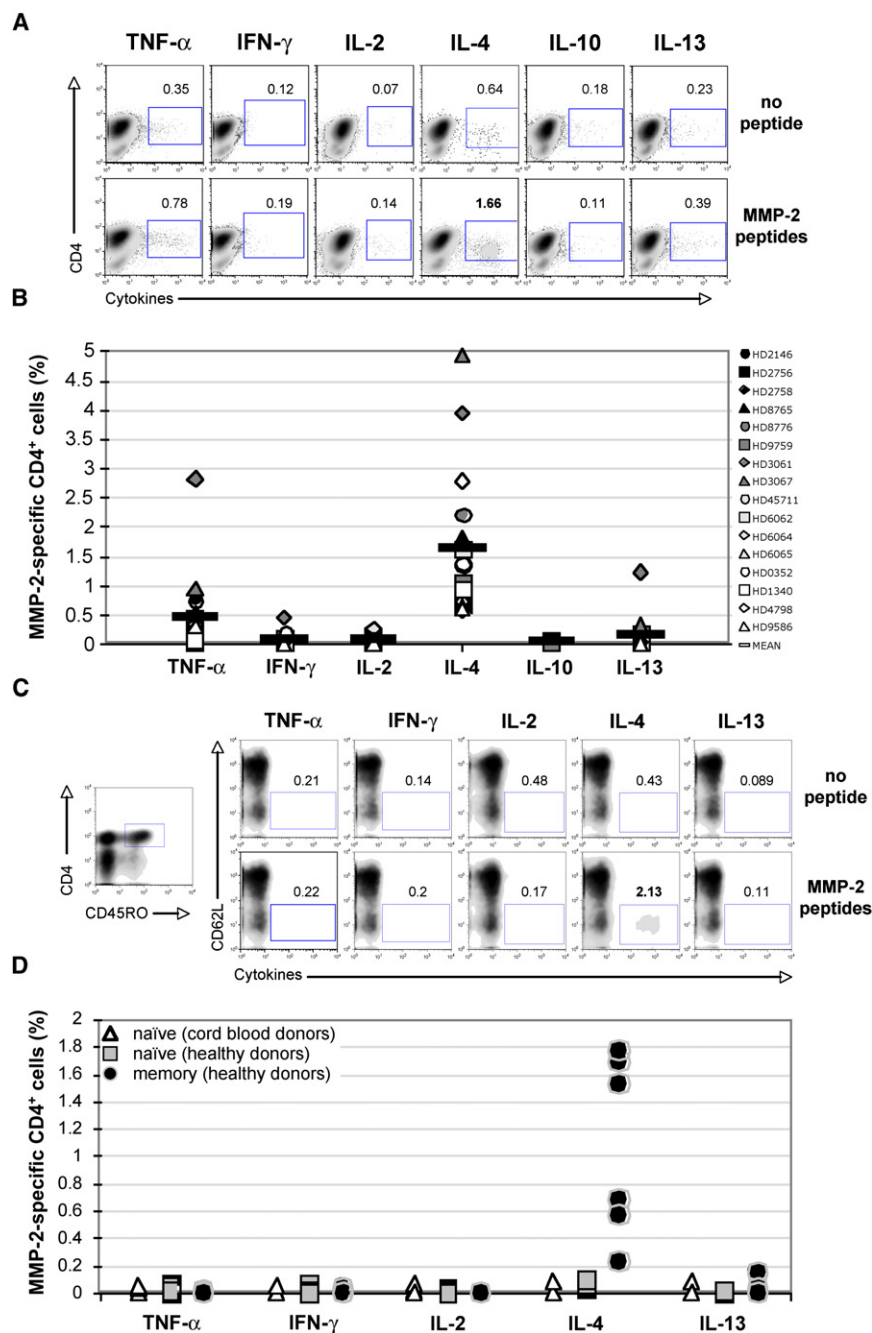


Figure 2. MMP-2-Specific Polyclonal CD4⁺ T Cell Responses in Healthy Donors

(A) Isolated CD4⁺/CD25⁻ T cells from 16 healthy donors were individually stimulated with the MMP-2 peptide pool (2 μ M) pulsed on the irradiated (35 grays) autologous CD4⁺ fraction. 12 days later, cells were restimulated, in an auto-presentation manner (no APCs), with the same peptides (2 μ M) for 6 hr, and assessed for cytokine production by intracellular staining. Density plots represent cytokine production by representative donor HD9759.

(B) Percentages of MMP-2-specific CD4⁺ T cells (% of producing CD4⁺ cells after peptide stimulation – % of producing CD4⁺ cells nonstimulated [background]) are shown for all donors. Percentage of secreting cells upon peptide stimulation was considered specific/positive when it exceeded by more than 2-fold the background (cytokine-secreting cells in the absence of peptide stimulation) and had more than 0.5% of responding cells (after background subtraction) for at least one cytokine.

(C) Ex vivo PBMCs from six healthy donors were stimulated by adding the MMP-2 peptide pool (2 μ M) onto the cells (no specific APCs were targeted). The gating strategy for visualizing memory CD4⁺ T cells is shown on the left plot. Density plots showing cytokine production of CD4⁺/CD45RO⁺/CD62L⁻ memory cells are represented for representative donor HD7957. Percentages of producing cells among CD4⁺/CD45RO⁺ gated cells are indicated.

(D) MMP-2-specific memory CD4⁺ T cell responses for all donors are represented. CD4⁺/CD45RA⁺/CD62L⁺ naïve cells derived from healthy (n = 6) and cord blood (n = 9) donors were used as controls.

See also Figure S2.

can be detected directly ex vivo, we stimulated PBMCs from six healthy donors with MMP-2 peptides and measured their cytokine responses. Responding gated CD4⁺ memory T cells (CD4⁺/CD45RO⁺/CD62L⁻) secreted IL-4 (mean = 1.09% \pm 0.61 of CD4⁺/CD45RO⁺ cells), but none of the other cytokines (Figures 2C and 2D). The absence of detectable TNF- α responses is likely to be due to the small percentages of MMP-2-specific CD4⁺ T cells, producing cytokine below the detection threshold. Gated naïve CD4⁺/CD45RA⁺/CD62L⁺ T cells, derived either from healthy donors or cord blood, were used as controls, and failed to respond to the MMP-2 peptides (Figure 2D). Therefore,

Nineteen CD4⁺ T cell clones responding to the MMP-2 peptide pool were subsequently characterized (as exemplified by clone HD9828/52) for epitope specificity (Figure S3A), CD4/CD8, TCR V β expression (Figure S3B) and HLA class-II isotype restriction (Figure S3C). Results for all CD4⁺ T cell clones are shown in Table S1. We looked for production of TNF- α , IL-4, and IFN- γ upon stimulation with individual peptides separately to assess epitope specificities. Nineteen CD4⁺ T cell clones recognized eleven distinct MMP-2-derived peptides, within the following amino acid positions: 1–20, 11–30, 21–40, 41–60, 161–180, 361–380, 551–570, 571–590, 601–620, 621–640, and 631–650

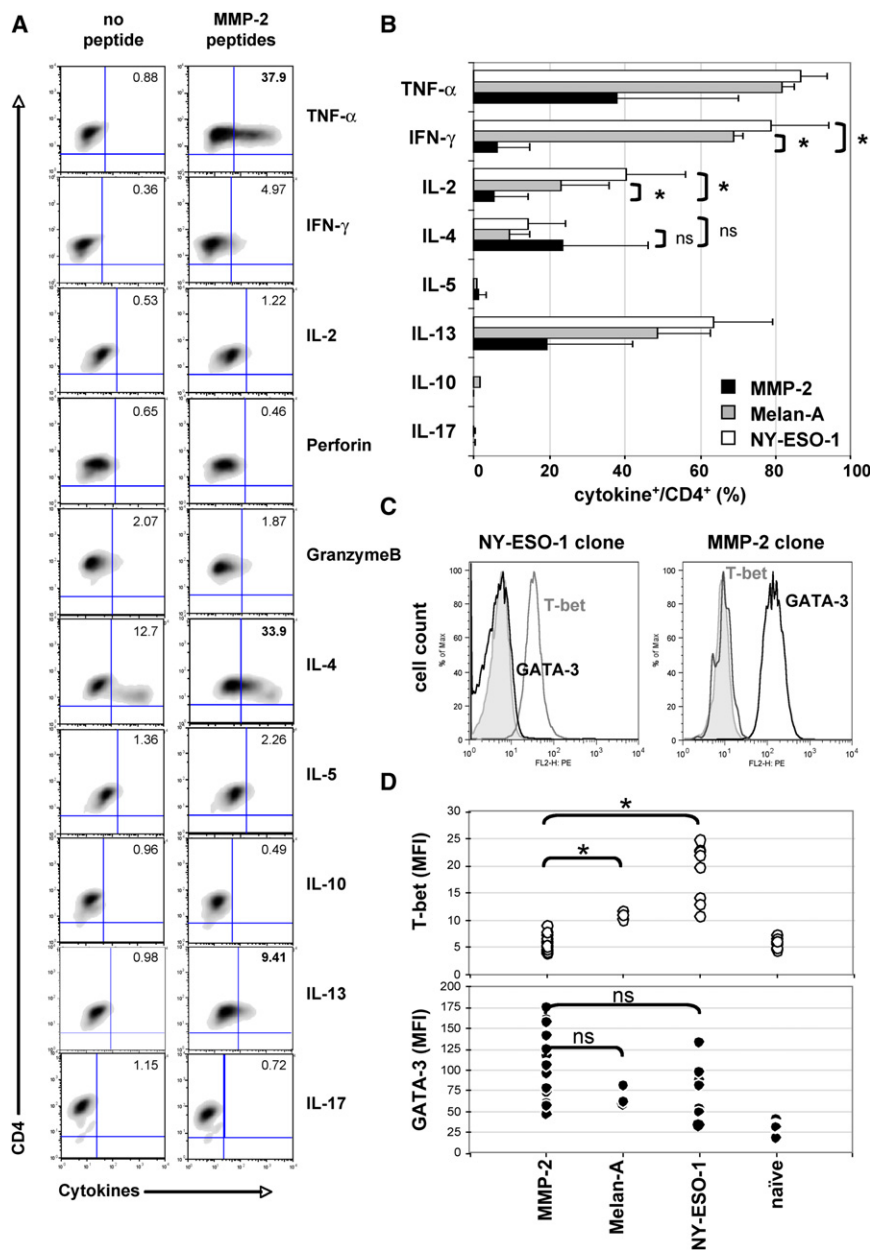


Figure 3. MMP-2-Specific CD4⁺ T Cells Display an Inflammatory T_H2 Phenotype

(A and B) CD4⁺ T cell clones were stimulated with peptides (2 μ M) for 6 hr. Cytokine production was assessed by intracellular staining (clone HD5950/MC2/43 is shown as an example) (A). The results for the 19 MMP-2-specific CD4⁺ clones are also shown (B, black bars). Three Melan-A/MART-1- and eight NY-ESO-1-specific CD4⁺ T cell clones were stimulated with peptides (2 μ M) for 6 hr before intracellular staining for cytokines (B, gray and white bars, respectively). Results are shown as mean \pm SD.

(C and D) T-bet and GATA-3 expression was measured by intracellular staining of MMP-2- (n = 19), Melan-A/MART-1- (n = 3) and NY-ESO-1-specific CD4⁺ T cell clones (n = 8). Histogram plots show a representative example for one NY-ESO-1-specific CD4⁺ T cell clone (HD3014/1) and one MMP-2-specific CD4⁺ T cell clone (HD5950/33).

(C) Isotype control is represented in tinted gray.

(D) MFIs for all CD4⁺ T cell clones are represented. Cord blood-derived CD4⁺/CD45RA⁺/CD62L⁺ naïve cells (n = 9) were used as a negative control. For each cytokine or transcription factor, analyses of variance were used to compare T cells specific for MMP-2, Melan-A and NY-ESO-1, and t tests were used for pairwise comparisons between groups. p values \leq 0.025 (*) were considered statistically significant using a Bonferroni correction for two comparisons.

See also Figure S3 and Table S1.

hand, that the whole protein is naturally processed and presented by DCs to T cells and, on the other hand, that MMP-2-specific human CD4⁺ T cells preferentially secrete IL-4 upon DC stimulation.

MMP-2-Specific CD4⁺ T Cells Display an Inflammatory T_H2 Phenotype

Because the majority of MMP-2-specific CD4⁺ T cell clones, upon peptide stimulation, secreted more IL-4 and TNF- α than IFN- γ (Figure S3A and not shown), we

(Figure S3A; Table S1). Antibodies toward most TCR V β chains were used to confirm clonality and distinguished at least 13 distinct clones among the original 19 CD4⁺ T cell clones (Figure S3B; Table S1), as >99% of each culture expressed only one V β chain (not shown). Blocking antibodies against HLA class II isotypes showed that most of the MMP-2-specific T cell clones were HLA-DR restricted (Figure S3C; Table S1).

To assess the physiological relevance of MMP-2-derived epitope presentation, CD4⁺ T cells from healthy donors were stimulated with autologous DCs loaded with whole MMP-2 protein. Twelve days later, we tested T cell responses to the peptide pool. Donors HD6026 and HD6028 had 0.12% and 0.17% of cells secreting IFN- γ as well as 0.73% and 1.99% secreting IL-4, respectively (Figure S3D). These results showed, on the one

tested them for production of a broader panel of cytokines to further assess their cytokine profile. MMP-2-specific T cell clones, as shown by the representative clone HD5950/MC2/43 (Figure 3A) or the 19 clones (Figure 3B), secreted mainly TNF- α (mean = 38.2% of secreting cells, after background subtraction), IL-4 (mean = 23.5%), and IL-13 (mean = 19.7%), but fewer cells produced IFN- γ (mean = 6.2%) and IL-2 (mean = 5.7%). This profile again corresponds to an inflammatory T_H2 phenotype (Ito et al., 2005; Soumelis et al., 2002).

Because of the unique inflammatory T_H2 profile of MMP-2-specific CD4⁺ T cells, we next examined CD4⁺ T cell responses to other MAA, under the same conditions. CD4⁺ T cells from healthy donors were stimulated with overlapping 20 amino acid long peptides spanning the length of the differentiation antigen

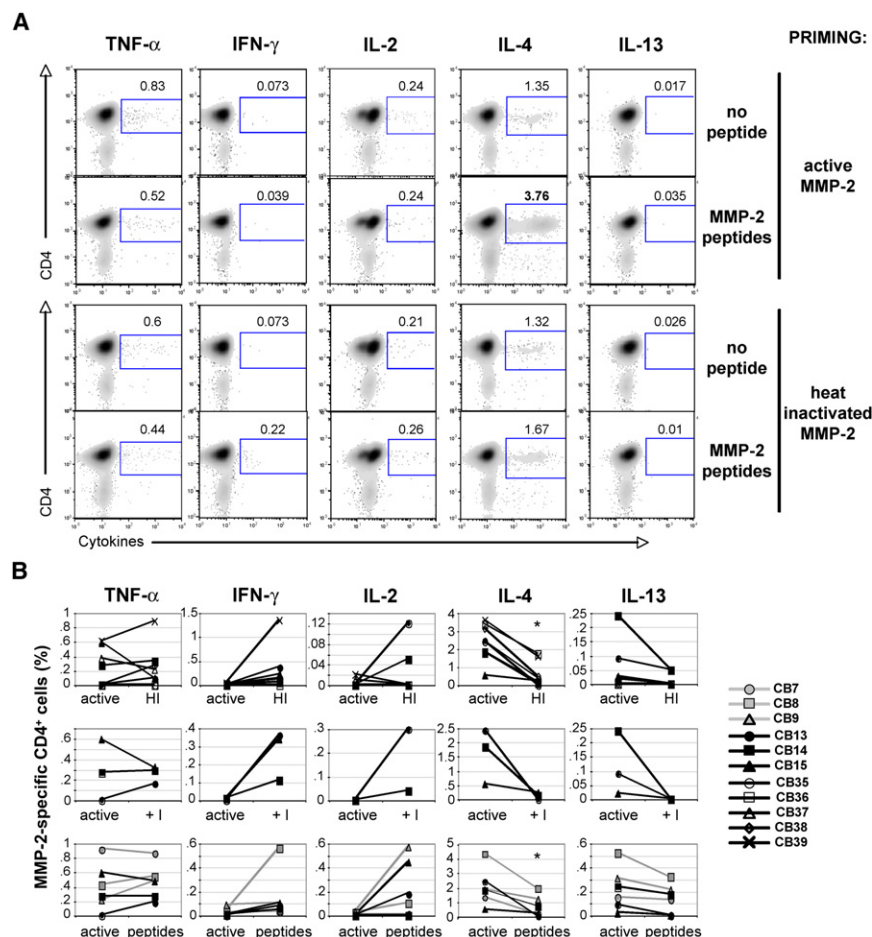


Figure 4. Effect of MMP-2 Enzyme on Specific CD4⁺ T Cell Differentiation

Core blood-derived CD4⁺/CD25⁻ cells from 11 donors were stimulated with irradiated autologous CD4⁺ cells loaded either with active MMP-2, heat-inactivated (HI) MMP-2 or with MMP-2 preincubated with a specific inhibitor (+ I). After 15 days, CD4⁺ T cells were stimulated with the MMP-2 peptide pool (2 μ M) for 6 hr before intracellular staining of cytokines.

(A) Density plots representing cytokine production by CD4⁺ T cells are shown for the representative donor CB35 primed to MMP-2 either in its active form or after heat-inactivation. Numbers indicate percentages of cytokine-producing cells upon MMP-2 peptide stimulation.

(B) Cytokines secreted by MMP-2-specific CD4⁺ T cells primed to inactive protein or peptides for all donors are shown and compared with MMP-2-specific CD4⁺ T cells generated after priming with active MMP-2. MMP-2 enzymatic activity was controlled (Figure S5C and S5D).

For each cytokine, three paired t tests were used to compare active MMP-2 to heat inactivated MMP-2, to MMP-2+I, and to MMP-2 peptides. *p* values ≤ 0.0167 (*) were considered statistically significant using a Bonferroni correction for three comparisons.

Active MMP-2 Enzyme Is Required for Differentiation of Naïve MMP-2-Specific CD4⁺ T Cells into Inflammatory T_H2 Cells

To determine whether the active full-length protein was required for MMP-2-specific CD4⁺ T cells to differentiate toward an inflammatory T_H2 phenotype,

Melan-A/MART-1 and the cancer testis antigen NY-ESO-1 sequences. Antigen-specific T cells were then enriched before generating antigen-specific CD4⁺ T cell clones. We obtained three CD4⁺ T cell clones specific for Melan-A/MART-1 (recognizing the epitopes P₂₁₋₄₀, P₅₁₋₇₀, and P₆₁₋₈₀) and eight for NY-ESO-1 (recognizing P₆₁₋₈₀, P₈₁₋₁₀₀, P₁₁₉₋₁₄₃, P₁₃₁₋₁₆₀, P₁₃₉₋₁₆₀, and P₁₆₁₋₁₈₀). Upon peptide stimulation, these clones secreted TNF- α (mean = 84.8% of secreting cells after background subtraction), IFN- γ (mean = 76.1%; overall ANOVA $p \leq 0.0001$, compared with MMP-2-specific T cell clones), IL-2 (mean = 35.0%; overall ANOVA $p \leq 0.0001$ compared with MMP-2-specific T cell clones), and IL-13 (mean = 58.5%), but low amounts of IL-4 (mean = 14.1%) (Figure 3B). To further characterize and compare MMP-2-, Melan-A/MART-1- and NY-ESO-1-specific T helper profiles, clones were stained for type 1 and type 2-associated transcription factors, T-bet and GATA-3, respectively. MMP-2-specific T cell clones expressed GATA-3 (mean MFI (mean fluorescence intensity) = 105) and insignificant levels of T-bet (mean MFI = 5.5), while Melan-A/MART-1- and NY-ESO-1-specific T cell clones expressed T-bet (mean MFI = 16.6) and lower amounts of GATA-3 (mean MFI = 72.3), confirming that CD4⁺ T cells recognizing MMP-2 exhibited a T_H2-like phenotype (Figures 3C and 3D). Cord blood-derived naïve CD4⁺ T cells, gated on CD4⁺/CD45RA⁺/CD62L⁺ cells, were used as a negative control (Figure 3D).

we primed naïve CD4⁺ T cells (from 11 cord blood donors) in vitro using autologous CD4⁺ cells loaded with different sources of MMP-2: overlapping peptides, or the protein in its active or inactive conformation. The CD4⁺ fraction was used as a source of APCs to include any cell type that could potentially be involved in priming. Peptide-specific responses of primed T cells were measured after 15 days. The percentage of MMP-2-specific CD4⁺ T cells secreting IL-4 (after background subtraction) was significantly higher when cells were primed with the active MMP-2 (mean = 2.38%), rather than with peptides (mean = 0.71%; mean of the individual differences = 1.34, $p \leq 0.013$) or inactivated MMP-2, whether inactivation occurred by heating (mean = 0.45% ; mean of differences = 1.80, $p \leq 0.0002$) or using a specific inhibitor (mean = 0.12% ; mean of differences = 1.47, ns) (Figures 4A and 4B). Furthermore, active MMP-2-primed cells produced less IFN- γ and IL-2 compared with inactive MMP-2 or peptides (Figures 4A and 4B). Thus the inflammatory TH2 lineage commitment of MMP-2-specific CD4⁺ T lymphocytes depends on the presence of active MMP-2 both as a source of antigen and as an endogenous TH2 conditioner.

Active MMP-2 Also Influences Other Tumor-Specific T Helper Differentiation

To evaluate whether active MMP-2 also affected CD4⁺ T cell differentiation specific for other MAA in a bystander fashion,

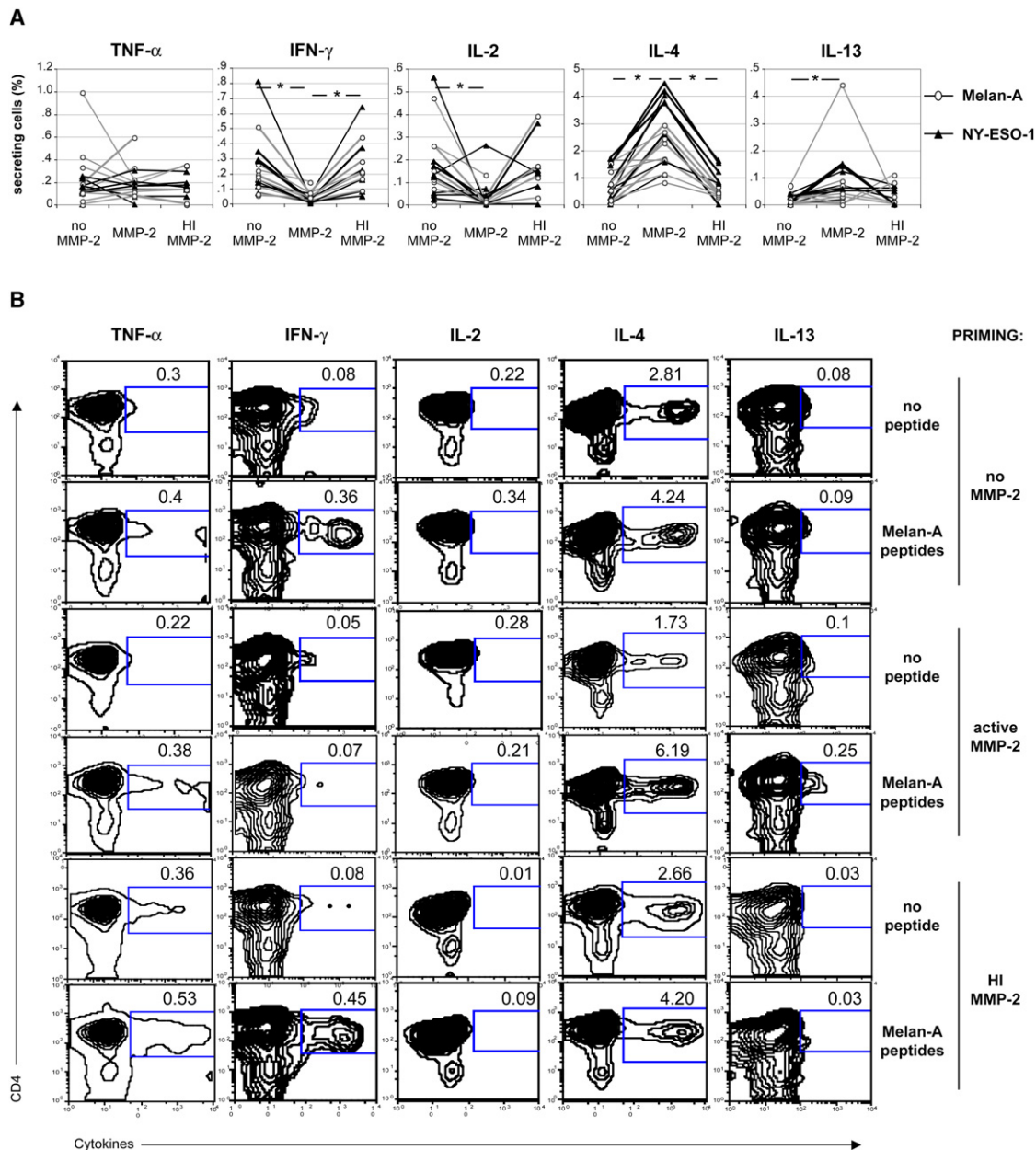


Figure 5. MMP-2 Influences Melan-A/MART-1- and NY-ESO-1-Specific T Helper Differentiation

(A) Cord blood-derived CD4⁺/CD25⁻ cells were stimulated with autologous poly(I:C)-matured DCs pulsed with overlapping peptides spanning either Melan-A/MART-1 or NY-ESO-1. Prior to poly(I:C) addition, DCs were incubated with or without MMP-2 (active or heat-inactivated) for 1 hr. MMP-2 enzymatic activity was controlled (Figure S5C and S5D). After 15 days, CD4⁺ T cells were stimulated with the Melan-A/MART-1 or NY-ESO-1 peptide pools (2 μ M) for 6 hr before intracellular staining of cytokines. Percentages of antigen-specific CD4⁺ T cells, secreting indicated cytokines, are represented for all donors.

(B) Contour plots showing percentages of cytokine-producing CD4⁺ T cells are shown for representative donor CB45 primed to Melan-A/MART-1. Percentage of secreting cells upon peptide stimulation was considered specific/positive when it exceeded by more than twofold the background (cytokine-secreting cells in the absence of stimulation) and had more than 0.5% of responding cells (after background subtraction) for at least one cytokine.

For each cytokine, two paired t tests were used to compare priming in the presence of active MMP-2 to either priming in the absence of active MMP-2 or with HI MMP-2. The Bonferroni adjustment was used for multiple comparisons. *p* values ≤ 0.025 (*) were considered statistically significant using a Bonferroni correction for two comparisons.

naïve CD4⁺ T cells from 10 cord blood donors were primed to Melan-A/MART-1 or NY-ESO-1 using autologous poly(I:C)-matured DCs pulsed with the corresponding overlapping peptides. After 15 days, primed CD4⁺ T cells secreted IFN- γ (mean = 0.23% of secreting cells after background subtraction)

and IL-2 (mean = 0.15%). CD4⁺ T cells specific for either tumor associated antigen, secreted little IL-4 and IL-13 (mean = 0.75% and 0.02%, respectively) (Figures 5A and 5B). On the other hand, when DCs were preincubated with active MMP-2 before maturation with poly(I:C), both Melan-A/MART-1- (Figures 5A

and 5B) and NY-ESO-1-specific (Figure 5A) primed CD4⁺ T cells significantly failed to produce type 1 cytokines ($p \leq 0.0004$ and $p \leq 0.0073$ for IFN- γ and IL-2, respectively) upon peptide restimulation (Figures 5A and 5B). Primed CD4⁺ T cells instead significantly produced more IL-4 (mean = 2.50%; mean of differences = 1.85, $p \leq 0.0001$) and IL-13 (mean = 0.08%; mean of differences = 0.07, $p \leq 0.0174$) (Figures 5A and 5B). In addition, CD4⁺ T cells primed using DCs preincubated with heat-inactivated MMP-2 significantly secreted similar cytokines than CD4⁺ T cells primed in the absence of MMP-2. Accordingly, the former CD4⁺ T cells (primed with HI MMP-2) were significantly different relative to CD4⁺ T cells primed in the presence of active MMP-2 (mean of differences = 0.21 with $p \leq 0.0007$ for IFN- γ , mean of differences = 0.11 with $p \leq 0.015$ for IL-2 and mean of differences = 1.92 with $p < 0.0001$ for IL-4) (Figures 5A and 5B). Therefore, pre-exposure to active MMP-2, but not to inactive MMP-2, confers DCs with the ability to skew the differentiation of naïve CD4⁺ T cells toward a T_H2-like phenotype, regardless of their antigen-specificity. Hence, MMP-2 could participate in the detrimental T_H2 skewing observed in certain tumors, including melanoma, thus impairing host antitumor immune responses (Botella-Estrada et al., 2005; Lauerova et al., 2002; McCarter et al., 2005; Minkis et al., 2008; Tatsumi et al., 2002).

MMP-2 Induces Inflammatory T_H2 Cells in a Basophil-Independent but DC-Dependent Manner

We next studied the cellular requirements underlying the inflammatory T_H2 polarization of MMP-2-specific cells. Basophils, recently described as APCs (Sokol et al., 2009), are thought to play an important role in T_H2 polarization by secreting factors such as IL-4 or thymic stromal lymphopoietin (TSLP) (Ito et al., 2005; Oh et al., 2007; Sokol et al., 2008). However, MMP-2 neither induced production of IL-4 nor TSLP by human basophils (Figures S4A–S4C). In addition, MMP-2 did not increase the expression of the basophil activation marker CD203c (Ocmant et al., 2007), further demonstrating that MMP-2 did not stimulate basophils (Figure S4A).

We next evaluated whether DCs could be involved in the polarization of MMP-2-specific CD4⁺ T cells. Expression levels by DCs of maturation markers CD40, CD80, CD83, CD86, HLA-DR, and CCR7 were not significantly affected by either active or inactive MMP-2 (not shown).

OX40L expressed on DCs can function as an inflammatory T_H2-skewing molecule (Ito et al., 2005). Both active and inactivated MMP-2 significantly increased OX40L expression on DCs ($n = 9$) irrespective of their maturation stage (Figures 6A and 6B). However, differentiation into inflammatory T_H2 cells required pre-exposure of DCs to MMP-2 in its active conformation only (Figure 5), suggesting the involvement, in the polarization mechanism, of another molecule specifically sensitive to active MMP-2.

Inhibition of IL-12 Production by MMP-2

Importantly, MMP-2-treated DCs produced significantly less T_H1-associated IL-12p70 (47.8% mean inhibition with 5 μ g/ml MMP-2; mean of differences = 41.7 with $p \leq 0.001$, $n = 11$ donors), in a dose-dependent manner, when stimulated with the TLR3 agonist poly(I:C). The reduction in IL-12p70 was not

observed using inactivated MMP-2, MMP-2 peptides, rhPEX (190 amino acid long C-terminal fragment of MMP-2 lacking protease activity) or controls including glycerol (vehicle control for MMP-2), DMSO (peptides diluted in DMSO) and a MMP-2-specific inhibitor alone (Figure 7A). To assess whether MMP-2 diminished IL-12p70 levels by inhibiting DC production/secretion or by directly degrading extracellular IL-12p70, we incubated rhIL-12 for 24 hr in the presence or absence of MMP-2. IL-12p70 levels remained the same in every condition (Figure S5A). Single chains IL-12p35 and IL-12p40 as well as trypsin-exposed IL-12p70 were not recognized by the CBA kit (Figure S5B), confirming its specificity for bioactive IL-12p70 and therefore strongly suggesting that IL-12 was not a substrate for MMP-2 and that instead MMP-2 directly inhibited DCs to produce IL-12p70. MMP-2 activity was controlled by measuring its capacity to degrade MCP-3 (Figure S5C). Furthermore, supernatants of MMP-2-exposed DCs did not affect IL-12p70 production by fresh DCs, suggesting that no cleaved part of a MMP-2 substrate is directly responsible for this effect (Figure S5E). Following poly(I:C) activation, $4.33\% \pm 0.94$ DCs expressed the IL-12p35 subunit, whereas only $1.5\% \pm 0.22$ expressed it when preincubated with active MMP-2 (Figure 7B). Thus, pre-exposure to active MMP-2 reduces DC production of IL-12p35 (mean inhibition = 65%), thereby preventing the formation of the bioactive IL-12p70. IL-12p40 production was not affected overall (Figure 7B). Optimal IL-12p35 transcription is thought to depend on STAT1 activation in human moDCs (Gautier et al., 2005). As expected, STAT1 phosphorylation was decreased when DCs were preincubated with active ($p \leq 0.0047$) but not inactive MMP-2 prior to poly(I:C) stimulation (Figures 7C and 7D), suggesting that active MMP-2 inhibits IL-12p35 production by preventing STAT1 phosphorylation.

MMP-2 binds integrins such as $\alpha v \beta 3$ (Brooks et al., 1996), $\alpha 1 \text{Ib} \beta 3$ (Choi et al., 2008), or other $\beta 2$ integrins (Stefanidakis et al., 2003) and the scavenger receptor CD91 (Emonard et al., 2004), all of which are expressed on human DCs. We therefore tested whether MMP-2 signaled through such molecules to block IL-12 production and induce OX40L expression. PEX peptide, known to prevent MMP-2 binding to integrins such as $\alpha v \beta 3$ (Brooks et al., 1998), and blocking mAbs specific for most of these receptors did not restore IL-12p70 production by MMP-2-exposed DCs (not shown), suggesting that other molecules may be involved.

STAT1 phosphorylation (Severa et al., 2006) and subsequent IL-12 production (Gautier et al., 2005) can be induced by type I IFN receptor triggering. We confirmed that IFN- β induced IL-12p70 production, as blocking mAbs to IFN- β and its receptor significantly inhibited IL-12p70 production and with a similar magnitude as MMP-2 (Figure S5F). We consequently tested involvement of this cytokine and its receptor in our system. IFN- β signaling through its receptor leads to STAT1 phosphorylation (Severa et al., 2006), which triggers IL-12p35 transcription and enhanced transcription of IFN- β itself through an amplification loop (Decker et al., 2005; Gautier et al., 2005). Accordingly, Poly(I:C)-matured DCs pre-exposed to active MMP-2 produced decreased levels of IFN- β (Figure 7E). Strikingly, we found that MMP-2-exposed DCs drastically lost expression of IFNAR1 chain of the receptor for type I IFNs ($n = 7$ donors, $p \leq 0.001$) (Figures 7F and 7G). Furthermore, recombinant rhIFNAR1

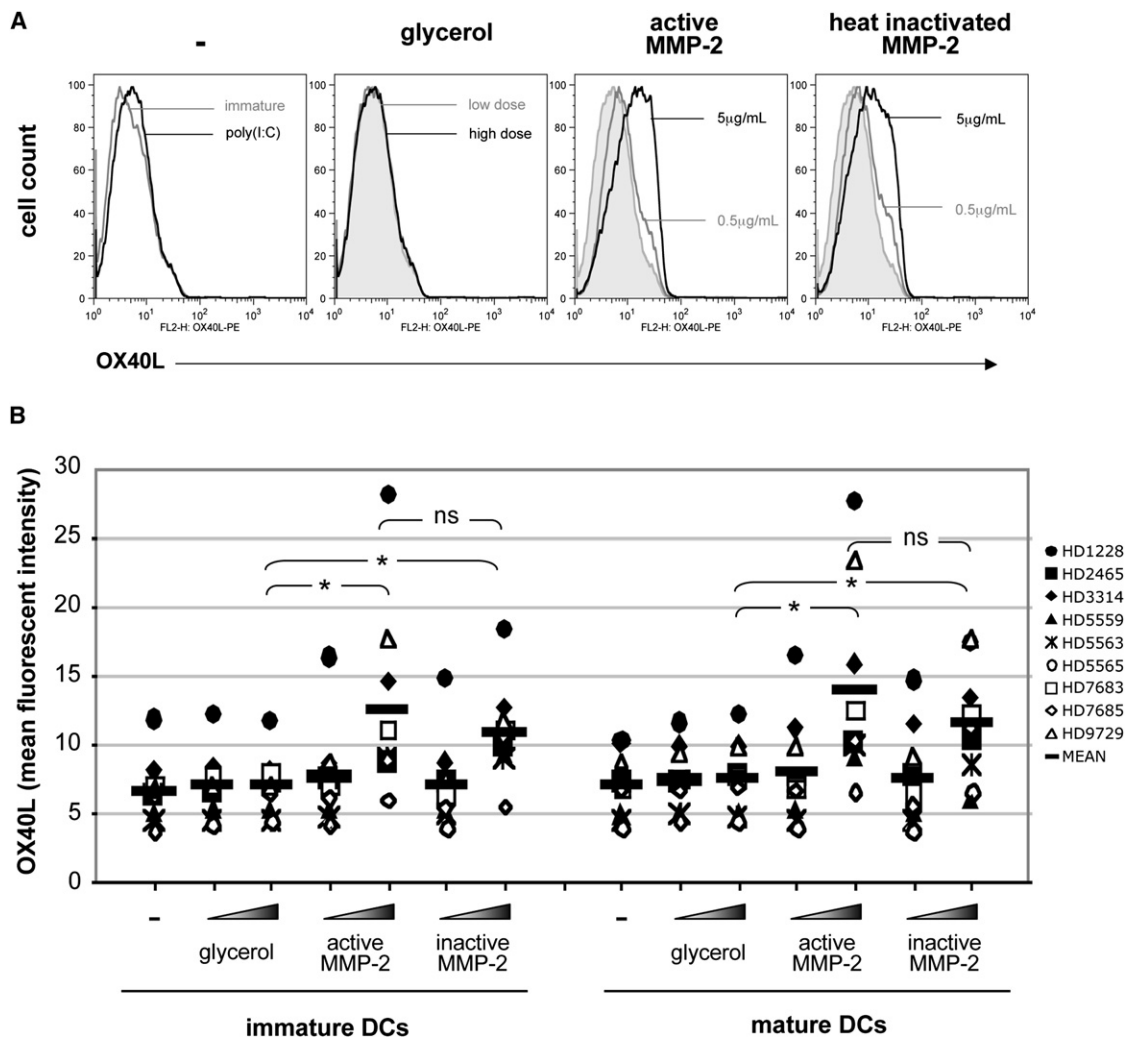


Figure 6. MMP-2 Protein Induces OX40L Expression on DCs

Immature and poly(I:C)-matured DCs were incubated with active or HI MMP-2 (0.5 or 5 μ g/ml) for 24 hr. MMP-2 enzymatic activity was controlled (Figure S5C and S5D). OX40L expression was assessed by surface staining.

(A) Histogram plots are shown for representative donor HD1228. Isotype control is represented in tinted gray.

(B) Mean fluorescent intensities are represented for all nine donors tested.

For immature and mature DCs, paired t tests were used to compare low and high values of glycerol, active MMP-2 and inactive MMP-2. p values ≤ 0.0167 (*) were considered statistically significant using a Bonferroni correction for three comparisons for each DC type.

See also Figure S4.

(≈ 110 kDa) incubated overnight with active MMP-2 was degraded by MMP-2 (Figure 7H). Therefore, MMP-2 cleaves IFNAR1 on DCs leading to loss of IL-12p35 production through reduction of STAT1 phosphorylation.

In summary, both active and inactive MMP-2 induce OX40L expression by DCs while active MMP-2 inhibits IL-12p35 production, and subsequent IL-12p70 formation by degrading IFNAR1.

MMP-2-Specific CD4⁺ T Cell Differentiation Depends on IL-12 and OX40L Expression by DCs during Priming

To test whether OX40L and/or IL-12p70 were directly responsible for inflammatory T_H2 polarization of MMP-2-specific T cells,

naïve CD4⁺ T cells from 19 cord blood donors were primed by autologous poly(I:C)-matured DCs pulsed with the active MMP-2 protein. As expected, primed MMP-2-specific CD4⁺ T cells secreted IL-4 and TNF- α corresponding to the inflammatory T_H2 phenotype observed so far. However, when exogenous rhIL-12 was added throughout priming, MMP-2-specific T cells partially reverted their phenotype toward a T_H1-like profile, i.e., secreting less IL-4 ($p \leq 0.0001$; $n = 19$) and more IFN- γ ($p = 0.022$; $n = 19$). These results are consistent with the concept that lack of IL-12p70 is critical for inflammatory T_H2 differentiation of MMP-2-specific CD4⁺ T cells (Figures 8A and 8B). When naïve T cells were primed in the presence of anti-OX40L, MMP-2-specific cells secreted less IL-4 ($p \leq 0.003$; $n = 10$) and more

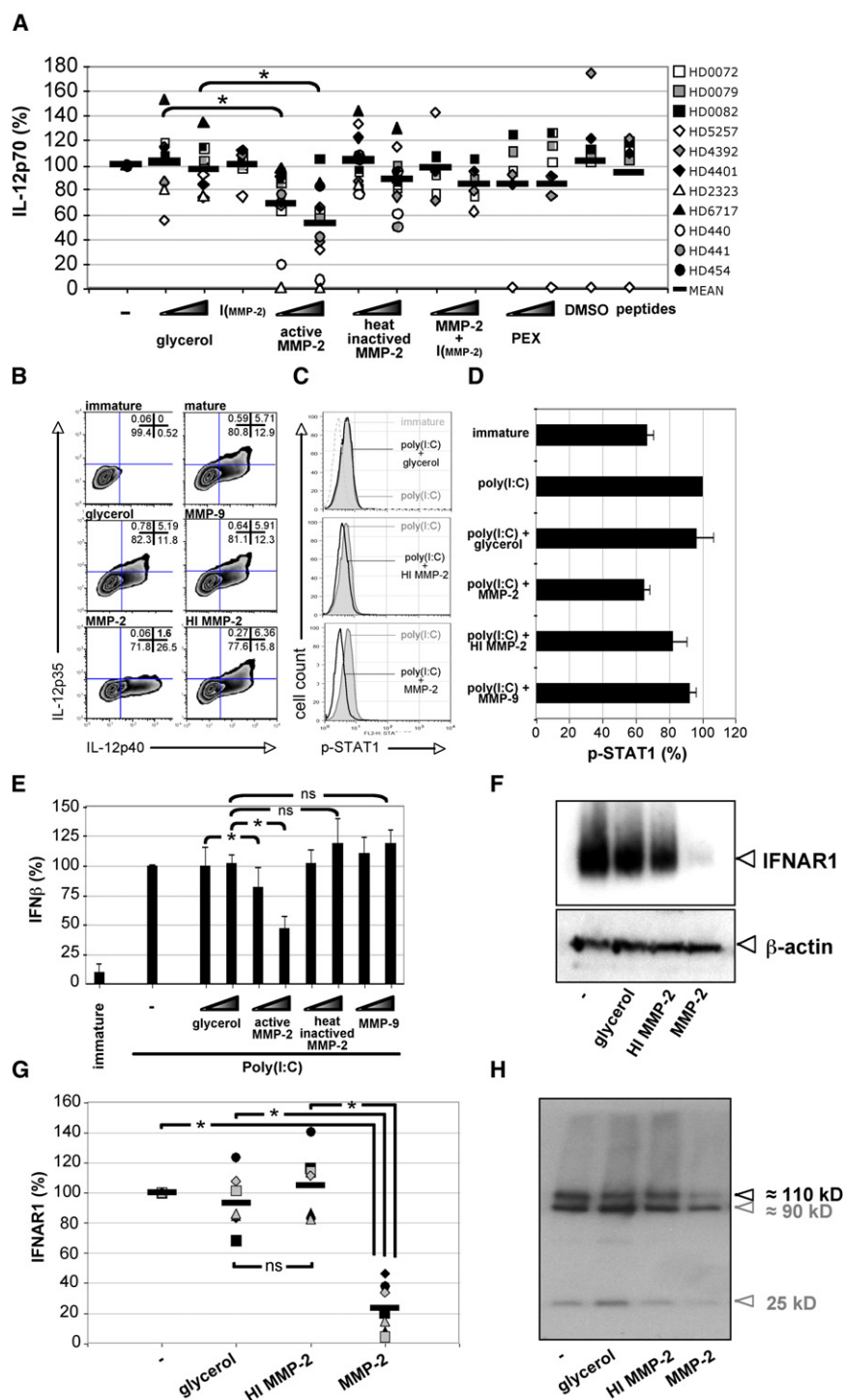


Figure 7. MMP-2-Dependent Mechanism Blocking IL-12 Production by DCs

Immature DCs were incubated with MMP-2 (0.5 or 5 μ g/ml) and poly(I:C)-matured 1 hr later. IL-12p70 levels were measured 16 hr later.

(A) Results are represented as a percentage of IL-12p70 levels, measured by CBA, produced when DCs were incubated with poly(I:C) only ($n = 11$). (B) For intracellular staining of IL-12p35 and IL-12p40 chains, Brefeldin A was added 4 hr after poly(I:C) activation and cells were stained after an additional 12 hr incubation. Numbers in the upper right quadrants are the percentages of each population for a representative donor ($n = 3$).

(C and D) Immature DCs were incubated with MMP-2 for 1 hr (5 μ g/ml) before poly(I:C) maturation. Intracellular staining of phosphorylated (Y701) STAT1 (P-STAT1) was performed 3 hr later. A representative donor (C) and results for all three donors (D) are shown. Active MMP-2 only significantly inhibits STAT1 phosphorylation ($p \leq 0.0047$). Results are shown as mean \pm SD.

(E) Immature DCs were incubated with MMP-2 and poly(I:C)-matured 1 hr later. IFN β levels were measured by ELISA 16 hr later. Results are represented as a percentage of IFN β levels produced when DCs were incubated with poly(I:C) only ($n = 9$). Results are shown as mean \pm SD.

(F and G) 10^6 DCs/lane were treated with 0.1 μ g MMP-2 or controls. Poly(I:C) was added 1 hr later. IFNAR1 protein expression was detected the next day by western blot analysis using a rabbit monoclonal antibody. A representative donor (F) and results for all seven donors (G) are shown.

(H) The rhIFNAR1 (5 μ g; ≈ 110 kDa) was incubated overnight with 0.3 μ g MMP-2 before performing western blot analysis for IFNAR1. The second band (around 90 kDa) and the third band (25 kDa) very likely correspond to either a degraded form of the protein still recognized by the Ab or a contaminant present in the commercial protein preparation and nonspecifically recognized by the Ab.

MMP-2 enzymatic activity was controlled (Figures S5C and S5D).

Statistical analysis: A: Two paired t tests were used to compare low levels of glycerol with low levels of active MMP-2 and high levels of glycerol with high levels of active MMP-2; E: For low and high levels, three comparisons were carried out (glycerol to active MMP-2, glycerol to HI MMP-2 and active MMP-2 to active MMP-9); G: three comparisons were carried out (glycerol to active MMP-2, glycerol to HI MMP-2 and active MMP-2 to HI MMP-2). For the two comparisons, p values ≤ 0.025 (*) were considered statistically significant; for the three comparison analyses, p values ≤ 0.0167 (*) were considered statistically significant. See also Figure S5.

IFN- γ ($p = 0.023$; $n = 10$) than CD4 $^+$ T cells primed without additional IL-12 (Figures 8A and 8B). Addition of both rhIL-12 and anti-OX40L during priming triggered MMP-2-specific CD4 $^+$ T cells secreting less IL-4 ($p \leq 0.002$; $n = 10$) and more IFN- γ ($p \leq 0.006$; $n = 10$) than anti-OX40L alone (Figure 8B), indicating

that both molecules are involved in MMP-2-specific CD4 $^+$ T cell polarization.

IL-4 is critical for T $_H$ 2 differentiation in many models (Sokol et al., 2008; Swain et al., 1990). We next primed naïve MMP-2-specific CD4 $^+$ T cells to active MMP-2 while blocking potential

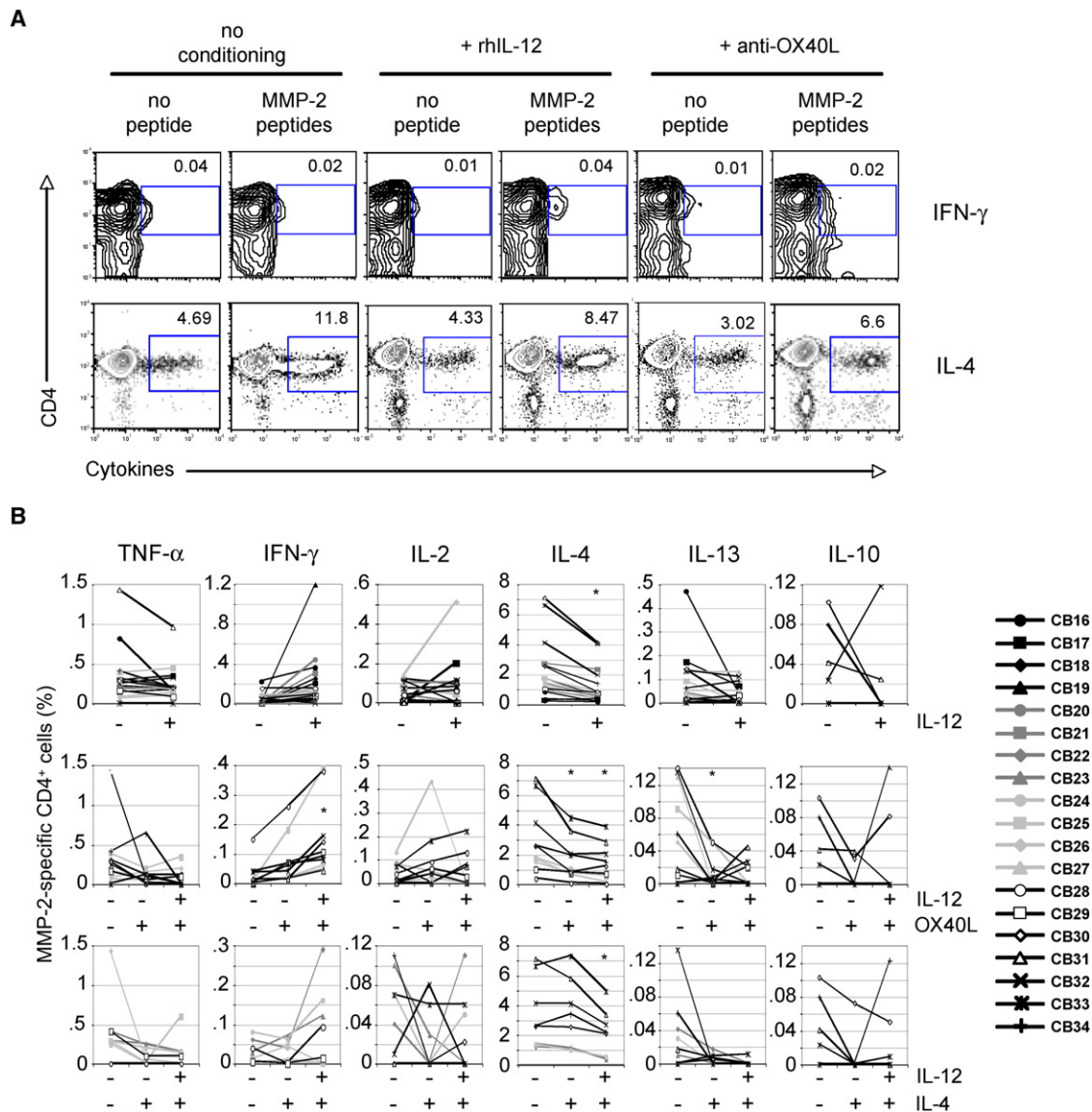


Figure 8. MMP-2-Specific CD4⁺ T Cell Differentiation Depends on IL-12p70 and OX40L Expression by DCs

(A) Cord blood-derived CD4⁺/CD25⁻ cells from 19 donors were stimulated with autologous DCs previously loaded with active MMP-2 (MMP-2 enzymatic activity was controlled; Figures S5C and S5D) for 15 days in the absence or in the presence of exogenous rhIL-12 (10 ng/ml) or blocking mAb for OX40L or IL-4 (10 μg/ml). CD4⁺ T cells were then stimulated with the MMP-2 peptide pool (2 μM) for 6 hr before intracellular staining of cytokines. Contour plots representing cytokine production by CD4⁺ T cells are shown for donor CB31 CD4⁺ T cells primed to MMP-2 alone, or together with rhIL-12, anti-OX40L mAb or anti-IL-4 mAb (not shown).

(B) Percentages of MMP-2-specific CD4⁺ T cells, secreting indicated cytokines, are represented for all donors.

For each cytokine, five paired t tests were used to compare “no conditioning” versus various conditions. p values ≤ 0.01 (*) were considered statistically significant using a Bonferroni correction for five comparisons.

DC-derived IL-4 with a specific mAb. Primed cells maintained an identical inflammatory T_H2 phenotype (n = 8) (Figure 8B), confirming that, in this T_H2 differentiation model, IL-4 is not a major determinant. Therefore, inflammatory T_H2 polarization of MMP-2-specific T cells is dependent upon both IL-12p70 and OX40L, but not on IL-4.

In summary, our data show that MMP-2 induces priming of T_H2 inflammatory CD4⁺ T cells, by diminishing IL-12 production (through degradation of type 1 IFN receptor) and inducing OX40L

expression by DCs. Therefore, MMP-2 acts as an endogenous type 2 conditioner in humans.

DISCUSSION

The first two subsets described of effector CD4⁺ T cells were the T_H1 and T_H2 subpopulations (Mosmann and Coffman, 1989). Both cells were shown to mediate anticancer functions (Nishimura et al., 1999), but IFN-γ-secreting T_H1 cells appeared

to be superior in inducing memory cytotoxic responses and strong cellular immunity (Nishimura et al., 2000). T_H1 cells are characterized by their production of IFN- γ and, to a lesser extent, IL-2. IL-12p70 produced by APCs, particularly activated DCs, as well as IFN- γ produced by NK cells and T cells, polarize $CD4^+$ T cells toward the T_H1 cell differentiation program through STAT1 and T-bet (Szabo et al., 2000). In contrast, T_H2 cells mediate host defense against extracellular parasites including helminths and are important in the induction and persistence of allergic diseases. Conventional T_H2 cells are classically defined as producers of IL-4, IL-5, IL-10, and IL-13 but the mechanism(s) underlying induction of T_H2 differentiation has remained unclear.

IL-4, by inducing GATA-3 expression, is usually described as essential for T_H2 differentiation (Sokol et al., 2008; Swain et al., 1990). Cellular sources of IL-4 for T_H2 differentiation have not been clearly established. Granulocytes such as basophils readily produce IL-4 after crosslinking of their Fc ϵ RI receptors and have been proposed to be responsible for T_H2 differentiation in several models (Falcone et al., 1996; Sokol et al., 2008). MMP-2 did not activate basophils (assessed by IL-4 and TSLP production as well as CD203c expression) (Figure S4A–S4C), suggesting that these cells were not involved in this model. Moreover, MMP-2-induced type 2 differentiation does not appear to depend on IL-4 as naïve MMP-2-specific $CD4^+$ T cells primed in the presence or in the absence of a blocking antibody specific for IL-4 gave rise to type 2 $CD4^+$ T cells with a similar profile (Figures 8A and 8B). This observation is in accordance with accumulating *in vivo* studies indicating that, in some settings, IL-4 is not essential for T_H2 differentiation (Everts et al., 2009; Jankovic et al., 2000; Steinfeldt et al., 2009). A default mechanism could also explain T_H2 differentiation, where the lack of T_H1 -polarizing signal, namely IL-12, would be sufficient and/or necessary (Minkis et al., 2008; Moser and Murphy, 2000). We found that active MMP-2 inhibited IL-12 production by DCs (Figures 7A and 7B), which was in part responsible for the MMP-2-induced type 2 polarization. Indeed, supplementing the priming cultures of MMP-2-specific $CD4^+$ T cells with rhIL12, even at doses as low as 1 ng/ml (not shown), induced lower percentages of IL-4-secreting T cells and more IFN- γ -secreting cells (Figures 8A and 8B). TSLP leads to T_H2 cell recruitment and allergic inflammation (Liu et al., 2007). Basophils (Sokol et al., 2008), epithelial cells (Soumelis and Liu, 2004), or DCs (Ito et al., 2005; Liu, 2006; Watanabe et al., 2004) can be sources of TSLP. TSLP contributes to T_H2 immunity either by inhibiting T_H1 differentiation, by acting directly on T cells to promote T_H2 differentiation or by activating DCs. Stimulation of DCs with TSLP, in the absence of IL-12p70, induces upregulation of the costimulatory molecule OX40L and can promote differentiation of naïve T cells into cells secreting IL-4, IL-5, IL-13, and TNF- α , named inflammatory T_H2 (Ito et al., 2005; Soumelis et al., 2002). MMP-2-specific $CD4^+$ T cells secreted IL-4, IL-13 and TNF- α , but no IL-10, consistent with such an inflammatory T_H2 profile. Interestingly, MMP-2 did not activate basophils to secrete TSLP (nor IL-4) (Figure S4). On the other hand, MMP-2 induced DCs to express OX40L (Figures 6A and 6B), in a TSLP-independent manner (not shown). We showed that inflammatory T_H2 differentiation of MMP-2-specific cells also relied on OX40L expression (Figures 8A and 8B). OX40 signaling in $CD4^+$ T cells

can directly induce type 2 lineage commitment by inducing NFATc1, which triggers IL-4 production and subsequent GATA-3 expression (So et al., 2006; Yu et al., 2009). MMP-2-induced type 2 differentiation likely works differently as we showed that IL-4 was not a major determinant in this model. Of note, addition of both rhIL-12 and anti-OX40L blocking mAb during priming induced MMP-2-specific $CD4^+$ T cells secreting significantly less IL-4 and more IFN- γ than anti-OX40L alone (Figure 8B), indicating that both molecules were involved in MMP-2-induced type 2 differentiation by triggering additive effects.

We found that MMP-2 degrades IFNAR1 (Figures 7F–7H), constituting one chain of the heterodimeric type I IFN receptor. The IFNAR1 chain, described here as a substrate for MMP-2, is essential for signaling (Decker et al., 2005). When cleaved, the type I IFN amplification loop is abrogated, explaining the reduction of IFN- β secreted by MMP-2-exposed DCs (Figure 7E). Type I IFN receptor signaling triggers STAT1 phosphorylation (Severa et al., 2006), which, in turn, also induces IL-12p35 transcription (Gautier et al., 2005). As a result, bioactive IL-12p70 formation is inhibited and cannot exert its function in T_H1 differentiation. Through this mechanism, MMP-2 acts as an endogenous type 2 “conditioner”.

TNF- α /IL10- T_H2 cells are thought to represent the pathogenic T_H2 cells that cause allergic inflammation, in contrast to the conventional IL-10-producing cells. Many potent allergens have intrinsic protease activity (Grobe et al., 1999; Kheradmand et al., 2002) and secreted proteases are essential for the infectious and reproductive cycles of helminths (McKerrow et al., 2006). Recent studies showed that the T2 ribonuclease derived from soluble egg antigens of the parasitic helminth *Schistosoma* triggers potent T_H2 responses by conditioning mouse DCs (Everts et al., 2009; Steinfeldt et al., 2009). One can speculate that the innate immune system might have evolved a mechanism to detect abnormal proteases associated with helminth infection that could then be triggered by other proteases, such as allergens or in this case MMP-2. Our results clearly showed that an enzyme-induced T_H2 -conditioning directly resides in the intrinsic enzymatic activity rather than solely in the active conformation of MMP-2.

MMP-2 also acts as a type 2 conditioner for $CD4^+$ T cells recognizing other MAA (Figures 5A and 5B), indicating that MMP-2 plays a dominant role in biasing the response against otherwise T_H1 -inducing tumor antigens. Our *in vitro* results suggest that increased MMP-2 secretion may locally influence the induction of immune responses and skew them toward type 2 responses. Accordingly, MMP-2-specific $CD4^+$ T cell responses tended to be found in TILs from patients exhibiting a poorer clinical outcome (Figure S1B). MMP-2 might therefore partially explain the observed prevalence, at least in some studies, of detrimental type 2 responses in various cancers including melanoma.

Our findings collectively support the idea that the protumoral MMP-2 protein represents a good candidate to target in immunotherapy to treat melanoma patients, since it can induce both broad $CD4^+$ and $CD8^+$ T cell responses. Furthermore, elucidation of mechanisms underlying T_H2 polarization, including the one identified in this study, opens the way to designing immune strategies for inducing effective antitumor T_H1 -like responses to treat cancer patients.

EXPERIMENTAL PROCEDURES

Human Tissue Specimens

Tumor-infiltrating lymphocytes (TILs) were provided by Pr. F. Jotereau and B. Dréno. Following informed consent, TILs were obtained from tumor-invaded lymph nodes of melanoma patients (stage IIb). These patients subsequently received autologous TILs and IL-2 infusions in a clinical trial (Labarriere et al., 2002). This protocol was approved by the Institutional Ethics Committee and registered with the regulatory state authority in France (Nantes). Moreover, TILs were expended in a specific genetic and cellular therapy unit (UTC; CHRU, Nantes, France) under good manufacturing practice (GMP) conditions. Blood from healthy donors was purchased from the New York Blood Center. Of note, all samples, from donors and patients, have been deidentified prior to the analysis.

Reagents

Purified human MMP-2 (mixture of the proenzyme [50%] and the active form [50%]) was purchased from Biomol. The MMP-2 enzyme was inactivated either by heating to 56°C for 45 min or by addition of the MMP-2 inhibitor III at 100 nM (Calbiochem) for 20 min. rhPEX was purchased from Genway. rhMMP-9 (Calbiochem) was used as a mixture of the proenzyme (50%) and the active protein (50%). Overlapping peptides (20 amino acid long overlapping of 10) spanning proMMP-2 sequence were from Proimmune (>80% pure). Lyophilized peptides were reconstituted in DMSO and were used either individually (2 μ M) or as a pool (2 μ M each). rhGM-CSF was purchased from Immunex. rhIL-12, rhIL-4, rhIL-7, and rhIL-2 were from R&D Systems. The kits used for basophil isolation and IFN- γ -secreting cell enrichment (Miltenyi Biotec) were used according to the manufacturer's instructions.

Antibodies

Allophycocyanin-conjugated antibody to CCR-7 (150503) was purchased from R&D Systems. Unconjugated (10 μ g/ml for blocking experiments) or phycoerythrin-conjugated antibody to OX40L (11C3.1) was from Biolegend. Blocking antibody to IL-4 (8F12; 10 μ g/ml) was from Acris. Antibodies to human V β chains covering 70% of the repertoire (iOTest Beta Mark; Beckman Coulter) were used according to the manufacturer's instructions. Fluorescein isothiocyanate-conjugated antibody to IL-12p35/IL-12p70 (B-T21) and IFNAR1 (EP899Y) were from Abcam. Phycoerythrin-conjugated antibodies to IL-12p40/IL-12p70 (C8.6), IL-10 (B-T10), CD203c (FR3-16A11) were from Miltenyi Biotec. Blocking antibody to HLA-DQ (SPV-L3; 20 μ g/ml) was from NeoMarkers. Blocking antibodies to HLA-DP (B7/21; 20 μ g/ml) or HLA-DR (L243; 20 μ g/ml), phycoerythrin-conjugated antibodies to phosphorylated (Y701) STAT1 (4a), GATA-3 (L50-823), IL-2 (MQ1-17H12), IL-4 (8D4-8), IL-5 (JES1-39D10), IL-13 (JES10-5A2), TNF- α (MAB11), IFN- γ (25723.11), perforin (δ G9), GranzymeB (GB11), CD40 (5C3), CD80 (L307.4), CD83 (HB15e), CD86 (IT2.2), HLA-DR (TU36), fluorescein isothiocyanate-conjugated antibodies to CD45RA (HI100) and CD45RO (UCHL1), and antibody to CD4 (RPA-T4) were purchased from BD Biosciences Pharmingen. Alexa fluor 488-conjugated antibody to IL-17 (eBio64DEC17), phycoerythrin-conjugated antibody to T-bet (4B10) and allophycocyanin-conjugated antibody to CD62L (DREG-56) were from eBioscience. Antibody to β -actin (C-2) was from Santa Cruz Biotechnology.

T Cell Culture, Stimulation and Priming

Peripheral blood mononuclear cells (PBMCs) were purified from healthy donor- (HD) or cord blood (CB) donor-derived buffy coats (New York Blood Center) by Ficoll-Paque Plus (GE Healthcare) centrifugation. CD4⁺/CD25⁻ cells were enriched (>90%) by magnetic cell sorting (Miltenyi Biotec) and primed/stimulated for 12–15 days either with irradiated (35 Gy) autologous CD4⁺ cells or with autologous mature DCs in IMDM (GIBCO) supplemented with 1 mM HEPES (Life Technologies), 2 mM L-glutamine (Sigma), streptomycin (100 U/ml)/penicillin (100 μ g/ml) (Sigma) and 5% heat inactivated pooled human serum (PHS; Valley Biomedical) in the presence of rhIL-2 (10 U/ml) and IL7 (5 ng/ml) (R&D Systems). Antigen presenting cells (CD4⁺ cells or DCs) were loaded either with peptides (2 μ M) or with the MMP-2 protein (10 μ g/ml) for 2 and 5 hr, respectively. DCs were matured using the TLR3 agonist poly(I:C) at 5 μ g/ml/10⁶ DCs (Amersham). To generate T cell clones, we originally relied on our published methodology (Godefroy et al., 2006,

2007), involving enrichment of IFN- γ -secreting cells upon short-term culture and peptide stimulation to generate MMP-2 responsive clones. Although MMP-2-specific cells could be isolated, it was realized that IFN- γ secretion was marginal compared with their secretion of IL-4 and TNF- α . IFN- γ -secreting cells in response to MMP-2 peptide pool were enriched by cytokine-guided magnetic cell sorting (Miltenyi Biotec) and cloned the following day by limiting dilution in the presence of irradiated allogeneic PBMCs, 1 μ g/ml phytohemagglutinin-L (Sigma) and 150 U/ml rhIL-2. Tumor infiltrating lymphocytes (TILs) were provided by Pr. F. Jotereau and B. Dréno. They were obtained from tumor-invaded lymph nodes of melanoma patients (stage IIb) and expanded ex vivo. These patients received autologous TILs and IL-2 infusions in a clinical trial (Labarriere et al., 2002). This protocol was approved by the Institutional Ethics Committee and registered with regulatory state authority in France (Nantes).

Dendritic Cell Preparation and Activation

PBMCs were purified from healthy- (HD) or cord blood- (CB) donors and plated at 40.10⁶ cells/100 ml/dish in complete IMDM with 5% PHS. Cells were allowed to adhere for 2 hr at 37°C. Nonadherent cells were removed. The monocyte-enriched fraction was supplemented with 100 U/ml rhGM-CSF and 300 U/ml rhIL-4 (R&D Systems) on days 0, 2, and 4. Immature DCs were harvested on day 5 and matured using poly(I:C) at 5 μ g/ml/10⁶ DCs (Amersham). Secretion of IL-12p70, TNF- α , IL-1 β , IL-6, IL-8, and IL-10 was assessed on both immature and mature DCs using the Human Inflammatory Cytokine Cytometric Bead Array (BD Pharmingen).

Enzyme-Linked Immunosorbent Assay

Activation of T cell clones (10,000 cells/100 μ l/well), polyclonal T cell populations (100,000 cells/100 μ l/well), DCs (50,000 cells/100 μ l/well), and basophils (10,000 cells/100 μ l/well) was determined by ELISA. IFN- γ (BioSource), TNF- α (BioSource), IL-4 (BioSource), TSLP (Quantikine; R&D Systems), IFN- β (VeriKine; PBL interferon source), and MCP-3 (DuoSet; R&D Systems) contents in supernatants were measured according to the manufacturer's instructions.

Intracellular Staining

T-bet and GATA-3 expression was measured on resting T cells. Cells were fixed (4% paraformaldehyde for 10 min at RT), permeabilized with 0.1% saponin, and stained for intracellular transcription factors. Cytokine production by T cells was also assessed by intracellular staining. T cells were stimulated with 2 μ M overlapping peptides. After 1 hr, 10 μ g/ml brefeldin A was added to the cells. Five hours later, T cells were stained for surface markers, fixed, permeabilized, and stained for intracellular cytokines (TNF- α , IFN- γ , IL-2, perforin, granzymeB, IL-4, IL-5, IL-10, IL-13, and IL-17). Antigen-specificity was defined by the percentage of cells secreting cytokine as long as it exceeded background (cytokine-secreting cells in the absence of peptide stimulation) by more than 2-fold and consisted of more than 0.5% of responding cells following subtraction of background for at least one cytokine. It was not uncommon to find relatively high background levels of IL-4 and TNF- α -producing T cells, likely due to the fact that these highly sensitive cells continued to produce cytokine up to 2–3 weeks after stimulation. For assessment of IL-4 production by basophils, brefeldin A was added simultaneously to stimulation. For intracellular staining of IL-12p35, IL-12p40, and phosphorylated STAT1, immature DCs were incubated with or without MMP-2 as described in figure legends. After 1 hr, 5 μ g/ml poly(I:C) was added to activate immature DCs. For monitoring IL-12 subunits, brefeldin A was added 4 hr after poly(I:C) addition. DCs were then cultured for 12 hr, fixed, permeabilized, and stained for intracellular IL-12. For detection of STAT1 phosphorylation on Y701, DCs were fixed 3 hr after poly(I:C) addition, permeabilized and stained. Data were acquired with FACScalibur cytometer (BD Biosciences) and analyzed using FlowJo software.

Western Blots

IFNAR1 and β -actin were detected by western blot analysis using rabbit and mouse mAbs, respectively. HRP-linked anti-rabbit (Cell Signaling) or anti-mouse (Amersham Biosciences) IgG were used as secondary Abs before chemiluminescent detection (ECL Plus, Amersham Biosciences).

Statistical Analysis

Separate analyses were performed for each experiment individually. Analyses take into account paired observations within donors when appropriate (e.g., MMP-2 versus no MMP-2, active versus inactive, active versus peptides). For three-group comparisons (i.e., MMP-2, Melan-A, NY-ESO-1), analyses of variance were performed for an overall comparison among independent groups, and t tests were then used for specific pairwise comparisons between groups. Within each analysis, p values were adjusted for multiple comparisons using a Bonferroni correction. For analyses in which each of two groups was compared with a third group (i.e., two comparisons, with no overall test of the three groups), two t tests were performed, using the Bonferroni adjustment for the two analyses. Two-sided statistical tests were performed at an overall alpha-level of 0.05, with adjustments for multiple comparisons, as described above. Details for each analysis are provided in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at doi:10.1016/j.ccr.2011.01.037.

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