

## Antigen-Specific CD4<sup>+</sup> T Cells Regulate Function of Myeloid-Derived Suppressor Cells in Cancer via Retrograde MHC Class II Signaling

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### Abstract

Myeloid-derived suppressor cells (MDSC) play a major role in cancer-related immune suppression, yet the nature of this suppression remains controversial. In this study, we evaluated the ability of MDSCs to elicit CD4<sup>+</sup> T-cell tolerance in different mouse tumor models. In contrast to CD8<sup>+</sup> T-cell tolerance, which could be induced by MDSCs in all the tumor models tested, CD4<sup>+</sup> T-cell tolerance could be elicited in only one of the models (MC38) in which a substantial level of MHC class II was expressed on MDSCs compared with control myeloid cells. Mechanistic investigations revealed that MDSCs deficient in MHC class II could induce tolerance to CD8<sup>+</sup> T cells but not to CD4<sup>+</sup> T cells. Unexpectedly, antigen-specific CD4<sup>+</sup> T cells (but not CD8<sup>+</sup> T cells) could dramatically enhance the immune suppressive activity of MDSCs by converting them into powerful nonspecific suppressor cells. This striking effect was mediated by direct cell–cell contact through cross-linking of MHC class II on MDSCs. We also implicated an Ets-1 transcription factor–regulated increase in expression of Cox-2 and prostaglandin E2 in MDSCs in mediating this effect. Together, our findings suggest that activated CD4<sup>+</sup> T cells that are antigen specific may enhance the immune suppressive activity of MDSCs, a mechanism that might serve normally as a negative feedback loop to control immune responses that becomes dysregulated in cancer. *Cancer Res*; 72(4): 928–38. ©2012 AACR.

### Introduction

It is now established that inadequate immune response in cancer is a critical element of tumor escape (rev. in ref. 1). Myeloid-derived suppressor cells (MDSC) play one of the major roles in tumor-associated immune abnormalities. This heterogeneous group of myeloid cells accumulates in tumor-bearing (TB) hosts and comprised pathologically activated precursors of granulocytes, macrophages, and dendritic cells. MDSCs are characterized by a potent ability to inhibit CD8<sup>+</sup> T-cell functions via different mechanisms (2–4). However, the main controversy exists about the antigen-specific nature of MDSC-mediated immune suppression and the role of MDSCs in CD4<sup>+</sup> T-cell suppression. Different studies described different effects of MDSCs on T-cell responses in cancer patients and TB mice (rev. in ref. 5). A number of studies showed that

MDSCs induced antigen-specific tolerance of CD8<sup>+</sup>, but not CD4<sup>+</sup> T cells (6–8). However, in different experimental systems, MDSC-mediated inhibition of IFN- $\gamma$  production by CD4<sup>+</sup> T cells (9–12). Similar controversy exists with the data obtained in cancer patients (13, 14); although, in most of the experiments with patients' peripheral blood MDSCs, the specific nature of T-cell suppression was not investigated (5).

The issue of the antigen-specific nature of MDSC effects on T cells is important for understanding the biology of immune defects in cancer. Accumulation of MDSCs, with potent nonspecific immune suppressive activity, in peripheral lymphoid organs could potentially result in profound systemic immune suppression. However, this is not the case in cancer patients or TB mice. In this study, we tried to address this question by investigating the ability of MDSCs to cause antigen-specific CD4<sup>+</sup> T-cell tolerance. We present our surprising findings that antigen-specific CD4<sup>+</sup>, but not CD8<sup>+</sup> T cells were able to dramatically affect the function of MDSCs by converting these cells from antigen-specific to nonspecific suppressors.

### Materials and Methods

#### Mice and reagents

All mouse experiments were approved by University of South Florida Institutional Animal Care and Use Committee. Female C57BL/6 mice (6–8 weeks of age) were obtained from the National Cancer Institute. OT-I (C57BL/6-Tg(TCR $\alpha$ TCR $\beta$ )1100mjb) and OT-II (C57BL/6-Tg(TcraTcrb)425Cbn/J) TCR-transgenic mice were purchased from Jackson Laboratories.

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IA<sup>b</sup> knockout mice B6.SJL(129)-*Ptprca*<sup>a</sup>/BoyAiTac *H2-Ab1*<sup>tm1Gru</sup> were purchased from Taconic Farms. C57BL/6 mice were injected s.c. with 5 × 10<sup>5</sup> EL-4 thymoma, MC38 colon carcinoma, B16F10 melanoma, or Lewis lung carcinoma (LLC) cells.

OVA-derived (H2K<sup>b</sup>, SIINFEKL), (IA<sup>b</sup>, ISQAVHAAHAEI-NEAGR) and control survivin-derived (LDRQRAKNKI; ref. 15) peptides were obtained from American Peptide Company. Lipopolysaccharides (LPS), incomplete Freund's adjuvant (IFA), and β-actin antibody were purchased from Sigma Chemical Co. SC58125 was purchased from Tocris Bioscience. The following antibodies were used to detect MHC class II and class I expression on MDSCs by flow cytometry (all from BD Biosciences): CD11b (M1/70), Gr-1 (RB6-8C5), Ly-6G (1A8), Ly-6C (AL-21), I-A<sup>b</sup> (25-9-17), and H-2K<sup>b</sup> (AF6-88.5).

### Cell isolation and generation

MDSCs were isolated from spleens of TB mice with biotinylated anti-Gr-1 antibody and MiniMACS columns (Miltenyi Biotec; GmbH). This resulted in more than 95% purity of Gr-1<sup>+</sup>CD11b<sup>+</sup> cells. In some experiments, MDSC subsets were sorted with FACS Aria cell sorter. T lymphocytes were isolated from spleens by T-cell enrichment columns (R&D Systems). Dendritic cells (DC) were generated from bone marrow, using GM-CSF (Invitrogen) and IL-4 (R&D Systems), as described before (15). DCs were activated on day 6 of culture, by overnight incubation, with LPS (100 ng/mL) and enriched by centrifugation over Nycoprep A gradient (Accurate Chemicals).

For flow cytometric evaluation of MHC class II on MDSCs in tumor tissues, tumors (and spleens in comparable experiments) were dissected and digested with 2 mg/mL collagenase XI (Sigma-Aldrich) for 45 minutes at 37°C. The digested tissue was passed through a 70-μm mesh, and erythrocytes were removed by hypotonic lysis and washed thoroughly to remove debris. The single cells were stained with antibodies for 20 minutes on ice.

### Adoptive cell transfer and immunization

A total of 4 × 10<sup>6</sup> to 5 × 10<sup>6</sup> of purified T cells from OT-I TCR or OT-II TCR transgenic mice were injected i.v. into naive C57BL/6 recipient mice. Two to 3 days later, these mice were injected i.v. with 4 × 10<sup>6</sup> to 5 × 10<sup>6</sup> MDSCs and, within an hour, immunized with 100 μg of specific peptides in IFA. Ten days later, cells from lymph nodes (LN) were restimulated, with specific or control peptides, and analyzed.

### Functional assays

The number of IFN-γ producing cells, in response to the stimulation with specific or control peptides (10 μg/mL), was evaluated in ELISPOT assay as described earlier (7). Each well contained 2 × 10<sup>5</sup> LN cells. The number of spots was counted in triplicate and calculated with an automatic ELISPOT counter (Cellular Technology, Ltd.). Cell proliferation, induced by antigen specific or CD3 (0.5 μg/mL) and CD28 (5 μg/mL) antibodies stimulation was evaluated by <sup>3</sup>H-thymidine incorporation, as described previously (7).

### Electromobility shift assay

Electromobility shift assay (EMSA) for NF-κB was conducted as described previously using <sup>32</sup>P-labeled probes

(16, 17). EMSA for Ets-1 was carried out according to manufacturer's protocol (Panomics). Briefly, nuclear extracts were prepared in hypertonic buffer containing 20 mmol/L HEPES (pH 7.9), 420 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20% glycerol, 1 mmol/L DTT, and protease and phosphatase inhibitors mixture. Extracts were normalized for the total protein, and 5 μg of protein was incubated with biotinylated Ets-1-specific probe GGAGGAGGGCTTGCTTGAGGAAGTATAAGAAT. Protein-DNA complex AntigenAxes were resolved by nondenaturing PAGE, transferred to a nylon membrane, and detected by streptavidin-horseradish peroxidase and a chemiluminescent substrate.

### Downregulation of Ets-1

MDSCs (10<sup>6</sup> cells) were mixed with 25 nmol/L Ets-1-specific or scrambled siRNA (Dharmacon) and transfection was carried out with Dharmacon siRNA transfection reagent. Cells were washed and cultured in wells with immobilized IA<sup>b</sup> antibody or immunoglobulin G (IgG) for 48 hours. Supernatants were collected and PGE-2 concentration was measured in ELISA.

### Quantitative real-time polymerase chain reaction

RNA was extracted with an RNeasy Mini kit and cDNA was synthesized with SuperScript III Reverse Transcriptase Kit (Invitrogen). PCR was conducted with 2.5 μL cDNA, 12.5 μL SYBR Master Mixture (Applied Biosystems), and targeted gene-specific primers. Amplification of endogenous β-actin, cyclophilin, and glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

### Flow cytometry

Flow cytometry data were acquired using a FACS LSR II (BD Biosciences) and analyzed with Flowjo software (version 8.9; Tree Star).

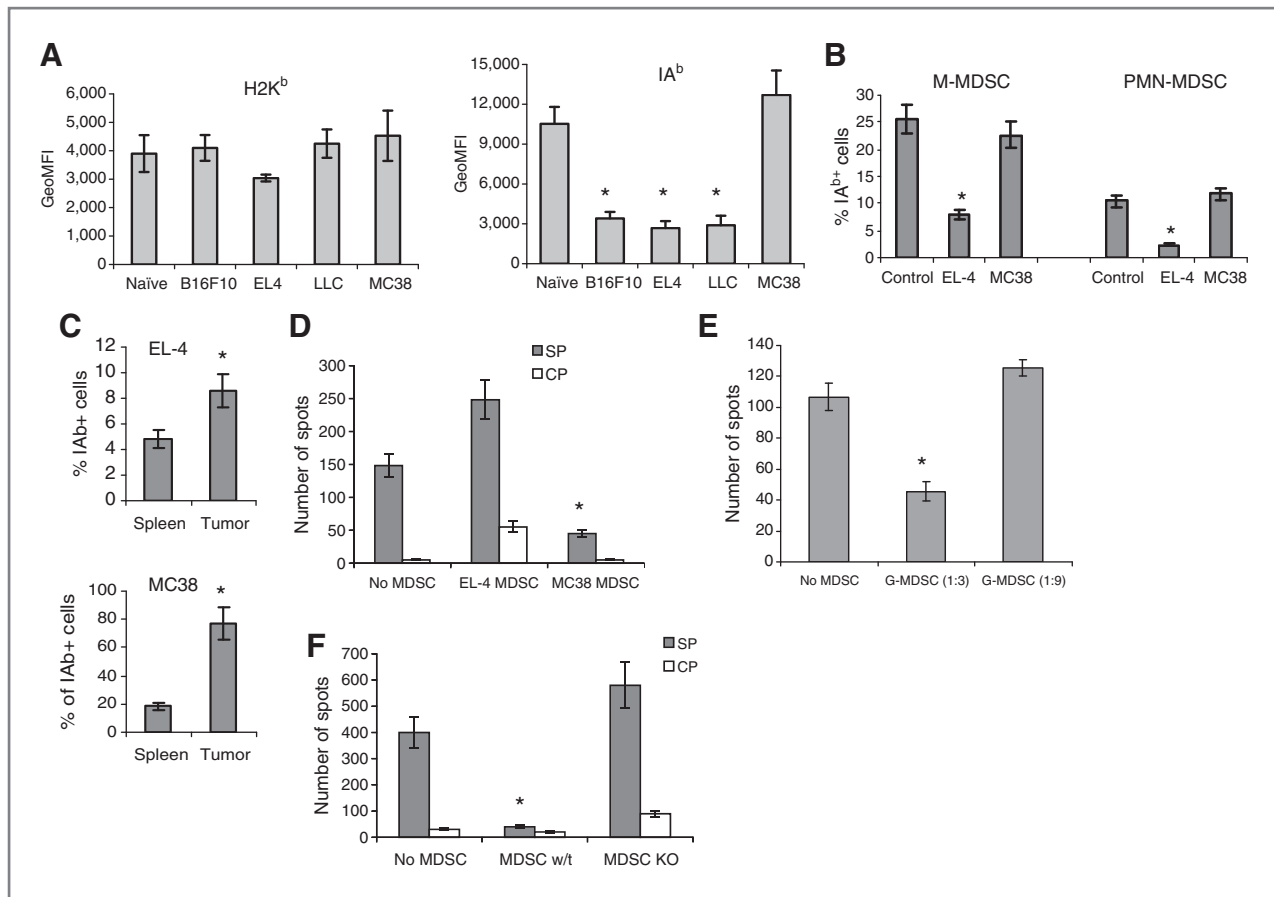
### Statistical analysis

Statistical analysis was conducted by a 2-tailed Student *t* test and GraphPad Prism 5 software (GraphPad Software Inc.) with significance determined at *P* < 0.05.

## Results

### MDSC-inducible CD4<sup>+</sup> T-cell tolerance depends on the expression of MHC class II

We tested the hypothesis that the contradictory data reported for MDSC effects on CD4<sup>+</sup> T-cell function could be linked with the expression of MHC class II on MDSCs. We measured MHC class I (H2K<sup>b</sup>) and class II (IA<sup>b</sup>) molecules on the surface of spleen Gr-1<sup>+</sup>CD11b<sup>+</sup> MDSCs in 4 different tumor models on C57BL/6 background: lung carcinoma, LLC; melanoma, B16F10; lymphoma, EL-4; and colon carcinoma, MC38. The dose of tumor cells was selected to provide for the development of equal size tumors, approximately 1.5 cm in diameter, within 3 weeks after injection. As a control, we used spleen Gr-1<sup>+</sup>CD11b<sup>+</sup> myeloid cells from naive C57BL/6 mice. Consistent with a previous report (18), MDSCs from all 4 models expressed normal levels of H2K<sup>b</sup> (Fig. 1A). In contrast, MDSCs from LLC, B16F10, and EL-4 TB mice showed a

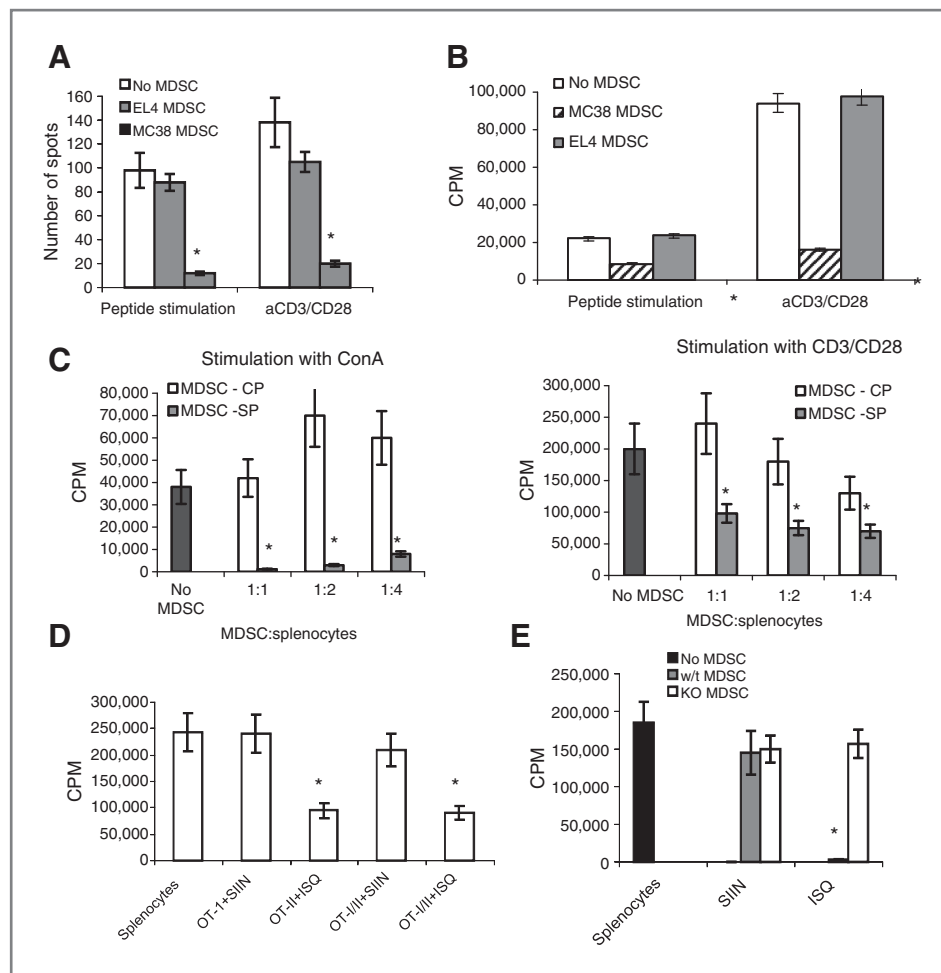


**Figure 1.** MDSC-inducible CD4<sup>+</sup> T-cell tolerance depends on MHC class II. **A**, expression of IA<sup>b</sup> and H2K<sup>b</sup> on MDSCs. Expression of H2K<sup>b</sup> (left) and IA<sup>b</sup> (right) was measured as geometric mean fluorescence intensity (MFI) within gated population of spleen Gr-1<sup>+</sup>CD11b<sup>+</sup> cells. Mean and SD from at least 4 mice in each model are shown. \*, statistically significant differences from control ( $P < 0.05$ ). **B**, the proportion of IA<sup>b</sup> cells among the populations of CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>-</sup> M-MDSCs or CD11b<sup>+</sup>Ly6C<sup>low</sup>Ly6G<sup>+</sup> PMN-MDSCs from spleens of TB or naïve mice. Each group includes 4 mice. **C**, the proportion of IA<sup>b</sup> cells among Gr-1<sup>+</sup>CD11b<sup>+</sup> MDSC isolated from spleens and tumor tissues. To obtain comparable results, both spleens and tumors were treated with collagenase to collect cells. Each group includes 4 mice. **D**, OT-II splenocytes were cultured for 48 hours with MDSCs from MC38 (MC38 MDSC) or EL-4 (EL-4 MDSC) TB mice at 3:1 ratio in the presence of specific (SP) or control (CP) peptides. The number of IFN- $\gamma$ -producing cells was calculated in triplicate in ELISPOT assay and presented as mean  $\pm$  SD per  $2 \times 10^5$  cells. Each group included 3 mice. \*, statistically significant differences from splenocytes incubated without MDSCs ( $P < 0.05$ ). **E**, CD11b<sup>+</sup>Ly6C<sup>low</sup>Ly6G<sup>+</sup> PMN-MDSCs were sorted from spleens of MC38 TB mice and added to OT-II splenocytes at indicated ratios. Cells were incubated with control or specific peptide and ELISPOT assay was conducted as described in Fig. 1D. Experiments were carried out in triplicates. Numbers of spots in the presence of control peptide were less than 5 (not shown). \*, statistically significant differences from values without MDSCs ( $P < 0.05$ ). **F**, MDSCs isolated from C57/BL6 w/t and IA<sup>b</sup> knockout (KO) MC38 TB mice were cultured with OT II splenocytes, and IFN- $\gamma$  production was measured in ELISPOT assay as described in Fig. 1D.

significantly lower expression of IA<sup>b</sup> than the control cells (Fig. 1A). The exception was MDSCs from MC38 TB mice, which had IA<sup>b</sup> expression similar to that of the control cells (Fig. 1A). The decrease in MHC class II expression on MDSCs was substantially smaller in TB mice on BALB/c background. MDSCs from MethA sarcoma-bearing mice showed control levels of MHC class II (IA<sup>d</sup>) expression, whereas only 50% decrease was observed in MDSCs from DA3 mammary carcinoma and CT26 colon carcinoma-bearing mice (data not shown).

For further experiments, we selected 2 tumor models: EL-4 (MDSCs with low IA<sup>b</sup> expression) and MC38 (MDSCs with normal IA<sup>b</sup> expression). Two subsets of MDSCs are currently identified: monocytic MDSCs (M-MDSC) and polymorphonuclear or granulocytic MDSCs (PMN-MDSC; Supplementary Fig.

S1). These subsets differ in surface markers, functional activity, and mechanisms of immune suppression (19, 20). We evaluated the proportion of IA<sup>b</sup> cells among spleen MDSCs from EL-4 and MC38 tumor-bearing mice. Control spleen myeloid cells with the same phenotype as M-MDSCs (CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>-</sup>) had significantly ( $P < 0.05$ ) higher proportion of IA<sup>b</sup> cells than control cells with the phenotype similar to PMN-MDSCs (CD11b<sup>+</sup>Ly6C<sup>low</sup>Ly6G<sup>+</sup>; Fig. 1B and Supplementary Fig. S1). In both MDSC populations from EL-4 tumor-bearing mice, the proportion of IA<sup>b</sup> cells was significantly decreased, whereas no differences were seen in MDSCs from MC38 tumor-bearing mice (Fig. 1B). Although our study was focused on spleen MDSCs and T-cell suppression in peripheral lymphoid organs, we also measured the levels MHC class II in Gr-1<sup>+</sup>CD11b<sup>+</sup> MDSCs in

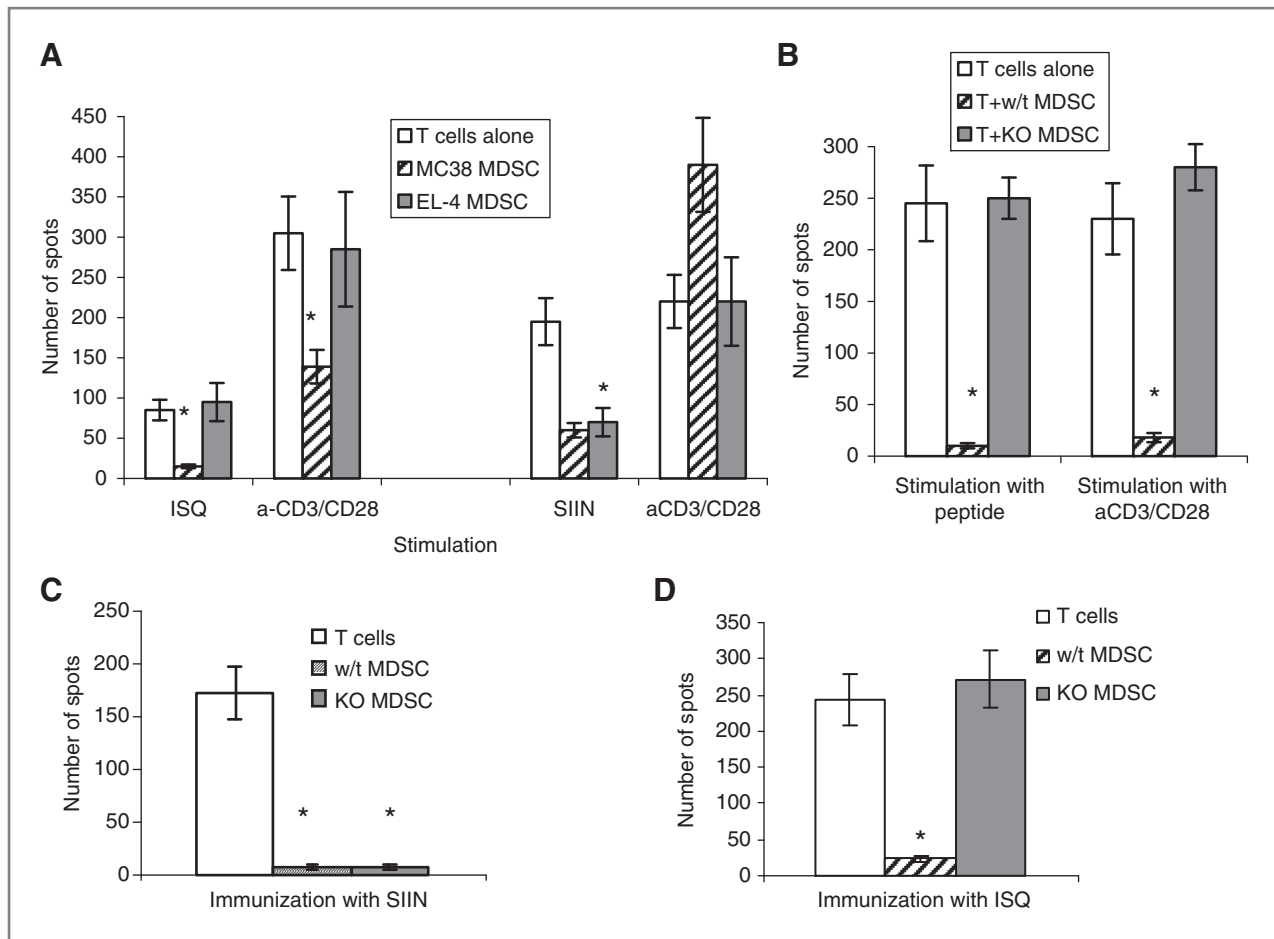


**Figure 2.** CD4<sup>+</sup> T cells cause conversion of MDSCs to nonspecific suppressors. A and B, CD4<sup>+</sup> T-cell tolerance *in vivo*. OT-II CD4<sup>+</sup> T cells were transferred *i.v.* into naïve C57BL/6 mice ( $5 \times 10^6$  cells per mouse). Two days later, mice were injected *i.v.* with  $5 \times 10^6$  MDSCs from MC38 or EL-4 TB mice. On the same day, mice were immunized with 100  $\mu$ g ISQ peptide in 100  $\mu$ L IFA. Ten days later, LN cells were isolated, stimulated with control or specific peptides or CD3/CD28 antibodies. IFN- $\gamma$  production was evaluated in ELISPOT assay (A) and cell proliferation by  $^3$ H-thymidine uptake (B). The values in cells stimulated with control peptides (CP) were subtracted from values in cells stimulated with specific peptide. Each experiment was carried out in triplicate and repeated twice. C, OT-II splenocytes and MDSCs from MC38 TB mice were cultured together at a 3:1 ratio with CP or specific peptide (SP). Forty-eight hours later, MDSCs were isolated and added to naïve splenocytes stimulated with either ConA (5  $\mu$ g/mL) or CD3/CD28 (0.5  $\mu$ g/5 $\mu$ g/mL) antibody. Cell proliferation was assessed in triplicate in  $^3$ H-thymidine uptake. Two experiments with the same results were carried out. D, OT-II CD4<sup>+</sup> and OT-I CD8<sup>+</sup> T cells were mixed at 1:1 ratio and cultured together with MDSCs from MC38 TB mice at a 3:1 ratio. Specific peptides SIIN and ISQ were added as indicated. MDSCs were isolated 48 hours later and cultured with naïve splenocytes stimulated with CD3/CD28 antibody. Cell proliferation was evaluated as described in Fig. 2A. E, experiments were carried out exactly as described in Fig. 2D. MDSCs were isolated from MC38 w/t and IA<sup>b</sup> knockout (KO) mice.

tumor sites. These cells had much higher MHC class II than MDSCs from spleens. This was observed in both tumor models (Fig. 1C).

Next, we asked whether MDSCs from EL-4 or MC38 tumor-bearing mice could inhibit CD4<sup>+</sup> T-cell responses. CD4<sup>+</sup> T cells from OT-II transgenic mice, which express TCR specific for IA<sup>b</sup> matched OVA-derived peptide (ISQ) were used. MDSCs were mixed with OT-II splenocytes, in the presence of control or specific peptides, and IFN- $\gamma$  production was evaluated 2 days later. MDSCs from EL-4 TB mice did not inhibit CD4<sup>+</sup> T-cell response to the specific peptide, whereas MDSCs from MC-38 TB mice caused a profound suppression (Fig. 1D). M-MDSCs are more potent immune suppressive cells than PMN-MDSCs

and usually display antigen nonspecific activity (19, 21). Because PMN-MDSCs represented a sizable majority of all MDSCs in MC-38 model, we tested their ability to suppress antigen-specific CD4<sup>+</sup> T cells. Sorted PMN-MDSCs showed potent ability to inhibit CD4<sup>+</sup> T-cell response to OVA-derived peptide (Fig. 1E). To clarify the role of the MHC class II molecule in MDSC effects, we used MDSCs isolated from IA<sup>b</sup><sup>-/-</sup> MC38-bearing mice. In contrast to their wild-type counterpart, these MDSCs did not inhibit the antigen-specific CD4<sup>+</sup> T-cell response (Fig. 1F). These data indicate that MDSCs in TB mice can induce antigen-specific CD4<sup>+</sup> T-cell suppression, as long as MDSCs express a sufficient level of MHC class II molecules.



**Figure 3.** Conversion of MDSC to nonspecific suppressors *in vivo*. A and B, a total of  $3 \times 10^6$  OT-II CD4<sup>+</sup> and  $3 \times 10^6$  OT-I CD8<sup>+</sup> T cells were mixed together and injected i.v. into C57BL/6 recipient mice. Mice received MDSCs from either EL-4 or MC38 TB mice 2 days later (A). Alternatively, MDSCs from IA<sup>b</sup> knockout (KO) MC38 TB mice were used (B). The mice were immunized with ISQ or SIIN peptide as indicated. Eight to ten days later, LN cells were isolated and stimulated with corresponding peptide or with anti-CD3/CD28 antibody. IFN- $\gamma$  ELISPOT assay was conducted in triplicate. Three experiments with the same results were carried out. C and D, a total of  $3 \times 10^6$  OT-II CD4<sup>+</sup> and  $3 \times 10^6$  OT-I CD8<sup>+</sup> T cells were mixed together and injected i.v. into C57BL/6 recipient mice. MDSCs were isolated from w/t and IA<sup>b</sup> KO MC38 TB mice and were transferred i.v. into recipient mice. These mice were immunized with either OT-I peptide-SIIN (C) or OT-II peptide-ISQ (D). Eight to ten days later, LN cells were isolated and restimulated with corresponding peptides. IFN- $\gamma$  ELISPOT assay was conducted. Each experiment included 3 mice and each assay was conducted in triplicate. Mean  $\pm$  SD from all experiments are shown. \*, statistically significant differences from values of T-cell activity without the presence of MDSCs ( $P < 0.01$ ).

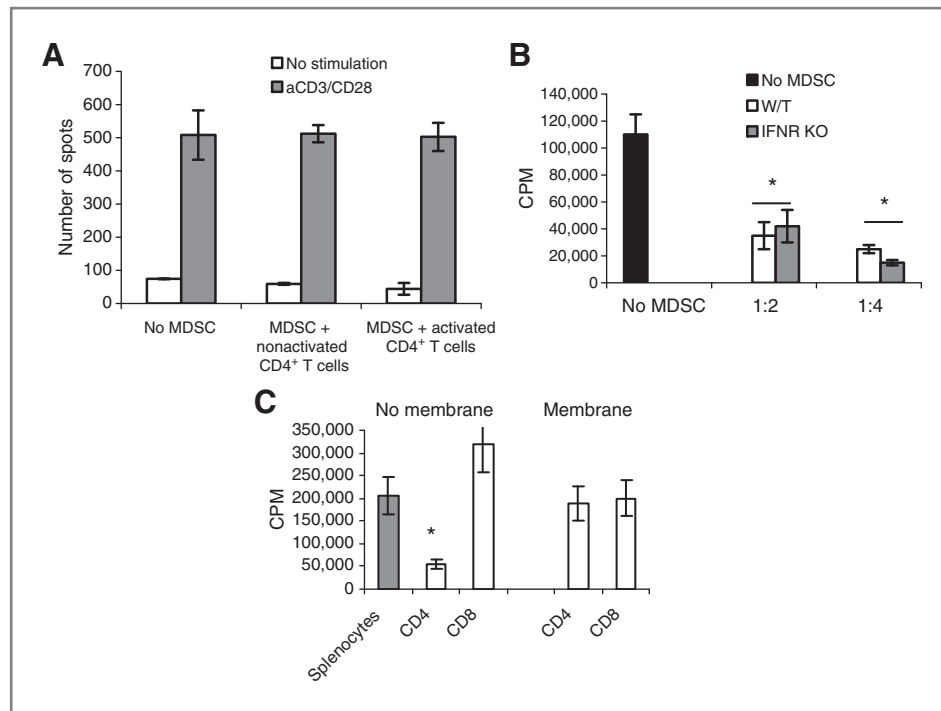
### CD4<sup>+</sup> T cells converted MDSCs to nonspecific suppressors

To investigate MDSC-induced CD4<sup>+</sup> tolerance *in vivo*, we used an experimental model that was previously developed for evaluation of the CD8<sup>+</sup> T-cell tolerance (7, 22). OT-II TCR transgenic T cells were transferred into naïve C57BL/6 mice. After 2 days, these mice were immunized s.c. with specific peptide (ISQ) in IFA. At the time of immunization, mice were injected i.v. with PBS (control) or with MDSCs isolated from spleens of EL-4 or MC38 TB mice. Ten days later, draining LNs were collected, and the cells were restimulated *in vitro* with cognate or irrelevant control peptides (Supplementary Fig. S2). MDSCs from MC38, but not EL-4 TB, mice induced CD4<sup>+</sup> T-cell tolerance (Fig. 2A and B).

Previous studies with OT-I CD8<sup>+</sup> T cells have shown that, although MDSCs caused peptide-specific tolerance of CD8<sup>+</sup> T

cells, they did not affect T-cell response to nonspecific stimuli (7, 22). To our surprise, when OT-II T cells were used, MDSCs, in addition to antigen-specific tolerance, inhibited nonspecific T-cell response to CD3/CD28 antibodies (Fig. 2A and B). We confirmed these observations *in vitro* by culturing MDSCs from MC38 TB mice for 48 hours with OT-II splenocytes in the presence of control or specific peptides. Gr-1<sup>+</sup> MDSCs were then reisolated from the cultures and added to splenocytes from naïve C57BL/6 mice, stimulated with either ConA or CD3/CD28 antibodies. MDSCs, preincubated with OT-II splenocytes in the presence of control peptide, did not inhibit nonspecific T-cell proliferation, whereas MDSCs preincubated in the presence of specific peptide acquired a potent immune suppressive activity (Fig. 2C). It was possible that the observed phenomenon was the result of the differences in functional activity of antigen-presenting cells between OT-I and OT-II mice. To





**Figure 4.** Mechanism of MDSC conversion to nonspecific suppressors. **A**, splenocytes from OT-II mice were cultured for 48 hours in complete medium (nonactivated T cells) or in the presence 5  $\mu$ g/mL ConA (activated T cells). T cells were then isolated and incubated for 48 hours with MDSCs from MC38 TB mice in the absence of peptides. MDSCs were then isolated and added to naïve splenocytes, stimulated with CD3/CD28 antibodies. IFN- $\gamma$  production was assessed in ELISPOT assay. Experiment was conducted in triplicate. Mean  $\pm$  SD in shown. **B**, MDSCs were isolated from w/t or IFN- $\gamma$ R knockout MC38 TB mice and cultured with OT-II CD4<sup>+</sup> T cells in the presence of the specific peptide. MDSCs were isolated 48 hours later and tested in their ability to suppress CD3/CD28 inducible activation of naïve T cells with different MDSC:splenocytes ratios. Proliferation was measured by <sup>3</sup>H-thymidine uptake. Two experiments were carried out. Each experiment was done in triplicates. \*, statistically significant differences from values of T-cell proliferation without the presence of MDSCs ( $P < 0.05$ ). **C**, OT-II and OT-I splenocytes were cultured with MDSCs from MC38 mice at a 3:1 ratio in the presence of corresponding specific peptides. MDSCs were either added to splenocytes directly (no membrane) or cultured on a semipermeable (0.45  $\mu$ m) membrane. MDSCs were isolated 48 hours later and added to naïve splenocytes at a 1:3 ratio. Cells were stimulated with CD3/CD28-specific antibody, and proliferation was measured in <sup>3</sup>H-thymidine uptake. Mean  $\pm$  SD from 3 experiments (each experiment in triplicate) is shown. \*, statistically significant differences from values of T-cell activity without the presence of MDSCs ( $P < 0.01$ ).

address this concern, CD4<sup>+</sup> T cells were isolated from OT-II mice; CD8<sup>+</sup> T cells were isolated from OT-I mice; and both were mixed with mature DCs generated from bone marrow of naïve mice and MDSCs from MC38 TB mice, in the presence of cognate peptides. MDSCs were reisolated 48 hours later and then added to naïve splenocytes, stimulated with CD3/CD28 antibodies. MDSCs preincubated with OT-I CD8<sup>+</sup> T cells did not suppress CD3/CD28-inducible T-cell proliferation, whereas MDSCs preincubated with OT-II CD4<sup>+</sup> cells acquired suppressive activity (Fig. 2D). This effect was reproduced when OT-II CD4<sup>+</sup> and OT-I CD8<sup>+</sup> T cells were mixed together. MDSCs acquired nonspecific suppressive activity only if T cells were stimulated with OT-II, but not OT-I-specific peptides (Fig. 2D). This effect was abrogated in MDSCs lacking IA<sup>b</sup> (Fig. 2E).

To verify these conclusions *in vivo*, OT-II CD4<sup>+</sup> T and OT-I CD8<sup>+</sup> T cells were mixed together at a 1:1 ratio and transferred to a C57BL/6 recipient, followed by the transfer of MDSCs from EL-4 or MC38 TB mice and immunization with cognate peptides (Supplementary Fig. S2). MDSCs from both EL-4 and MC38 TB mice caused peptide-specific tolerance, after immunization with OT-I peptide. No inhibition of CD3/CD28 inducible T-cell proliferation was observed (Fig. 3A). In contrast,

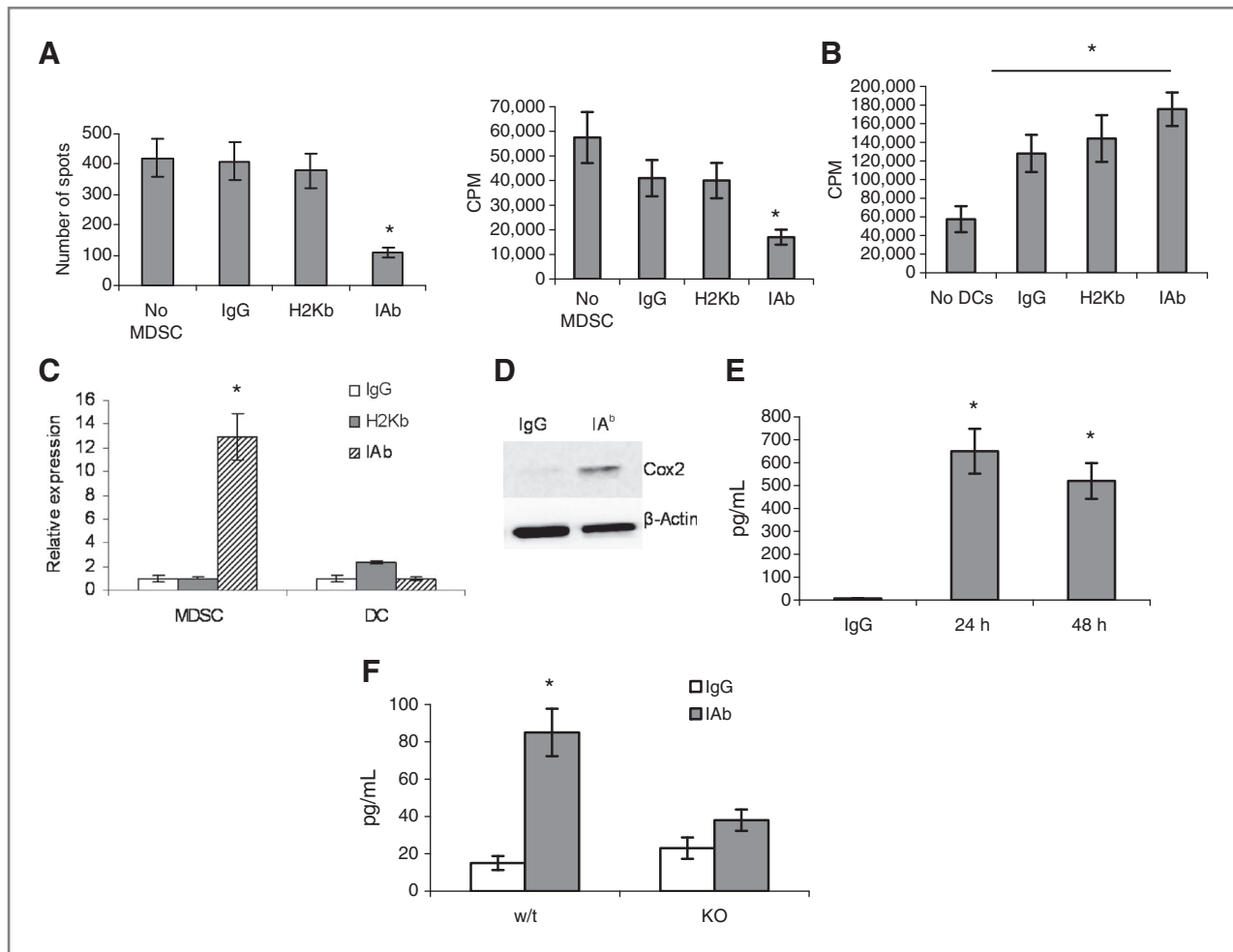
MDSCs from MC38 TB mice induced CD4<sup>+</sup> T-cell tolerance, which was associated with the inhibition of T-cell responses to CD3/CD28 antibodies (Fig. 3A). This effect was completely abrogated when MDSCs from IA<sup>b-/-</sup> MC38 TB mice were used (Fig. 3B).

We asked whether expression of MHC class II on MDSCs contributed to CD8<sup>+</sup> T-cell tolerance *in vivo*. OT-I or OT-II T cells were transferred into recipient C57BL/6 mice, together with MDSCs from wild-type (w/t) or IA<sup>b-/-</sup> MC38 TB mice, followed by immunization with corresponding peptides. Both, w/t and IA<sup>b-/-</sup> MDSCs induced CD8<sup>+</sup> T-cell tolerance equally well, whereas only w/t MDSCs, but not IA<sup>b-/-</sup> cells, induced tolerance of CD4<sup>+</sup> T cells (Fig. 3C and D).

Thus, antigen-specific CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells, were able to convert MDSCs to nonspecific suppressor cells *in vitro* and *in vivo* and this effect was dependent on MHC class II.

#### CD4<sup>+</sup> T-cell-inducible conversion of MDSCs is mediated via cross-linking of MHC class II

We asked whether nonspecific activation of CD4<sup>+</sup> T cells was sufficient to convert MDSCs to nonspecific suppressors. CD4<sup>+</sup> T cells from OT-II mice were activated by 48 hours

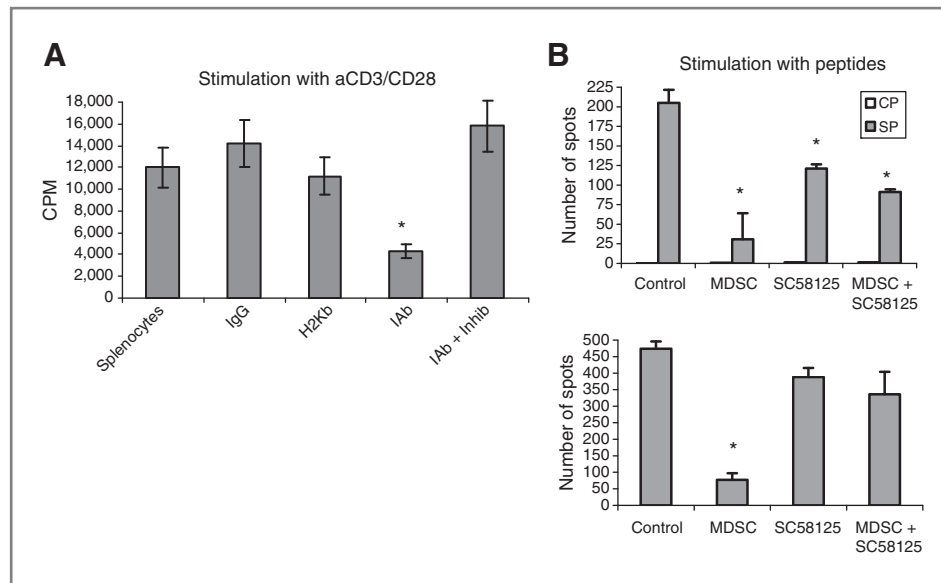


**Figure 5.** Ligand of IA<sup>b</sup> on MDSCs convert these cells to nonspecific suppressors. **A**, MDSCs isolated from MC38 TB mice were cultured for 48 hours with immobilized IgG, H2K<sup>b</sup>, or IA<sup>b</sup> antibodies. MDSCs were then harvested and added to naïve splenocytes stimulated with CD3/CD28 antibodies. IFN- $\gamma$  ELISPOT (left) and proliferation (panel) assays were conducted. Cumulative results from 4 experiments (each in triplicate) are shown. \*, statistically significant differences from values of T-cell activity without the presence of MDSCs ( $P < 0.05$ ). **B**, experiments were conducted essentially as in Fig. 5A except DCs were used instead of MDSCs. **C**, MDSCs isolated from MC38 TB mice or bone marrow–derived DCs were cultured with immobilized IgG, H2K<sup>b</sup>, or IA<sup>b</sup> antibodies. Four hours later, RNA was isolated and relative expression of *Cox-2* was analyzed by qRT-PCR. Three experiments, in triplicate, were carried out. **D**, MDSCs from MC38 TB mice were cultured on plates with immobilized IgG or IA<sup>b</sup> antibody for 24 hours. Whole-cell lysate was collected and *Cox-2* level was detected in Western blot. **E**, MDSCs isolated from MC38 TB mice were cultured with immobilized IgG or IA<sup>b</sup> antibody for 24 or 48 hours. The concentration of PGE2 was measured in supernatants in ELISA. Two experiments (each in triplicate) with the same results were carried out. The results of 1 experiment are shown. \*, statistically significant differences from IgG control ( $P < 0.001$ ). **F**, experiments were carried out as described above. MDSCs were isolated from w/t and IA<sup>b</sup> KO mice and cultured for 24 hours with either immobilized control IgG or IA<sup>b</sup> antibody. PGE2 was measured in supernatants by ELISA. Two experiments, in triplicate, were carried out. \*, statistically significant differences from values in MDSCs cultured with IgG ( $P < 0.01$ ).

incubation of splenocytes with ConA. T cells were then isolated and incubated for 48 hours in the absence of specific peptide, with MDSCs from MC38 TB mice, followed by MDSCs isolation. MDSCs were then added to naïve splenocytes, stimulated with CD3/CD28 antibodies. In the absence of specific peptide, preactivated CD4<sup>+</sup> T cells failed to convert MDSCs to nonspecific suppressors (Fig. 4A). To test the possibility that increased IFN- $\gamma$  production from activated CD4<sup>+</sup> T cells could be responsible for the MDSC conversion, we established MC38 tumors in mice IFN- $\gamma$  receptor (IFN- $\gamma$ R) knockout mice. MDSCs from these mice lose the ability to respond to IFN- $\gamma$  by upregulating *iNOS*

expression (data not show). Pretreatment of w/t and IFN- $\gamma$ R<sup>-/-</sup> MDSC with OT-II splenocytes, in the presence of specific peptide, caused equal conversion of MDSCs to nonspecific suppressors (Fig. 4B), indicating that CD4<sup>+</sup> T cells effect on MDSCs was not mediated by IFN- $\gamma$ . Furthermore, MDSC conversion required direct cell–cell contact because incubation of MDSCs with OT-II splenocytes, separated by a semipermeable membrane, did not cause the MDSC conversion (Fig. 4C).

Because the effect of CD4<sup>+</sup> T cells on MDSCs required expression of IA<sup>b</sup>, we asked whether the cross-linking of MHC class II on MDSCs without the presence of CD4<sup>+</sup> T cells would



**Figure 6.** CD4<sup>+</sup> inducible conversion of MDSCs is mediated by Cox2/PGE2. **A**, MDSCs isolated from MC38 TB mice were incubated with immobilized IgG, H2K<sup>b</sup>, or IA<sup>b</sup> antibodies for 48 hours with and without 5  $\mu$ mol/L COX-2 inhibitor (SC58125) as indicated. After incubation, MDSCs were isolated, washed, and cultured with naive splenocytes stimulated with CD3/CD28 antibodies. Proliferation was measured by <sup>3</sup>H-thymidine uptake in triplicate. Typical result of 3 experiments is shown. \*, statistically significant differences from values of splenocytes cultured without MDSCs ( $P < 0.05$ ). **B**, MDSCs from MC38 TB mice were used in experimental model of CD4<sup>+</sup> T-cell tolerance as described in Fig. 1D and E. SC58125 (10 mg/kg) was injected i.p. on days 1, 3, 5, and 7 after MDSC administration. LN cells were collected on day 10 and stimulated with control peptide (CP), specific peptide (SP), or CD3/CD28 antibodies. IFN- $\gamma$  production was evaluated in ELISPOT assay. Experiment was carried out in triplicates.

recapitulate the effect of T cells. To address this question, control IgG-, H2K<sup>b</sup>-, and IA<sup>b</sup>-specific antibodies were immobilized on plates. MDSCs from MC38 TB mice were cultured for 48 hours on those plates and then collected and added to naive splenocytes stimulated with CD3/CD28-specific antibodies. MDSCs incubated with IA<sup>b</sup>, but not with control IgG or H2K<sup>b</sup> antibody, suppressed nonspecific T-cell activation (Fig. 5A). In contrast, preincubation of immature DCs resulted in an increase of T-cell responses to stimulation with CD3/CD28 antibodies (Fig. 5B).

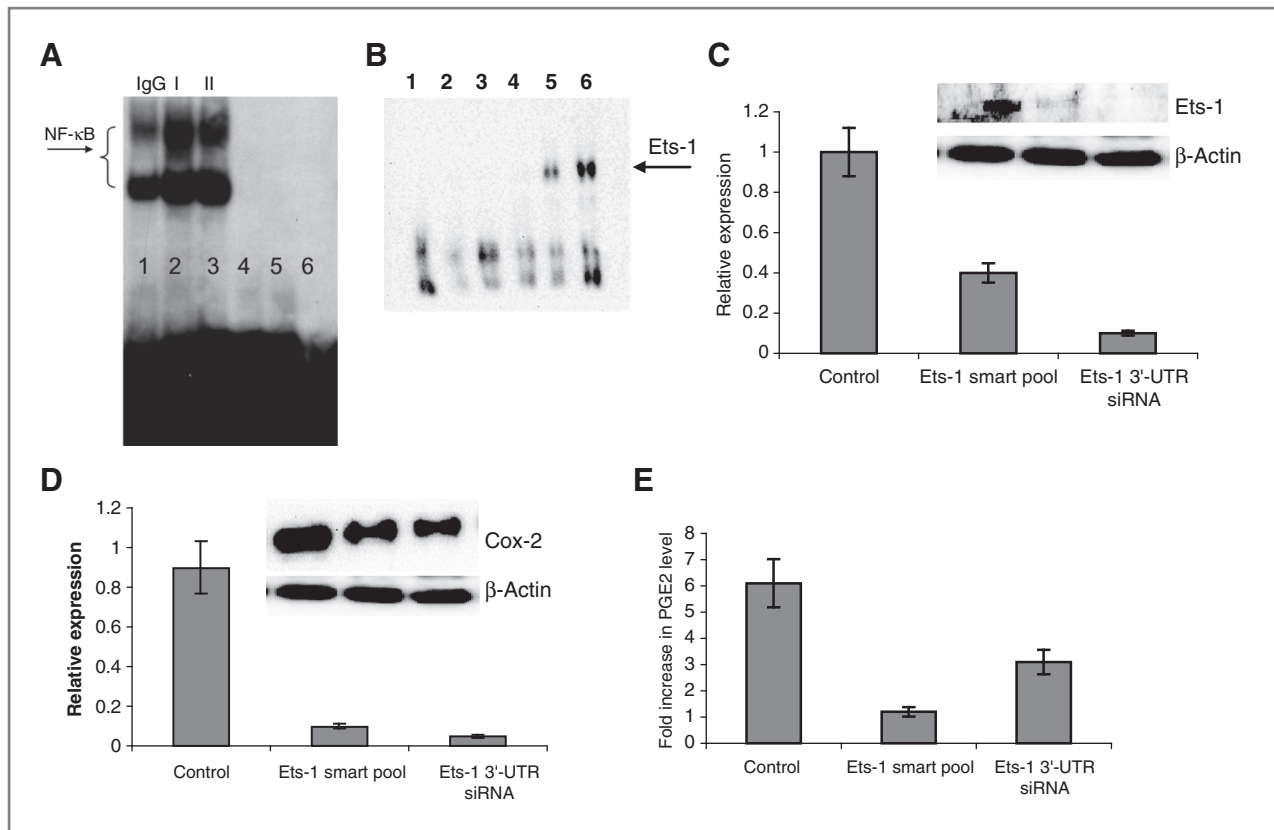
Then, we focused on the possible mechanism of this effect. Using the experimental system described above, we screened different factors known to be involved in MDSC-mediated immune suppression. Cross-linking of IA<sup>b</sup> on MDSCs did not result in upregulation of iNOS, arginase, or reactive oxygen species production in MDSCs (data not shown). However, it caused dramatic upregulation of *Cox-2* expression (Fig. 5C) and increased expression of Cox2 protein (Fig. 5D). This effect was not observed in DCs (Fig. 5C). *Cox-2* is critically important for the synthesis of prostaglandin E2 (PGE2), an immune suppressive factor known to be produced by MDSCs (23–25). Cross-linking of IA<sup>b</sup> resulted in dramatic upregulation of PGE2 production by MDSCs (Fig. 5E). This effect was not seen in MDSCs lacking IA<sup>b</sup> (Fig. 5F). Thus, our data indicated that retrograde signaling via MHC class II in MDSCs may result in upregulation of *Cox-2* and PGE-2.

Downregulation of PGE2 synthesis with selective Cox2 inhibitor SC58125 completely abrogated the ability of IA<sup>b</sup> ligation to convert MDSCs into nonspecific suppressors (Fig.

6A). To test this concept *in vivo*, OT-II T cells and MDSCs from MC38 TB mice were transferred to tumor-free recipient mice followed by immunization with specific peptide. Half of the mice were treated with i.p. injections of COX-2 inhibitor and the remaining half with vehicle alone (control). Seven days later, the response of LN T cells to stimulation with specific peptide and CD3/CD28 antibodies was measured. Cox-2 inhibitor only partially reduced the ability of MDSCs to induce antigen-specific CD4<sup>+</sup> T-cell tolerance, whereas nonspecific inhibition of T-cell responses was completely abrogated (Fig. 6B).

What could be the molecular mechanism of this effect? NF- $\kappa$ B and Ets family transcription factor Ets-1 were previously implicated in regulation of *Cox-2* expression (26–29). We tested their possible involvement in our experimental system. MHC class I and class II antibodies induced similar activation of NF- $\kappa$ B in MDSCs (Fig. 7A and Supplementary Fig. S3), which argued against its specific role in MHC class II-mediated PGE-2 regulation. Cross-linking of MHC class II caused upregulation of Ets-1 activity (Fig. 7B). Cross-linking of H2K<sup>b</sup> did not have the same effect (Supplementary Fig. S4). To test possible role of Ets-1 in upregulation of *cox2* and PGE2 by MHC class II cross-linking, we downregulated Ets-1 using 2 different siRNA (Fig. 7C). MDSCs then were cultured on immobilized anti-IA<sup>b</sup> antibody and the level of expression of *cox2* as well as PGE2 production was evaluated. Blockade of Ets-1 in MDSCs with specific siRNA resulted in significant decrease in *cox2* expression caused by MHC class II ligation (Fig. 7D) and abrogation of PGE-2 production by these cells (Fig. 7E).





**Figure 7.** Upregulation of Ets-1 in MDSCs by  $IA^b$  cross-linking on MDSCs is responsible for increased production of  $PGE_2$ . **A.** MDSCs were isolated from spleens of MC38 TB mice and incubated for 16 hours with immobilized IgG-,  $H2K^b$ -, or  $IA^b$ -specific antibodies. NF- $\kappa$ B activity was measured with EMSA. Lines 1 to 3 NF- $\kappa$ B-specific probe: 1, MDSCs incubated with IgG; 2,  $H2K^b$ ; or 3,  $IA^b$ -specific antibody. Lanes 4 to 6 controls: 4, NF- $\kappa$ B probe alone; 5, MDSCs after incubation with  $IA^b$  antibody in the presence of 10-fold excess of unlabeled probe; 6, the same in the presence 20-fold excess of unlabeled probe. **B.** MDSCs were isolated from MC38 TB mice and cultured for 16 hours with immobilized  $IA^b$  antibody or control IgG. Nuclear extracts were prepared and EMSA was carried out with Ets-1-specific probe. Lanes: 1, Ets-1 probe no cells; 2, cells no probe; 3, cells cultured with IgG and labeled Ets-1 probe with 2 $\times$  excess of unlabeled Ets-1 probe; 4, as line 3 but without unlabeled probe; 5, cells cultured with  $IA^b$  and Ets-1-labeled probe with 2 $\times$  excess of unlabeled Ets-1 probe; 6, as line 5 but without unlabeled probe. Two experiments with the same result were carried out. **C.** MDSCs from MC38 TB mice were transfected with either control nonspecific cyclophilin B pool siRNA, ETS1-specific 3'-UTR/ORF smart pool siRNA, or ETS1 specific 3'-UTR single siRNA. Cells were incubated with immobilized  $IA^b$  for 48 hours. RNA and proteins were isolated. Relative expression of *ets-1* was analyzed (in triplicates) using qRT-PCR (2 experiments with the same results were carried out). The level of Ets-1 protein was evaluated in Western blotting (inset). **D.** MDSCs from MC38 TB mice were transfected with Ets-1-specific or control siRNAs and incubated with immobilized  $IA^b$  for 48 hours. Expression of *cox2* mRNA (mean  $\pm$  SD of triplicates) and COX2 protein (inset) was measured by qRT-PCR and Western blot, respectively. Two experiments with the same results were carried out. **E.** PGE-2 levels were assessed in triplicate by ELISA. Mean  $\pm$  SD are shown. Three experiments with the same results were carried out. In Fig. 7C and D, differences between specific Ets-1 siRNA and control siRNA were statistically significant ( $P < 0.05$ ).

## Discussion

This study was designed to clarify the issue of antigen-specific  $CD4^+$  T cells tolerance caused by MDSCs because it was apparent that the effect of MDSCs on  $CD4^+$  T cells depend on the nature of the tumor model used (5). We found that the ability of MDSCs to induce antigen-specific  $CD4^+$  T-cell tolerance *in vivo* was dependent on the expression of MHC class II. In most tumor models studied, expression of MHC class II molecules on MDSCs was lower than in myeloid cells with the same phenotype from tumor-free mice. In some models (B16F10, EL-4, LLC), it was dramatically lower, in some (DA3, CT26) it was only slightly (less than 2-fold) lower, and in some (MC38, MethA) it was not substantially different from naïve mice. Similar variability in MHC class II expression was described

in some human studies. In melanoma, MDSCs are characterized as MHC class II (HLA-DR)<sup>low</sup> cells, some (albeit rather low) expression of MHC class II on MDSCs was reported in patients with leukemia and several solid tumors (30–33). This may explain some of the contradictory data about the effect of MDSCs on  $CD4^+$  T-cell function. The exact mechanism of MHC class II regulation in MDSCs is not yet clear. It is possible that STAT3 may play a major role in this effect because many cytokines produced by tumors one way or another may trigger STAT3 signaling in myeloid cells and upregulation of STAT3 is a common finding in myeloid cells in TB hosts (34–36). On the contrary, it is known that upregulation of STAT3 results in reduction of MHC class II expression in DCs (17, 37, 38).

Our data are consistent with previously reported mechanism of  $CD8^+$  T-cell tolerance caused by MDSCs, which

dependent on MHC class I, required cell–cell contact and was mediated via peroxynitrite release by MDSCs (22). The unexpected finding in our study was the observation that interaction of MDSCs with antigen-specific CD4<sup>+</sup> T cells dramatically changes the nature of MDSC-mediated suppression. MDSCs acquired the ability to inhibit T-cell functions, regardless of the presence of the antigen. This effect was not observed with CD8<sup>+</sup> T cells and was dependent on the expression of MHC class II. Importantly, antigen-specific interaction apparently was critical for this phenomenon as without the presence of specific peptide, conversion was not observed. Our data have shown that this effect required MHC class II cross-linking, which leads to upregulation of *cox2* and PGE<sub>2</sub>, which were previously implicated in MDSC-mediated immune suppression (23–25).

Previously, it has been shown that lymphocyte-activated gene-3, a CD4-related transmembrane protein, interacts with MHC class II and inhibits DC activation (39). MHC class II dimerization plays a role in the production of proinflammatory molecules by myeloid cells. These functions of MHC class II have been shown to engage various intracellular signaling events, including activation of the signaling protein PLC, the kinases Src, Syk, and PKC, and the mitogen-activated kinases p38 and Erk (40). In a recent study, it has been shown that interaction of MHC class II with staphylococcal enterotoxins triggers a MyD88-mediated signaling mechanism that resulted in activation of NF- $\kappa$ B (41). NF- $\kappa$ B on the other hand has been shown to regulate *Cox2* expression (42). MHC class II molecules can also have a cross-talk with TLR (43) or co-stimulatory CD40 molecules (44). TLRs could act as adaptor receptors, influencing the responses induced by MHC class II molecules (44). MHC II cross-linking by agonistic antibodies induces an immunoreceptor tyrosine-based activation motif (ITAM)-mediated inhibitory signaling pathway, involving Fc $\gamma$ R $\gamma$ - and

ERK-mediated recruitment of SHP-1 that suppresses DC maturation and immunostimulatory capacity (39). Thus, NF- $\kappa$ B transcription factor, which is downstream from most of these pathways was most likely choice for the factor-regulating *cox2* expression in MDSCs. However, our experiments did not support this hypothesis. We turn our attention to another transcription factor previously implicated in regulation *cox2*–Ets-1. Cross-linking of IA<sup>b</sup> resulted in upregulation of Ets-1 in MDSCs. Downregulation of Ets-1 abrogated increased *cox2* expression and PGE<sub>2</sub> production caused by IA<sup>b</sup> ligation. These data suggest that Ets-1 may play a major role in retrograde MHC class II signaling in MDSCs that resulted in PGE<sub>2</sub> synthesis.

Although the exact molecular mechanism of regulation of *cox2* expression in MDSCs needs to be clarified, this study, for the first time, has shown that activated antigen-specific T cells can potentiate immune suppressive activity of MDSCs by converting these cells to nonspecific suppressors and thus limiting the ability of the host to mount potent immune response in tumor-bearing hosts.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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