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Defective MHC Class II Presentation by Dendritic Cells Limits CD4 T Cell Help for Antitumor CD8 T Cell Responses

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Cancer immunosurveillance failure is largely attributed to insufficient activation signals and dominant inhibitory stimuli for tumor Ag (Tag)-specific CD8 T cells. CD4 T cell help has been shown to license dendritic cells (DC), thereby having the potential for converting CD8 T cell responses from tolerance to activation. To understand the potential cooperation of Tag-specific CD4 and CD8 T cells, we have characterized the responses of naive TCR transgenic CD8 and CD4 T cells to poorly immunogenic murine tumors. We found that whereas CD8 T cells sensed Tag and were tolerized, the CD4 T cells remained ignorant throughout tumor growth and did not provide help. This disparity in responses was due to normal Tag MHC class I cross-presentation by immature CD8α+ DC in the draining lymph node, but poor MHC class II presentation on all DC subsets due to selective inhibition by the tumor microenvironment. Thus, these results reveal a novel mechanism of cancer immunosubversion, in which inhibition of MHC-II Tag presentation on DC prevents CD4 T cell priming, thereby blocking any potential for licensing CD8α+ DC and helping tolerized CD8 T cells.

MHC-II presentation by DC. A similar discrepancy was observed in the endogenous, polyclonal T cell populations. This introduces the novel possibility that the tumor microenvironment selectively inhibits DC-mediated MHC-II presentation, thereby limiting CD4 T cell help and causing tolerogenic anti-tumor CD8 T cell responses.

Materials and Methods

Mice, cell lines, and reagents

OVA<sub>97–257</sub>-H-2Kb-specific, TCR transgenic OT-I mice and OVA<sub>323–339</sub>/I-Ab-specific, TCR transgenic OT-II mice were a gift from F. Carbone (University of Melbourne, Melbourne, Australia). OT-1PL and OT-IIPL mice were generated through crossing OT-I or OT-II mice (respectively) with Thy1-congenic B6.PL-Thy1a/Cy (Thy1.1) mice (The Jackson Laboratory) and breeding to homozygosity. OT-II Rag<sup>1<sup>-/-</sup> were generated by crossing OT-1PL to C57BL/6Rag-1 tm1Mom (Rag1-deficient, 2216) mouse (The Jackson Laboratory). Pmel mice (B6.Cg-Thy1a/CyTg(TeraTcrb)R6Rst/JC57BL/6Ncr) were purchased from The Jackson Laboratory. C57BL/6Ncr and CD45.1-congenic B6 (B6.Ly5.2) mice were purchased from the National Cancer Institute. Experiments were conducted under specific pathogen-free conditions at the University of Minnesota, and were performed in compliance with relevant laws and institutional guidelines and with the approval of the Institutional Animal Care and Use Committee at the University of Minnesota. EL4 and E.G7 (EL4 transfected with a secreted form of OVA) cells were maintained by in vitro culture in complete RPMI 1640 medium (1) without or with 250 μg/ml G418 (respec-

Tumor CD8 T cell responses.

Adoptive transfer and tumor challenge

Ninety-five percent pure, naive CD8<sup>+</sup> T cells were obtained from OT-IPL or Pmel mice, as previously described (1). OT-II T cells were purified using a CD4<sup>+</sup> T cell enrichment kit (Miltenyi Biotec) to >97% purity. Cells were then labeled with CFSE, and 1 × 10<sup>5</sup> cells were i.v. injected into recipient mice. Twenty-four to 48 h later, recipients were s.c. injected with either 2 × 10<sup>5</sup> B16.F10 or B16.OVA cells in PBS or E.G7 cells in PBS. Tumor area was analyzed by taking the product of perpendicular tumor size measurements using calipers. In some experiments, 50 μg of OVA<sub>333–349</sub> and LPS was injected i.v. into day 7 E.G7-bearing or day 10 B16.OVA-bearing animals. In some experiments, 10<sup>5</sup> freeze/thawed or irradiated B16.OVA cells were injected, and T cells were analyzed for CFSE dilution 4 days later. In certain experiments, B16.F10 cells were injected, followed by adoptive transfer of T cells on day 8 or 9 postinjection, followed by intratumoral injection of indicated doses of LPS-free OVA protein (Sigma-Aldrich) on day 10. As control, tumor-free mice were injected with OVA protein into heavy musculature of the upper tibia, into plantar surface of the hind foot (footpad), or s.c. Alternatively, 50 μg of LPS-free Ezaed fluorescent protein or EzGFP (gift from M. Jenkins, University of Minnesota, Minneapolis, MN) was injected without prior T cell transfer. In certain experiments, animals were first perfused, followed by isolation of LNs, spleen, lungs, liver, bone marrow, and tumor tissues.

Flow cytometric and statistical analysis

Mice were sacrificed at indicated times, and single-cell suspensions from tissues were obtained through homogenization. Transferred cells were identified using CD8<sup>+</sup> or CD4<sup>+</sup> specific staining and congenic markers for Thy1.1 and/or CD45.2, and sometimes with Vα2 TCR-specific Ab. Cell counts were obtained using PHK26 reference microbeads (Sigma-Aldrich). Detection of granzyme B or IFN-γ production was done, as described previously (1). Foxp3 staining was done using the Foxp3 Staining Buffer Set and anti-Foxp3-allophycocyanin Ab, according to manufacturer’s specifications (eBioscience). All stained cells were collected on either a FACS-Calibur flow cytometer or LSR-II flow cytometer (BD Biosciences), and analyzed using FlowJo software (Tree Star). Statistical significance was calculated by using two-tailed t tests, or one-way ANOVA. Value of p representation: <0.05 is represented by one asterisk; <0.01 by two asterisks; <0.001 by three asterisks.

DC purification and in vitro proliferation

DLNs or tumors of B16.OVA-bearing mice were harvested and treated with 400 U/ml collagenase D (Roche Applied Science) solution, and incubated for 25 min at 37°C. EDTA was added to yield a 10 mM solution for 1 min, followed by a PBS wash. In some experiments, CD11c<sup>+</sup> cells were isolated by incubating with anti-CD11c1 beads, followed by positive enrichment (Miltenyi). For intracellular IL-12p40 staining, DC were incubated in brefeldin A (1 μg/ml) during colagenase digestion and for an additional 4 h in complete RPMI 1640 medium with brefeldin A at 37°C and 5% CO<sub>2</sub> (BD Biosciences). To separate CD8α<sup>+</sup> DC, cell suspensions were first depleted of T, NK, and B cells using Thy1.2-, DX-5-, and B220-specific Abs and anti-FITC beads. Remaining cells were then stained with anti-CD8α FITC, incubated with anti-FITC beads, and separated into CD8α<sup>+</sup> fractions by LS column separation. A total of 2 × 10<sup>6</sup> DC was then incubated with 3 × 10<sup>6</sup> CFSE-labeled OT-I and OT-II T cells in wells (96-well plates) for 4 days at 37°C.

Results

Tag-specific CD4 T cells do not alter antitumor CD8 T cell responses

We have recently shown that adoptive transfer of 1.5 × 10<sup>6</sup> naive OT-I T cells does not alter tumor progression due to poor expansion and weak activation, caused by insufficient provision of a secondary signal cytokine during priming (22) (F. Popescu and M. F. Mescher, manuscript in preparation). Because CD4 T cells have the ability to enhance production of third signal cytokines and co-stimulatory molecules by DC, and therefore can enhance CD8 T cell activation, we adoptively cotransferred 1 × 10<sup>6</sup> purified, naive OT-I T cells and OT-II T cells and s.c. challenged the mice with E.G7 or B16.OVA tumor 1 day later. Surprisingly, tumor growth was not significantly altered in either tumor model when both OT-I and OT-II T cells were cotransferred (Fig. 1). The small difference in B16 growth at day 20 was not reproducible in other experiments. Examination of the OT-I response in the DLN at the earliest time of visible tumor detection revealed that OT-I T cells up-regulated surface CD69 expression in an Ag-specific manner, because CD4 T cells have the ability to enhance production of third signal cytokines and costimulatory molecules by DC, and therefore can enhance CD8 T cell activation, we adoptively cotransferred 1 × 10<sup>6</sup> purified, naive OT-I T cells and OT-II T cells and s.c. challenged the mice with E.G7 or B16.OVA tumor 1 day later. Surprisingly, tumor growth was not significantly altered in either tumor model when both OT-I and OT-II T cells were cotransferred (Fig. 1). The small difference in B16 growth at day 20 was not reproducible in other experiments. Examination of the OT-I response in the DLN at the earliest time of visible tumor detection revealed that OT-I T cells up-regulated surface CD69 expression in an Ag-specific manner, because CD4 T cells do not provide sufficient help and activation caused by insufficient provision of a
both DLN and the tumor site, and a majority of OT-I cells at the tumor site produced granzyme B (GrzB) (Fig. 2, D and E). Importantly, no differences in CD69 expression, clonal expansion, or cytokine production were seen when OT-II T cells were cotransferred (Fig. 2, A and C–E), indicating that OT-II T cells did not help OT-I T cell responses. Similar results were obtained throughout the course of tumor growth (data not shown).

**FIGURE 2.** OT-I T cell antitumor responses. A total of $1 \times 10^6$ OT-I T cells was either adoptively transferred alone or in combination with OT-II T cells. B16.OVA and B16.F10 cells were injected 1 day later in opposite flanks. E.G7 and EL4 were injected 3 days postadoptive transfer in opposite flanks. On day 7 post-E.G7/EL4 injection and day 9 post-B16.OVA/B16.F10 injection, DLN and tumor samples were harvested and OT-I T cells were analyzed for surface CD69 expression (A) and CFSE dilution (B). DLN and tumor samples were analyzed for total OT-I T cell numbers (C). OT-I T cells were examined for intracellular IFN-$\gamma$ (D) and GrzB protein levels (E) after a 4-h in vitro restimulation with OVA257-264 and GolgiStop. Results are represented as mean ± SD. Data are representative of at least three independent experiments.

**FIGURE 3.** Pmel T cell antitumor responses. A total of $1 \times 10^6$ CFSE-labeled Pmel T cells was adoptively transferred alone or in combination with OT-II T cells, followed by a s.c. E.G7/EL4 or B16.OVA/B16.F10 opposite-flank tumor inoculation. On day 7 post-E.G7/EL4 injection and day 9 post-B16.OVA/B16.F10 injection, DLN and tumor samples were harvested and Pmel T cells were analyzed for surface CD69 expression (A), CFSE dilution (B), total cellular recovery (C), and intracellular expression of GrzB without peptide restimulation (D). Results are represented as mean ± SD. Data are representative of two independent experiments.
CD8 T cells respond similarly to secreted OVA and cytoplasmic endogenous TAggs

Because the majority of TAggs are derived from cytoplasmic and not secreted proteins, we compared the responses of OT-I T cells to Pmel transgenic CD8 T cells specific for a melanocyte-specific intracellular protein gp100, which is expressed in the B16 melanoma. As seen with the OT-I T cells, at the earliest time of visible tumor growth, DLN Pmel T cells up-regulated surface CD69 expression and proliferated, as assessed by CFSE dilution in response to both B16.F10 and B16.OVA tumors, but not to the E.G7 tumor (Fig. 3, A and B). Additionally, Pmel T cells produced GrzB at the tumor site, indicating at least partial activation, but did not clonally expand by >2- to 3-fold and did not affect tumor growth (Fig. 3, C and D, and data not shown), suggesting that the level of third signal cytokines available at the DLN is insufficient for supporting survival and full differentiation (22). Importantly, as with OT-I T cells, Pmel responses were not significantly altered by a cotransfer of OT-II T cells (Fig. 3, A, C, and D). Similar results were obtained

FIGURE 4. OT-II T cell antitumor responses. A–C, 1 × 10⁶ CFSE-labeled OT-II T cells were adoptively transferred, followed by a s.c. E.G7/EL4 or B16.OVA/B16.F10 opposite-flank tumor inoculation. On day 7 post-E.G7/EL4 and day 9 post-B16.OVA/B16.F10 inoculation, DLN were harvested and OT-II T cells were analyzed for surface CD69 expression (A), CFSE dilution (B), and total cellular recovery (C). D and E, Naive, day 7 E.G7-bearing, or day 10 B16.OVA-bearing were i.v. injected with 50 μg of OVA₃₂₃₋₃₃₉ and LPS. Total OT-II cellular recovery was analyzed 4 days later (Post-Imm) in the spleen (D) and tumor (E) samples and compared with preimmunization levels (Pre-Imm). Results are represented as mean ± SD. Data are representative of at least three independent experiments.

FIGURE 5. T cell sensitivity does not govern differential responsiveness to TAg. OT-I and OT-II T cells were transferred into tumor-free or B16.F10 tumor-bearing hosts. On day 10 of tumor growth, the animals were injected with 0.5 μg of OVA protein into the footpad, i.m., s.c., or i.t. Four days later, DLN were harvested and analyzed for OT-I (gray) and OT-II (white) CFSE dilution profiles (A). Percentage of cells undergoing division to indicated amounts of OVA protein was quantified and compared between s.c. injected tumor-free and i.t. injected tumor-bearing animals for OT-I (B) and OT-II (C) T cells. OVA (1 μg) was injected s.c. into tumor-free, i.t., or s.c. into contralateral side of tumor-bearing animals (D). Inhibition of proliferation was measured as percentage of decrease in CFSE-diluted cells as compared with tumor-free responses. Results are represented as mean ± SD. Data are representative of at least two independent experiments.
throughout the course of tumor growth (data not shown). These results indicated that cytoplasmic and secreted TAgS are efficiently cross-presented in tumor DLN on MHC-I, but lead to CD8 T cell tolerance, and cannot be helped by even a relatively large population of TAg-specific CD4 T cells. Additionally, the similarity of Pmel and OT-I T cell responses validated OVA as a relevant artificial TAg model.

**OT-I T cells do not respond to a cognate tumor challenge**

To investigate the reason for lack of helper activity of CD4 T cells, we analyzed the responses of OT-II T cells in the DLN, spleen, and tumor. In contrast to the OT-I and Pmel responses, OT-II T cells did not up-regulate surface CD69 expression in the tumor DLN, did not dilute CFSE, did not undergo clonal expansion, and did not infiltrate the tumor, indicating a complete lack of a response to tumor-secreted OVA protein (Fig. 4, A–C and E). Similar results were obtained at later time points (data not shown). To examine whether OT-II T cells were defective in responding to Ag in tumor-bearing animals, we immunized tumor-bearing or tumor-free animals by i.v. injection of OVA323–339 and LPS and analyzed the clonal expansion of cells on day 4 postimmunization. As expected, substantial OT-II expansion occurred in tumor-free animals (Fig. 4D). Importantly, OT-II T cells expanded identically in the spleens of both E.G7 and B16.OVA tumor-bearing animals compared with tumor-free controls, and infiltrated the tumor site, showing that OT-II T cells remain naive throughout tumor growth and are not tolerized (Fig. 4, D and E). This suggested that the reason for poor OT-II T cell responses was a lack of sufficient OVA presentation on MHC-II in the tumor DLN.

**T cell sensitivity does not govern differential responsiveness to TAg**

OT-I and OT-II T cells have different TCR affinities and therefore differing abilities to respond to low levels of MHC-peptide complex (33). It was therefore possible that the differential antitumor responses were simply due to low levels of MHC-I- and MHC-II-presented OVA protein. We therefore compared the ability of OT-I and OT-II T cells to respond to different doses of OVA protein injected into various solid tissues and s.c. sites in naive mice or intratumorally (i.t.) into B16.F10-bearing animals. We found that in naive hosts, OT-II T cells divided to a similar or greater extent than OT-I T cells to low doses of footpad, i.m., or s.c. injected OVA protein (Fig. 5, A–C), demonstrating efficient presentation of soluble proteins on MHC class II for all routes of administration (34). In contrast, the OT-II responses were dramatically decreased at low Ag concentrations when OVA was injected directly i.t. compared with the same amount in naive hosts, whereas the OT-I responses remained largely unaltered (Fig. 5, A–C). Interestingly, the inhibition of OT-II responses in tumor-bearing animals was restricted only to the local tumor site (B16-local) and was not observed in the contralateral side, distal s.c. injection (Fig. 5D). This evidence suggests that the differing responses of OT-I and OT-II T cells are not due to intrinsic cell differences, but rather due to specific inhibition of MHC-II presentation of TAg in the DLN by the local tumor microenvironment.

**Polyclonal CD4 and CD8 T cell responses recapitulate transgenic T cell responses**

Adoptive transfer allows in vivo detection of early responses by CD8 and CD4 T cell before extensive clonal expansion. However, transfer of high numbers of T cells introduces the variable of intraclonal competition, which can change the kinetics, activation requirements, and outcome of responses to highly immunogenic Ags (23, 35–37). In the case of the antitumor responses, we do not observe large expansion and activation of CD8 T cells at any time point during tumor growth, indicating that decreasing the transfer cell numbers would only decrease the ability to detect sufficient cell numbers for statistical analysis. Nevertheless, we examined the endogenous polyclonal T cell populations for evidence of preferential CD8 T cell activation that would indicate that the observed differential OT-I and OT-II T cell responses are not unique to the transgenic T cells. We first separated the CD4 lymphocytes into T regulatory (Treg) and potential TAg-specific Th (non-Treg) cells by means of intracellular Foxp3 staining (38), and compared the total numbers of endogenous non-Treg CD4 T cells and CD8 T cells in the spleens and tumors of naive or tumor-bearing animals. To minimize variability, we expressed the total cell numbers as a

![FIGURE 6. Endogenous T cell antitumor responses. Naive or day 14 B16.OVA-bearing animal spleens and tumor tissues were harvested and analyzed for the ratio of total numbers of endogenous CD4+Foxp3− to CD8+ T cells (A). Tumor-free or B16.F10-bearing animals were perfused, and LN, spleen, liver, lungs, bone marrow, and tumor tissues were isolated and examined for the ratio of infiltrating CD4 and CD8 T cells (B). CD69 staining was used to calculate the fold increase in CD69 expression of tumor-infiltrating compared with spleen-derived naive CD4+Foxp3− and CD8+ T cells (C). CD4+ T cells from tumors and spleens of naive and tumor-bearing (Tum) animals were analyzed for surface expression of CD69 and intracellular expression of Foxp3 (D). Numbers represent percentage of total cells in each quadrant. Results are represented as mean ± SD. Data are representative of at least three independent experiments.](http://www.jimmunol.org/)
ratio of non-Treg CD4 T cells to CD8 T cells. We found no difference in the relative numbers of these cells in spleens of tumor-bearing animals when compared with naive animals, with the ratio being ~1.5, indicating that non-Treg CD4 T cells numerically dominate in this tissue. However, at the tumor site, the ratio dropped to ~0.5, i.e., twice as many CD8 T cells compared with non-Treg CD4 T cells were present in the tumor (Fig. 6A). In addition, CD4 T cells clearly numerically dominated over CD8 T cells in other lymphoid and nonlymphoid peripheral tissues, such as lymph nodes, liver, lungs, and bone marrow, indicating that the specific decrease in CD4 T cell tumor infiltration is not an artifact of solid tissue infiltration (Fig. 6B). Interestingly, the ratio of Treg CD4 T cells to CD8 T cells did not dramatically change between tumor-free and tumor-bearing animals in spleen or tumor sites, indicating that only the non-Treg CD4 T cell population is inhibited from responding to the tumor (data not shown). Additionally, examination of surface CD69 expression revealed that a much higher percentage of CD8 T cells up-regulated CD69 expression than non-Treg CD4 T cells when normalized to basal CD69 expression in naive spleens (Fig. 6C). Because CD4 T cells require MHC-II to sense presented Ag, it would not be surprising that CD4 T cells would see less Ag at the tumor, where the majority of cells are MHC-II-negative tumor cells. However, in contrast to the non-Treg CD4 T cells, the Foxp3+ Treg population was predominantly CD69+ at the tumor site, arguing that enough MHC-II-peptide complexes exist at the tumor site for up-regulation of CD69 expression (Fig. 6D). These results indicate that the endogenous polyclonal T cells behave similarly to the transgenic T cells, with the CD8 T cells responding to a much greater extent compared with non-Treg CD4 T cells.

Although DC do not present TAg to CD4 T cells, DLN CD8α+ DC efficiently cross-present TAg to CD8 T cells

To investigate the presentation capacity of DLN DC to CD4 and CD8 T cells, we isolated CD11c+ and CD11c− cells from B16.OVA DLN and incubated them ex vivo with CFSE-labeled OT-I and OT-II T cells for 4 days. Only the CD11c+ fraction elicited proliferative responses in OT-I T cells. However, neither fraction was capable of eliciting detectable OT-II proliferation. Both subsets were capable of eliciting OT-I and OT-II responses when OVA257–264 and OVA257–339 peptides were added to the culture, indicating that there was no intrinsic T cell defect and that sufficient MHC class II was present to elicit proliferation (Fig. 7A). DC that migrate from the inflamed tissue and resident LN DC have different capacities to prime CD8 and CD4 T cells (18, 19). Although CD8α+ DC have been implicated to be the principal DC subset responsible for efficient cross-presentation of exogenous soluble proteins on MHC-I, the migratory interstitial DC (CD11b+CD8α−) are thought to be responsible for sustained presentation to CD4 T cells in skin DLN (39, 40). We therefore separated CD11c+ cells of B16.OVA DLNs into CD8α+ and CD8α− fractions and performed the in vitro proliferation assay. We found that whereas the CD8α− DC subset caused robust OT-I proliferation, neither DC subset was capable of causing proliferation of OT-II T cells unless OVA protein was added to the culture medium (Fig. 7B). Because it was possible that tumor-infiltrating DC (TIDC) may be able to present Ag on MHC-II, but are unable to migrate to DLN, or that they process Ag too quickly for presentation to CD4 T cells, we examined whether TIDC were capable of MHC-II OVA presentation directly ex vivo. We found that TIDC were unable to cause OT-II proliferation unless additional OVA protein was added to the culture medium (Fig. 7C).

Because cellular associated proteins have been shown to be specifically targeted for cross-presentation, it was possible that normally secreted OVA was taken up in association with dying tumor cells, or that DC were cross-dressing with MHC class I-peptide complexes directly from dying tumor cell membranes (33, 41, 42). These Ag acquisition pathways could then lead to the observed phenotype of CD8, but not CD4 T cell priming. However, injection of up to 1 × 107 freeze/thaw-killed or irradiated dying tumor cells failed to elicit responses in either OT-I or OT-II T cells, indicating that cell-associated OVA protein and DC cross-dressing do not
substantially contribute to CD8 T cell priming (Fig. 7D, and data not shown). Even though enough Ag was available to stimulate OT-I responses, as seen in vivo and in vitro, DLN CD8α+ DC were predominantly phenotypically immature, as assessed by MHC-II expression, CD86, CD80, CD40 levels, and IL-12 production (Fig. 8), which could explain why the in vivo response is tolerogenic and does not control tumor progression. Together these results indicate that whereas the immature CD8α+ DLN DC competently cross-present TAg on MHC-I, neither TIDC, migratory interstitial DC, nor resident CD8α+ DC are able to efficiently present the same Ag on MHC-II in tumor-bearing animals.

Inhibition of MHC-II presentation in migratory DC in tumor DLN
To directly visualize differences in MHC-II presentation in naive and tumor-bearing hosts, we used the EAgFP fluorescent protein system that allows detection of cells with internalized soluble protein, and where the MHC-II presentation can be assessed using the YAe Ab staining to detect I-Ab-E52–68 peptide complex (39, 43).

We injected EAgFP either s.c. into naive or i.t. into B16.F10-bearing animals and examined DC complex formation in DLN 24 h later by isolating CD11c+ DC and staining with the YAe Ab. Naive DLN had a population of migratory DC (CD11c+, CD11b+, CD8α+, CD4+), but not resident DC (data not shown), which had high levels of internalized fluorescent protein and stained brightly by the YAe Ab, indicating that normal Ag processing for MHC-II presentation took place (39). In contrast, there was a significant reduction in GFP+ migratory DC from naive or tumor-bearing DLN for MFI of I-Aβ (C). Results are represented as mean ± SD. Data are representative of two independent experiments.
protein when compared with GFP+ cells from naive animals (Fig. 9, A and B). Interestingly, GFP+ cells in both naive and tumor-bearing DLN displayed similarly high levels of MHC-II, which were directly comparable to mature and not immature (based on MHC-II staining) CD11b+ DC (Fig. 9C). These experiments show that the migratory DC phenotype appears to be different from the resident CD8α+ DC in tumor DLN (compare Figs. 8A and 9C). This potentially suggests that whereas the migratory DC Ag delivery and MHC-II presentation machinery, but not the overall surface MHC-II levels, are directly inhibited in tumor-bearing animals, the immature CD8α+ DC MHC-I presentation machinery remains largely intact to cause CD8 T cell tolerance.

Discussion
Evasion of the immune system is currently regarded as the seventh hallmark of cancer progression, with some of the more recently discovered strategies affecting DC differentiation and maturation (44, 45). Considering that the steady-state tumor microenvironment does not contain strong stimuli for DC maturation, it becomes heavily dependent on CD4 T cell help, ablation of which would lead to tolerant antitumor CD8 T cell responses (44, 45). It is therefore not surprising that the progressing tumor would evolve mechanisms to inhibit this last safeguard against CD8 T cell tolerance.

By examining TAg-specific naive CD4 and CD8 T cell responses to two tumor cell lines, we found that the CD8 T cells rapidly responded to a s.c. challenge by recognizing cross-presented cytoplasmic and secreted TAg, but were tolerant in nature and did not alter tumor growth. We have previously shown that this is in part due to low levels of signal three cytokines available for CD8 T cells during priming in DLN (22). In contrast to the CD8 responses, OT-II TAg-specific CD4 T cells remained completely ignorant and naive throughout tumor progression. Even though OT-II CD4 T cells have been reported to have poor responsiveness to low levels of MHC-II-peptide complexes (33), we found that they responded equivalently or better than OT-I CD8 T cells to immunization with soluble OVA into various solid and s.c. sites, but their response was dramatically decreased to i.t. OVA administration. Interestingly, OT-II responses remained intact in tumor-bearing animals if OVA administration was administered at a distal from the tumor site. These results indicated that T cell response differences were due to a localized tumor-specific mechanism for selective inhibition of MHC-II-restricted TAg presentation, and not due to T cell intrinsic differences, a mutation of tumor OVA protein, or DC cross-dressing themselves with tumor membrane-derived MHC-I complexes (42).

Examination of polyclonal endogenous T cells indicated that they respond similarly to transgenic T cells, with CD8 T cells infiltrating the tumor site in greater magnitude and specificity than CD4 T cells. We additionally found that Treg CD4 T cells are not primed by this subset due to limiting TAg levels and overall low MHC-II levels (18, 19). By using soluble EoGFp protein, we were able to directly visualize MHC-II presentation inhibition in tumor DLN in comparison with tumor-free controls. We found both a decreased frequency of migratory DC carrying the fluorescent protein, as well as decreased levels of intracellular protein and surface MHC-II-peptide complex on DC in tumor DLN. Interestingly, the migratory DC displayed functionally high levels of total MHC-II, potentially displaying self Ags and evoking Treg responses, as witnessed at the tumor site. Put together, selective inhibition of the migratory DC MHC-II presentation machinery coupled with CD8α+ DC-limited MHC-II presentation capacity could lead to a blockade in antitumor CD4 T cell responses. However, it is also possible that MHC-II presentation machinery in both migratory and resident LN DC is affected by the tumor microenvironment.

Although the majority of studies agree that adoptively transferred CD8 T cells undergo tolerance and do not control tumor growth, there are some examples in the literature of naive CD8 T cells controlling tumor progression without immunization. Recently, Boissonnas et al. (47) have shown that 1 × 107 transferred naive OT-I CD8 T cells undergo efficient activation and can control E.G7 tumor growth. However, such a high transfer number has been shown to cause a CD4 T cell help-independent activation of CD8 T cells in both nontumor and tumor models (23, 36). Additionally, several reports show that naive CD4 T cells respond to a cognate tumor challenge and help CD8 T cell responses without immunization (23, 48, 49). However, these same studies also show clear dependence of the effective antitumor responses on the high transfer number of CD4 and CD8 T cells. In our hands, adoptive transfer of 1 × 106 transgenic T cells does not result in CD8 T cell clonal expansion and control of tumor growth, indicating that the transfer numbers are insufficient for effective CD8 T cell activation to occur independently of CD4 T cell help. This is additionally supported by the fact that the low precursor frequency tumor-specific endogenous T cells appear to mirror the transgenic T cell response.

In addition, certain leukemia and prostate tumor models show relatively robust tolerogenic CD4 T cell activation to TAg (50, 51). One difference between the systems is that CD4 T cell responses described in our model represent responses to tumor-specific and not tumor-associated Ags, as described in the prostate model (50). More importantly, it is likely that in liquid tumors, Ag levels in the blood and lymph are higher than if they were derived from a solid tumor, thereby having greater access to resident spleen and lymph node DC. In agreement with this, transgenic and endogenous CD4 T cells undergo a tolerogenic response to the E.G7 thymoma when it is injected i.p. and grows in ascites fluid (52). This tolerance can be reversed through CTLA-4 blockade, which allows for CD4 T cell help-dependent control of the tumor by CD8 T cells. This

LACK OF TAg PRESENTATION TO CD4 T CELLS

We found that the tumor DLN CD8α+ DC subset was responsible for cross-presenting TAg for CD8 T cell priming, with neither the CD8α+ nor the CD8α+ DC subset being able to cause CD4 T cell activation. Interestingly, CD8 T cell responses to both cytoplasmic and secreted tumor proteins were fundamentally identical, indicating that cross-presentation of different TAgs by DLN DC is a relatively efficient process. Moreover, we were able to characterize the CD8α+ DC subset in tumor DLN to be functionally immature, explaining the tolerogenic nature of the CD8 T cell response. Because MHC-II presentation by the CD8α+ DC subset is not as efficient as cross-presentation, it is possible that CD4 T cells are not primed by this subset due to limiting TAg levels and overall low MHC-II levels (18, 19). By using soluble EoGFp protein, we were able to directly visualize MHC-II presentation inhibition in tumor DLN in comparison with tumor-free controls. We found both a decreased frequency of migratory DC carrying the fluorescent protein, as well as decreased levels of intracellular protein and surface MHC-II-peptide complex on DC in tumor DLN. Interestingly, the migratory DC displayed functionally high levels of total MHC-II, potentially displaying self Ags and evoking Treg responses, as witnessed at the tumor site. Put together, selective inhibition of the migratory DC MHC-II presentation machinery coupled with CD8α+ DC-limited MHC-II presentation capacity could lead to a blockade in antitumor CD4 T cell responses. However, it is also possible that MHC-II presentation machinery in both migratory and resident LN DC is affected by the tumor microenvironment.
further supports the notion that provision of CD4 T cell help critically depends on CD4 T cell activation by APCs. It is also important to note that these particular studies did not examine both the CD8 and CD4 T cell response kinetics, which introduces the possibility that unsynchronized CD8 and CD4 T cell responses may occur (50, 51). Therefore, CD4 T cells could respond much later than CD8 T cells and not be able to provide help during priming, causing tolerance in CD8 T cells. Moreover, the majority of the aforementioned studies use either fairly immunogenic tumor cell lines, or poorly characterized spontaneous tumor models in which immunogenicity is difficult to control (23, 49, 51, 53). Thus, although clearly not universal to all tumor models, our experimental system describes the behavior of low T cell precursor frequencies in response to certain poorly immunogenic, solid tumors.

Overall, these results point to a novel mechanism of tumor immune evasion, in which the tumor microenvironment causes aberrant DC functionality by selectively restricting MHC-II presentation by activated interstitial DC that migrate from the tumor site to the draining lymph nodes, while leaving cross-presentation by immature, lymph node resident CD8er DC virtually intact. The decrease in the ability of DC to present TAg to CD4 T cells priming. However, it is possible that even if CD4 T cells were activated, CD4 T cell help would not be sufficient to rescue CD8 T cell tolerance induction, because more than one dominant inhibitory mechanism might be in place in tumor-bearing animals. Nevertheless, lack of MHC class II presentation on DC does represent the earliest block in the helper pathway, and hence, is at least in part responsible for tolerance induction in CD8 T cells.

Although the mechanisms of impairment of DC presentation capacity are currently unresolved, the tumor microenvironment is known to harbor a milieu of suppressive cytokines derived from tumor cells themselves, or produced by infiltrating Treg CD4 T cells, or by infiltrating tumor cells. It is also possible that even if CD4 T cells were activated, CD4 T cell help would not be sufficient to rescue CD8 T cell tolerance induction, because more than one dominant inhibitory mechanism might be in place in tumor-bearing animals. Nevertheless, lack of MHC class II presentation on DC does represent the earliest block in the helper pathway, and hence, is at least in part responsible for tolerance induction in CD8 T cells.

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Disclosures

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