Mechanism of T Cell Tolerance Induced by Myeloid-Derived Suppressor Cells

Srinivas Nagaraj, Adam G. Schrum, Hyun-Il Cho, Esteban Celis and Dmitry I. Gabrilovich

*J Immunol* 2010; 184:3106-3116; Prepublished online 8 February 2010;
doi: 10.4049/jimmunol.0902661
http://www.jimmunol.org/content/184/6/3106

---

**References**

This article *cites 38 articles*, 20 of which you can access for free at:
http://www.jimmunol.org/content/184/6/3106.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2010 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Mechanism of T Cell Tolerance Induced by Myeloid-Derived Suppressor Cells

Srinivas Nagaraj, Adam G. Schrum,‡ Hyun-II Cho,† Esteban Celis,‡ and Dmitry I. Gabrilovich‡

Ag-specific T cell tolerance plays a critical role in tumor escape. Recent studies implicated myeloid-derived suppressor cells (MDSCs) in the induction of CD8<sup>+</sup> T cell tolerance in tumor-bearing hosts. However, the mechanism of this phenomenon remained unclear. We have found that incubation of Ag-specific CD8<sup>+</sup> T cells, with peptide-loaded MDSCs, did not induce signaling downstream of TCR. However, it prevented subsequent signaling from peptide-loaded dendritic cells. Using double TCR transgenic CD8<sup>+</sup> T cells, we have demonstrated that MDSC induced tolerance to only the peptide, which was presented by MDSCs. T cell response to the peptide specific to the other TCR was not affected. Incubation of MDSCs with Ag-specific CD8<sup>+</sup> T cells caused nitration of the molecules on the cell surface. These data demonstrate profound nonresponsiveness to the specific peptide, whereas nitrotyrosine-negative CD8<sup>+</sup> T cells responded normally to that stimulation. MDSCs caused dissociation between TCR and CD3<sub>ε</sub> molecules, disrupting TCR complexes on T cells. Thus, these data describe a novel mechanism of Ag-specific CD8<sup>+</sup> T cell tolerance in cancer. The Journal of Immunology, 2010, 184: 3106-3116.

In recent years, Ag-specific T cell tolerance was identified as one of the major mechanisms of tumor escape to immune destruction (1-3). Ag-specific nature of tumor nonresponsiveness explains the fact that tumor-bearing hosts are not capable of maintaining tumor-specific immune responses while still responding to other immune stimuli (4-6). T cell tolerance in cancer has been shown to be mediated by host APCs (7, 8), although the nature of these APCs remained unclear. Recent studies have provided evidence that myeloid-derived suppressor cells (MDSCs) may represent the major population of APCs responsible for induction of Ag-specific CD8<sup>+</sup> T cell tolerance in cancer (reviewed in Ref. 9).

MDSCs are the group of myeloid cells comprised of hematopoietic progenitor cells and precursors of macrophages, dendritic cells (DCs), and granulocytes. These cells are part of normal hematopoiesis but dramatically expanded and activated in many types of cancer in mice and men. In mice, MDSCs share common phenotypic markers Gr-1<sup>+</sup>CD11b<sup>+</sup>, although in recent years, several other markers have been suggested to describe specific subsets of these cells (9). Most importantly, MDSCs also share a common trait: high potential to suppress T cell responses. The potential mechanisms by which MDSC can contribute to T cell nonresponsiveness in cancer are starting to emerge. There is now sufficient evidence demonstrating the ability of MDSC to induce Ag-specific CD8<sup>+</sup> T cell tolerance in cancer (10, 11). MDSCs can pick up Ags, migrate to peripheral lymphoid organs, and process and present these Ags to T cells (5, 12, 13). Arginase, NO, and reactive oxygen species were all implicated in MDSC-mediated T cell suppression (9). However, the molecular mechanism of CD8<sup>+</sup> T cell tolerance induced by MDSCs remained unclear. In the current study, we tried to address this question.

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 wk of age) were obtained from the National Cancer Institute, Bethesda, MD. OT-1 TCR-transgenic (Tg) mice [C57BL/6-Tg(TCR<sub>α</sub>) 1100mjb] were purchased from The Jackson Laboratory (Bar Harbor, ME). Double TCR-Tg mice were described elsewhere (14). To establish tumors, C57BL/6 mice were injected s.c. with 5 × 10<sup>6</sup> EL-4 or MC38 cells. A2L2 and 66.3 tumors were established by injecting s.c. 5 × 10<sup>5</sup> cells in BALB/c mice.

OVA-derived (H-2<sup>k</sup>, SIINFEKL), lymphocytic choriomeningitis virus (LCMV)-specific KAVYNFWTC, rNEUp66 (TYVPANASL), and control H-2<sup>k</sup> (RAHYNVITF) peptides were obtained from American Peptide Company (Sunnyvale, CA). IFA and LPS were purchased from Sigma-Aldrich (St. Louis, MO). All Abs used for flow cytometry were obtained from BD Pharmingen (San Diego, CA) except anti-nitrotyrosine (NT) (Millipore, Bedford, MA), and anti-pZAP-70 (eBioscience, San Diego, CA).

Cell isolation and generation

MDSCs were isolated from spleens of tumor-bearing mice using biotinylated anti-Gr-1 Ab and MiniMACS columns (Miltenyi Biotec, Auburn, CA). More than 95% of Gr-1<sup>+</sup> cells were also CD11b<sup>+</sup>. T lymphocytes were isolated from spleens using T cell enrichment columns (Miltenyi Biotec). Female C57BL/6 mice were injected s.c. with 5 × 10<sup>5</sup> 105 EL-4 or MC38 cells.

<ref>Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00</ref>
Adaptive cell transfer and immunization

A total of 4.5 × 10^6 of purified T cells from OT-1 TCR or double TCR-Tg mice were injected i.v. into naive C57BL/6 recipient mice. Two to three days later, these mice were injected i.v. with 4–5 × 10^6 MDSC and within an hour immunized s.c. with 100 μg specific peptides in IFA. Ten days later, cells from lymph node (LN) were restimulated with specific or control peptides and analyzed.

To produce Neu-specific T cells, BALB/c mice were primed with 100 μg plasmid pECl-17-neu, which is previously described (16), followed immediately by electroporation of the injected area (95 V, four pulses of 65 ms with repoling) using an Electrop Square Porator device (model TX830; BTX, San Diego, CA). Two weeks later, 100 μg synthetic peptide (NEU166 (TYYVANASL) was injected i.v. in combination with 50 μg polyethylene-polylysine (Hiltonol), which was kindly provided by Dr. Andres Salazar (Onco-

var, Washington, DC). Eight days from the last immunization, mice were euthanized and used for further experiments. Mice demonstrated >40% of rNeU166-specific CD8 T cell responses by tetramer staining analysis.

B3Z assay

B3Z is a lacZ-inducible CD8+ T cell hybridoma expressing TCR specific for OVA257-264 (SIINFEKL), presented on the murine H2Kb MHC class I molecule. LPS-matured DCs or MDSCs from H-2Kb mice were loaded with SIINFEKL or control peptide (10 μg/ml) for 90 min and then cultured with B3Z for 3 h at 37°C in a 5% CO2 atmosphere. After that, cells were washed, and β-galactosidase activity was detected in live cells by using fluorescein di-b-β-galactopyranoside and propidium iodide according to the manufacturer’s protocol (Invitrogen).

Functional assays

The number of IFN-γ-producing cells in response to stimulation to the specific or control peptides (10 μg/ml) was evaluated in an ELISPOT assay performed as described earlier (11). Each well contained 2 × 10^4 LN cells. The number of spots was counted in triplicate and calculated using an automatic ELISPOT counter (Cellular Technology, Shaker Heights, OH). Cell proliferation induced by Ag-specific or anti-CD3 (0.5 μg/ml) and anti-CD28 (5 μg/ml) Abs stimulation was evaluated using 3H thymidine incorporation as described previously.

Intracellular calcium concentration

A total of 5 × 10^6 OT-1 T cells were suspended in 2% FBS-RPMI 1640 and stained with 3 μg/ml Fluo-4 and 5 μg/ml fura-red for 30 min at 37°C. Later, cells were washed twice with RPMI 1640, and 1.5 × 10^6 cells were resuspended in 1 ml serum-free RPMI 1640 and warmed at 37°C before use. These cells were cocultured with LPS-activated DCs or MDSCs from H-2Kb mice loaded with SIINFEKL and control peptide (10 μg/ml) for 90 min and then cultured with B3Z for 3 h at 37°C in a 5% CO2 atmosphere. After that, cells were washed, and β-galactosidase activity was detected in live cells by using fluorescein di-b-β-galactopyranoside and propidium iodide according to the manufacturers protocol (Invitrogen).

Confocal microscopy

Splenocytes from double TCR (DT) mice were cultured with specific peptide in the presence of MDSCs (at a 3:1 ratio) on a poly-d-lysine–coated glass-bottom culture dish (MatTek, Ashland, MA). The plates were labeled with anti-αβ TCR APC, anti-Vβ 8.1 TCR Alexa 555, and anti-NT Alexa 488. Cells were viewed with a DMI6000 inverted Leica TCS AOB5 SP5 tandem-scanning confocal microscope with an X40/1.30 NA oil immersion objective (Leica Microsystems, Deerfield, IL). Tunable 488 Argon and 546 and 633 laser lines were applied to excite the samples using AOBS line scanning. The presence of Ag-specific CD8+ T cells, OT-1 T cells were labeled with CFSE prior to transfer into the recipients. Proliferation of Ag-specific CD8+ T cells was evaluated 3 d postimmunization. Ag-specific T cells in control mice actively proliferated (Fig. 1F). The proportion of pentamer-positive CD8+ T cells in MDSC-treated mice was substantially lower than in control mice. However, pentamer-positive CD8+ T cells demonstrated a similar level of proliferation to that

Measuring subunit ratios within the TCR/CD3 complex via immunoprecipitation of multiprotein complexes detected by flow cytometry

T cells were purified from 48 h cultures that had OT-1 splenocytes alone or splenocytes with OVA-pulsed MDSC, followed by analysis by immunoprecipitation of multiprotein complexes detected by flow cytometry (IPFCM) as described (17). Briefly, T cells were lysed in 1% Digiton iso-
tonic lysis buffer, and postnuclear lysates were incubated with IP mAbs specific for either CD3ζ (mAb H146) or Vβ5 (mAb MB9-4) to immunoprecipitate native TCR/CD3 complexes. Captured complexes were probed in parallel with PE-conjugated nonspecific hamster Ig or mAbs specific for CD3ζ (mAb 6B10), TCR-β (H57) or Vα2 (B20.1). Geometric mean fluorescence intensity (MFI) was used to estimate the relative subunit quantities in the captured complexes as described in the text and in previous work (17).

Flow cytometry

Flow cytometry data were acquired using FACScalibur flow cytometer and analyzed with FlowJo software version 8.8.6 (Tree Star, Ashland, OR). For intracellular staining, cells were labeled with anti-CD8 and anti-Vα2 Abs, fix, and permeabilized according to the manufacturer’s protocol (BD Pharmingen, San Diego, CA).

Statistical analysis

Most of the data were analyzed using the two-tailed Mann-Whitney U test. Where warranted, the two-tailed unpaired t test was used. All p values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad PRISM 5 software (GraphPad, La Jolla, CA).

Results

Signaling in CD8+ T cells exposed to MDSCs

To investigate the mechanism of MDSC-induced CD8+ T cell tolerance, we initially used the experimental system described previously (5, 11). T cells were isolated from Tg Tg-1 mice expressing TCR specific for OVA-derived peptide SIINFEKL (OT1) and transferred into naive C57BL/6 recipients. Two days later, MDSCs isolated from EL-4 tumor-bearing mice were injected, and mice were immunized with OT1 peptide in IFA (Fig. 1A). LN cells were collected 10 d after T cell transfer and restimulated with control or specific peptides. CD8+ T cell response was evaluated in IFN-γ ELISPOT assay. This experimental system allows for evaluation of the direct effect of MDSC on CD8+ T cells in tumor-free mice by avoiding confounding influence of the presence of tumor on T cell function. Previous studies have determined that MDSCs were undetectable in tumor-free mice 5 d after adoptive transfer (11, 18). Thus, restimulation of LN cells in this model was performed in the absence of MDSCs. In contrast to LN cells from control mice that demonstrated potent responses to the specific peptide, LN cells from mice that received MDSCs were not able to respond to the peptide (Fig. 1B). To assess the effect of MDSCs on the presence of Ag-specific CD8+ T cells, we used staining with SIINFEKL/Kb pentamers (Fig. 1C). In control mice, the proportion of pentamer-positive CD8+ T cells increased dramatically postimmunization, whereas in mice that received MDSCs, it was much lower and quickly returned to the level observed in non-immunized mice (Fig. 1D). Although MDSC administration did not affect the total number of CD8+ T cells, it reduced the total number of pentamer-positive CD8+ T cells (Fig. 1E). To investi- gate the mechanism of the decreased accumulation of Ag-specific CD8+ T cells, OT-1 T cells were labeled with CFSE prior to transfer into the recipients. Proliferation of Ag-specific CD8+ T cells was evaluated 3 d postimmunization. Ag-specific T cells in control mice actively proliferated (Fig. 1F). The proportion of pentamer-positive CD8+ T cells in MDSC-treated mice was substantially lower than in control mice. However, pentamer-positive CD8+ T cells demonstrated a similar level of proliferation to that
in control mice. This may suggest that decreased accumulation of Ag-specific CD8+ T cells could be caused by decreased ability of these cells to bind Ag and expand during initial phase of antigenic stimulation rather than nonspecific inhibition of T cell proliferation (Fig. 1F).

Next we asked whether MDSC affected TCR signaling in CD8+ T cells. We used H2Kb T cell hybridoma (B3Z) that has been engineered to express OT-1–specific TCR linked to β-galactosidase (19). MDSCs and DCs (derived from bone marrow and used as positive control) were loaded with OT1 peptide and then cultured with B3Z cells. DCs caused dramatic activation of TCR-mediated signaling, whereas MDSCs failed to stimulate the B3Z cells (Fig. 2A, 2B). When MDSCs were incubated with T cells together with DCs, it resulted in significant reduction in the response (Fig. 2B). Similar results were obtained during the analysis of calcium mobilization in OT-1 T cells (Fig. 2C). However, calcium ionophore ionomycin caused substantial Ca2+ mobilization in T cells even in the presence of MDSCs (Fig. 2D).

To specifically evaluate potential effect of MDSCs on TCR signaling in CD8+ T cells, peptide-loaded DCs and MDSCs were cultured with OT-1 T cells, and several tyrosine kinases and adaptor molecules associated with TCR signaling were evaluated.
by flow cytometry. DCs induced a substantial increase in the levels of pCD3ζ, p56lck, pZAP-70, and pERK1/2 (Fig. 2E). In contrast, MDSCs did not upregulate any of these molecules (Fig. 2E). The addition of MDSCs to DCs did not affect the activation of T cells as was evaluated by the level of pZAP70 (Fig. 2F, top panel). However, if DCs and MDSCs were cultured together with T cells for 3 h, this resulted in a substantial reduction in the levels of pERK1/2 and pZAP-70 (Fig. 2F, bottom panel).

**MDSCs induce dissociation of TCR complex**

One of the important mechanisms regulating T cell responses to peptide/MHC class I complexes is the integrity of the TCR complex. To investigate the effect of MDSCs on the integrity of the TCR complex, splenocytes from OT-1 mice were cultured for 48 h with specific peptide in the presence or absence of MDSCs. MDSCs did not cause a substantial decrease in the expression of TCRVβ, Vβ5, or Vα2 on the surface of T cells (Fig. 3A). We studied the...
subunit composition of the TCR/CD3 complex using a recently developed method of IP-FCM (17), which allows subunit interaction analysis in primary T cells that are only available in limited quantities. First, we found no evidence of MDSC-mediated selective loss of CD3ξ-chain in T cells, because similar quantities of CD3ξ could be immunoprecipitated and detected regardless of the presence or absence of MDSC (Fig. 3B). However, TCRβ:CD3ξ complexes from T cells cultured with MDSC displayed only 50–74% of the levels found in complexes isolated from T cells that were cultured alone (Fig. 3B, 3C). This decrease in subunit interaction was detected to a similar extent regardless of which subunit was immunoprecipitated and which was probed. Similarly, the TCRα:CD3ξ interaction from MDSC-cocultured T cells was 29% of the level measured in complexes from T cells cultured without MDSCs (Fig. 3B, 3C). Thus, incubation of T cells with MDSC induced a decrease in CD3ξ association with both TCR α and β subunits, although the total quantity of all three subunits was unaffected.

To assess physical interaction between CD8 and TCR, we used FRET assay, which allows for a measurement of distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. Splenocytes from OT-1 mice were cultured with MDSC in the presence of specific peptide for 48 h. After that time, cells were stained with anti-CD8-PE or anti-CD3-PE Abs (electron donor) and anti-TCR-Vα2-APC (electron acceptor). Cells were analyzed by confocal microscopy for FRET-SE as described in Materials and Methods. FRET-SE was calculated in 10–16 regions with high intensity of fluorescence on different T cells, and mean ± SDs are shown.
significantly reduced the level of FRET between CD8 and TCR as well as between CD3 and TCR (Fig. 3D), which was indicative of reduced physical interaction between these molecules.

**CD8+ T cell tolerance is specific to only the epitope presented by the MDSC**

To further investigate the mechanism of MDSC-mediated CD8+ T cell tolerance, we used C57BL/6 DT-Tg CD8+ T cells that co-express the TCR for OT1 and the TCR for the LCMV gp33-derived epitope KAVYNFATC (gp33) on the same cell (14). MDSCs were isolated from EL-4 tumor-bearing mice and cultured with splenocytes from DT mice in the presence of OT1 peptide. In control, splenocytes were cultured with OT1 peptide without MDSC. After 48 h incubation, T cells were purified and mixed with splenocytes from naive C57BL/6 mice and stimulated with control, OT1, or gp33 peptides. The number of IFN-γ–producing cells was evaluated in ELISPOT assay. Consistent with previous observations (5), MDSCs induced CD8+ T cell responses between these molecules. After the initial culture with MDSCs, was not affected (Fig. 4A). However, the response of CD8+ T cells to gp33 peptide, which was not present during the initial culture with MDSCs, was not affected (Fig. 4A).

We then reversed the experiment and cultured MDSC and splenocytes from DT Tg mice with gp33-derived peptide followed by T cell isolation and stimulation with three peptides described above. Under these experimental conditions, the CD8+ T cells retained their response to OT1 peptide but showed an inhibited response to the gp33 peptide (Fig. 4B).

To test these findings in vivo, T cells were isolated from DT mice and transferred into naive recipients. Two days later, MDSCs isolated from tumor-bearing mice were injected, and mice were immunized with either OT1 or gp33 peptide in IFA. Mice were boosted with peptides 6 d later, and the response of LN cells to stimulation with these peptides was evaluated in ELISPOT assay 7 d after second immunization. Because MDSCs were undetectable in spleens or LN 5 d after adoptive transfer into tumor-free recipients, second immunization was performed in the absence of these cells. Control mice immunized with OT1 peptide demonstrated a potent response to this peptide and a weak response to gp33 peptide. Control mice immunized with gp33 peptide showed potent responses to gp33 peptide and lower response to OT1 peptide (Fig. 4C). MDSC transfer prior to first immunization with OT1 abrogated CD8+ T cell response to the specific (OT1) peptide. In striking contrast, CD8+ DT T cells from the mice that were immunized the first time with OT1 peptide in the presence of MDSCs and then reimmunized with gp33 peptide (after the MDSCs were gone) demonstrated a potent response to gp33 peptide, whereas the response to OT1 peptide remained suppressed (Fig. 4C). Taken together, these data indicate that MDSCs induce CD8+ T cell tolerance involving only the TCR specific to the peptide presented by MDSCs. The ability of the same cells to respond to stimulation with the peptide that was not presented by MDSCs was not affected.

To evaluate the effect of MDSCs on TCR signaling in DT CD8+ T cells, splenocytes from these mice were cultured for 48 h with or without MDSCs in the presence of OT1 peptide. After that time, splenocytes were washed, rested in serum-free medium for 90 min, and then incubated with DCs loaded with SIINFEKL or gp33 peptides. The levels of pZAP-70 and p56lck were evaluated in the CD8+ T cells by intracellular staining using flow cytometry. Pre-incubation of T cells with MDSCs dramatically reduced the ability of CD8+ T cells to upregulate pZAP-70 and p56lck in response to DCs loaded with OT1 peptide. In contrast, response of T cells to gp33 peptide that was not present in culture with MDSCs was not affected (Fig. 4D).

We then tested the effect of tumor-associated Ags on DT T cells. Mice were implanted with EG7 tumor (EL-4 tumor cells expressing OVA) or EL-4 tumors as control. Fifteen days later, when tumors reached 1 cm in diameter, mice were injected i.v. with 5 × 10^6 DT T cells. Seven days postinjection, LN cells were isolated, stimulated with OT1 or gp33 peptides, and response was evaluated in proliferation assay. LN cells from EL-4 tumor-bearing mice showed potent response to both OT1 and gp33 peptide (Fig. 4E). LN cells from EG7 tumor-bearing mice demonstrated similar response to gp33 peptide. In contrast, the response to OT1 peptide was significantly inhibited (Fig. 4E). These data were consistent with the results obtained from experiments with MDSCs and demonstrated that in tumor-bearing mice, immune suppression in peripheral lymphoid organs was specific for the Ag presented on tumor cells.

We investigated whether Ag-specific CD8+ T cell tolerance can be observed in another model of tumor-bearing mice without use of adoptive transfer of MDSC and Tg T cells. A2L2 cells were made from parental mammary carcinoma 66.3 cells by transfection of a rat NEU-expressing vector (20). When tumors reached 1 cm in diameter (~2 wk after tumor inoculation), the mice were injected i.v. with 4 × 10^7 T cells isolated from BALB/c mice that were immunized with a synthetic peptide representing an H2-Kd–restricted rat NEU CD8 T cell epitope (21). Tetramer analysis of the spleen cells from the vaccinated mice demonstrated that ~40% of CD8+ T cells expressed TCR specific for rat NEU-derived peptide (Fig. 5A). Seven days after the adoptive transfer of the CD8+ T cells into the tumor-bearing mice, the LN cells were isolated and stimulated in vitro with the relevant rat NEU-derived or control peptides. CD8+ T cells responses were evaluated using IFN-γ ELISPOT and proliferation assays. In the absence of T cell transfer, LN cells from neither A2L2 nor 66.3 tumor-bearing mice responded to stimulation with the specific peptide. Ag-specific T cells retained significant peptide-specific response after adoptive transfer to 66.3 (rat NEU-negative) tumor-bearing mice, whereas no response was detected in T cells after transfer to A2L2 (rat NEU-positive) tumor-bearing mice (Fig. 5B, 5C). At the same time, T cells from 66.3 and A2L2 tumor-bearing mice showed a similar level of response to stimulation with CD3/CD28 Abs (Fig. 5D), indicating that the observed differences in Ag-specific responses between these tumor-bearing mice were not due to the differences in the global T cell suppression.

**Mechanism of T cell tolerance induced by MDSCs**

How can MDSCs induce tolerance in DT CD8+ T cells to only one TCR? We have previously demonstrated that nitration of TCR complex by MDSCs could be one of the major factors of CD8+ T cell tolerance (5). We asked whether this mechanism can explain the specificity of T cell tolerance. To study this mechanism in DT mice, we used three-color confocal microscopy. The OT1-specific Vα2 TCR was stained with APC-conjugated Ab (magenta), gp33-specific Vβ 8.1 TCR with Ab conjugated with Alexa 555 (red), and NT with Ab conjugated with Alexa 488 (green) (Fig. 6A). Stimulation of T cells with DCs loaded with OT1 peptide resulted in polarization of Vα2 TCR (arrows), whereas Vβ8.1 TCR remained largely intact. Under these conditions, no positive NT staining was evident (Fig. 6B). On the other hand, incubation of DT T cells with MDSCs and OT1 peptide resulted in preferential polarization of Vα2 TCR with positive NT staining observed only at the site of polarization (Fig. 6C). To investigate whether similar effect could be observed with MDSCs that process tumor-associated Ag, MDSCs were isolated from EG7 tumor-bearing mice and incubated for 48 h with DT T cells. Cells were labeled with anti-NT and either anti-Vα2 Abs that recognize TCR specific for OVA-derived epitope that is present in EG-7
tumor cells or with anti-Vβ 8.1 Abs that recognize TCR specific for LCMV-derived peptide not present in tumor. After 48 h, incubation T cells were purified and mixed at 1:5 ratio with splenocytes from naive C57BL/6 mice and stimulated with either control, OT1, or gp33 peptides. The number of IFN-γ-producing cells was evaluated in quadruplicate in an ELISPOT assay. Each experiment was performed three times. *Statistically significant (p < 0.05) differences between control CD8+ T cells and T cells incubated with MDSCs. C, T cells were isolated from DT mice and were transferred into naive C57BL/6 recipients. MDSCs isolated from tumor-bearing mice were injected 2 d later, and mice were immunized once with OT1 peptide at day 2 and a second time 6 d later with 100 μg OT1 or gp33 peptide in IFA. Control mice received no MDSCs and were immunized on day 2 and 6 d later with OT1 or gp33 peptide. Seven days after the second immunization, response of LN cells to restimulation with control (CP), OT-1, or gp33 peptides was evaluated in an ELISPOT assay. Each group included three mice. *Statistically significant (p < 0.05) differences between cells stimulated with control and specific peptides. D, DT cells were cultured with or without MDSCs from EL-4 tumor-bearing in the presence of the 10 μg/ml OT1 peptide. After 48 h, splenocytes were washed, rested in serum-free medium for 90 min, and then incubated with DCs loaded with OT1 or gp33 peptides. Cells were labeled with anti-CD8, anti–pZAP-70, and p56lk and their expression evaluated using flow cytometry in triplicate. Mean ± SD are shown. *Statistically significant (p < 0.05) differences between control T cells and T cells incubated with MDSC. E, Mice were implanted s.c. with 2×10^5 EG7 or EL-4 tumor. Fifteen days later when tumors reached 1 cm in diameter, mice were injected i.v. with 5×10^6 DT T cells. Seven days postinjection, LN cells were isolated stimulated with control, OT1, or gp33 peptides, and response was evaluated in [3H]thymidine assay. Mean ± SD are shown. *Statistically significant difference between cells stimulated with OT1 and gp33 peptides.

FIGURE 4. MDSCs induce tolerance to only one specific TCR in T cell-expressing dual TCR. A and B, Splenocytes were isolated from DT mice and cultured with MDSCs isolated from EL-4 tumor-bearing mice at 1:4 ratio in the presence of 10 μg/ml OT1 (A) or gp33 peptides (B). As control, splenocytes were cultured with specific peptide without MDSCs. After 48 h, incubation T cells were purified and mixed at 1:5 ratio with splenocytes from naive C57BL/6 mice and stimulated with either control, OT1, or gp33 peptides. The number of IFN-γ-producing cells was evaluated in quadruplicate in an ELISPOT assay. Each experiment was performed three times. *Statistically significant (p < 0.05) differences between control CD8+ T cells and T cells incubated with MDSCs. C, T cells were isolated from DT mice and were transferred into naive C57BL/6 recipients. MDSCs isolated from tumor-bearing mice were injected 2 d later, and mice were immunized once with OT1 peptide at day 2 and a second time 6 d later with 100 μg OT1 or gp33 peptide in IFA. Control mice received no MDSCs and were immunized on day 2 and 6 d later with OT1 or gp33 peptide. Seven days after the second immunization, response of LN cells to restimulation with control (CP), OT-1, or gp33 peptides was evaluated in an ELISPOT assay. Each group included three mice. *Statistically significant (p < 0.05) differences between cells stimulated with control and specific peptides. D, DT cells were cultured with or without MDSCs from EL-4 tumor-bearing in the presence of the 10 μg/ml OT1 peptide. After 48 h, splenocytes were washed, rested in serum-free medium for 90 min, and then incubated with DCs loaded with OT1 or gp33 peptides. Cells were labeled with anti-CD8, anti–pZAP-70, and p56lk and their expression evaluated using flow cytometry in triplicate. Mean ± SD are shown. *Statistically significant (p < 0.05) differences between control T cells and T cells incubated with MDSC. E, Mice were implanted s.c. with 2×10^5 EG7 or EL-4 tumor. Fifteen days later when tumors reached 1 cm in diameter, mice were injected i.v. with 5×10^6 DT T cells. Seven days postinjection, LN cells were isolated stimulated with control, OT1, or gp33 peptides, and response was evaluated in [3H]thymidine assay. Mean ± SD are shown. *Statistically significant difference between cells stimulated with OT1 and gp33 peptides.
CD8\(^+\) T cell tolerance is specific for tumor-associated Ags. A, Ag-specific CD8\(^+\) T cells postimmunization with rNeu-derived peptide. BALB/c mice were primed with 100 \(\mu\)g plasmid pEC1–170\(\text{neu}\), followed immediately by electroporation of the injected area. Two weeks later, 100 \(\mu\)g synthetic peptide rNEUp66 was injected i.v. in combination with 50 \(\mu\)g anti-CD40 mAb and 50 \(\mu\)g polyinosinic-polycytidylic acid. Eight days after the last immunization, spleens were harvested and labeled with NEUp66-specific tetramer and anti-CD8 Ab and were evaluated by flow cytometry. CD8\(^+\) T cells were gated. B and C, Mice were injected with 2 \(\times\) 10\(^6\) 66.3 and A2L2 cells. Ten days later, mice were injected i.v. with 5 \(\times\) 10\(^6\) rNEU-specific T cells isolated from immunized mice. Seven days later, the LN cells were restimulated with rNEUp66 peptide (SP) or control peptide (CP). IFN-\(\gamma\) production was seen. In contrast, NT-positive CD8\(^+\) T cells had substantially higher response to gp33 peptide than to OT1 peptide. Response was evaluated in IFN-\(\gamma\) ELISPOT assay (B) and proliferation by \(^{3}\)H]thymidine uptake (C). LN cells were restimulated with specific (SP) or control (CP) peptides (B, C) or CD3/CD28 Abs (D). T cell proliferation was measured by \(^{3}\)H]thymidine uptake. Results presented as mean ± SD from four mice. *Statistically significant \((p < 0.05)\) differences between the groups.

Discussion

MDSCs were recently identified as a major factor involved in immune suppression associated with cancer, infectious diseases, sepsis, and trauma (9). However, the mechanism of this phenomenon remains poorly understood. The question is how CD8\(^+\) T cells exposed to MDSCs in vitro and in vivo can retain response to nonspecific stimuli (5, 11). Molecular events leading to activation of Ag-specific T cells are well described. TCR ligation of the peptide/MHC complex results in phosphorylation of ITAMs in CD3 polypeptides through the Src kinases Lck and Fyn. The phosphorylated ITAMs recruit ZAP-70, which leads to its phosphorylation. ZAP-70 phosphorylates linker for activation of T cells, which in turn, via several different pathways, induces Ca\(^{2+}\) influx and activation of Ras with subsequent trigger of an MAPK cascade that includes activating JNK, ERK, and p38 MAPKs (22, 23). This pathway could be negatively regulated via regulatory tyrosine kinases and phosphatases (24). One of the possible mechanisms of negative regulation of T cell activation was suggested by Germán’s group (25), which demonstrated that a constant feature of TCR occupancy by peptide/MHC regardless of their affinity to TCR is activation of Lck-dependent phosphorylation of inhibitory Src homology region 2 domain-containing phosphatase 1 (SHP-1). In addition, low-affinity TCR epitopes promote an association of phosphor–SHP-1 with the TCR and that binding causes the loss of phospho–ζ-chain, which may lead to its downregulation. High-affinity epitopes can overcome this feedback inhibition by activation of ERK that phosphorylates Lck and abrogates the binding of phospho–ζ-chain, thereby protecting the TCR from signaling in response to low-affinity peptide/MHC complexes to a low-affinity interaction (5). Therefore, we hypothesized that a similar mechanism of T cell tolerance could be employed during MDSC–T cell interaction.

Our experiments, however, argue that negative signaling is not responsible for MDSC-mediated CD8\(^+\) T cell tolerance. MDSCs loaded with specific peptide, although they did not trigger TCR signaling in CD8\(^+\) T cells, prevented signaling in response to activation of T cells with peptide-loaded DCs. However, MDSCs suppressed the T cell response only to the peptide that was presented by these cells, whereas these T cells retained potential...
FIGURE 6. MDSC causes nitration of the surface molecules on CD8+ T cells. A–E, DT T cells were isolated and labeled with OT-1–specific APC-conjugated anti-Vα2 TCR Ab (magenta), gp33-specific Alexa 555-conjugated anti-Vβ 8.1 TCR Ab (red), and Alexa 488-conjugated anti-NT Ab (green) (original magnification ×400). A, Typical staining of double Tg CD8+ T cells is shown (left: gp33 TCR; right: OT-1 TCR). B, Double Tg T cells were stimulated with DC loaded with OT-1 peptide. Arrow denotes polarization of Vα2 TCR. C, Double Tg T cells were incubated with MDSCs and OT-1 peptide. Arrows represent regions of positive NT staining and site of Vα2 TCR polarization. Scale bar, 5 μm. D, Double Tg T cells were incubated for 48 h with MDSCs isolated from EG-7 tumor-bearing mice and then stained with APC-conjugated anti-Vα2 TCR Ab (magenta) and Alexa 488-conjugated anti-NT Ab (green) or PE-Cy5–conjugated anti-Vβ 8.1 TCR Ab (red) and Alexa 488-conjugated anti-NT Ab (green). Scale bar 5 μm. E, Typical example of cell sorting gates of NT-positive CD8+ T cells. MDSCs from EL-4 tumor-bearing mice were cultured for 48 h with splenocytes from OT-1 Tg mice in the presence of the SIINFEKL (10 μg/ml). Forty-eight hours later, cells were stained with DAPI, anti-CD8 Alexa 647, and anti-NT Alexa 488 and sorted by flow cytometry. F, MDSCs from EL-4 tumor-bearing mice were cultured for 48 h with splenocytes from OT-1 Tg mice in the presence of the SIINFEKL (10 μg/ml). Forty-eight hours later, cells were sorted and added at 1:5 ratio to naive C57BL/6 splenocytes and cultured for 24, 48, or 72 h prior to stimulation for 3 d with specific OT-1 peptide (SP) in a [3H]thymidine incorporation assay (G). A total of 2 ng/ml IL-2 was added to the 48- and 72-h cultures. Each experiment was performed in triplicate and repeated twice. Mean ± SDs are shown. H, MDSCs from EL-4 tumor-bearing mice were cultured for 48 h with splenocytes from OT-1 Tg mice in the presence of the SIINFEKL (10 μg/ml). Forty-eight hours later, cells were stained with DAPI, anti-CD8, anti-CD8 APCs, and CD8+Vα2+ cells were sorted. In control, cells were stained with DAPI, anti-CD8, and isotype control IgG, and all CD8+ T cells were sorted. Cells were then cultured with naive splenocytes at 1:10 ratio with 2 ng/ml IL-2. These cells were rested for 24 or 48 h and then stimulated with control or specific (SIINFEKL) peptide and evaluated in triplicates in [3H]thymidine incorporation assay. I, Double Tg T cells were incubated with MDSC for 48 h in the presence of SIINFEKL peptide. CD8+ NT+ or NT- cells were sorted as described above, mixed with naive splenocytes and stimulated with either OT1 or gp33 peptides. The number of IFN-γ producing cells was evaluated in quadruplicates in ELISPOT assay.
response to the peptide specific for the other TCR. We previously have demonstrated that MDSCs caused tyrosine nitration of TCR and CD8 molecules on T cells by releasing peroxynitrite (5). Interaction of MDSCs and CD8+ T cells in the presence of specific peptide results in polarization of TCR and formation of synapse where, apparently, MDSCs caused nitration of the surface protein on T cells. The critical role of this process for T cell non-responsiveness was confirmed by the fact that only NT-positive CD8+ T cells failed to respond to subsequent stimulation with specific peptide. In contrast, polarization of TCR not specific for the peptide was much less pronounced. Therefore, their exposure to peroxynitrite released by MDSCs could be much lower than that of Ag-specific TCR. This may explain why CD8+ T cells retained the ability to respond to the peptide not presented by MDSCs. Nitration of surface molecules on T cells was not permanent and probably limited by the turnover of the molecules. In our experiments, 3 d culture in vitro in absence of MDSCs was sufficient to completely recover the response of CD8+ T cells to the specific peptide.

Conformational changes in the TCR-CD3 complex play a critical role in T cell signaling (26, 27). Our data suggest that MDSCs alter integrity of the TCR-CD3 complex in CD8+ T cells as well as physical interaction between CD8 and TCR. Dissociation between TCR and CD3ζ would invariably prevent TCR-mediated signaling. Moreover, these T cells would be unable to respond to Ag presented by APCs. Only when new TCR and CD3 molecules are synthesized could this effect be reversed. This defect is attributed only to TCR involved in the interaction with the peptide presented by MDSCs; therefore, other TCR-CD3 complexes on the same cell remained relatively intact and could provide signaling in response to stimulation with APCs loaded with appropriate peptide. This mechanism can explain the inability of the immune system to generate tumor-specific CD8+ T cells even in the environment rich in tumor-associated Ags. Instability of TCR-CD3 complexes caused by MDSCs represents a novel mechanism of T cell tolerance in cancer. However, it has been recently implicated in CD8+ T cell tolerance caused by persistence of self-Ag (28). Encounter with peripheral self-Ags rendered T cells tolerant to self, but these cells responded normally to the virus-specific TCR. Tolerant TCR complexes were structurally less stable than functional complexes and selectively exhibited proximal signaling defects (28). Thus, alterations in the stability of TCR complexes may represent a general mechanism of T cell tolerance observed in different pathological and physiological conditions. However, it may not be applicable to the inhibition of the effector cytotoxic T cells in tumor sites. A recent study has demonstrated that lack of lytic function of tumor-infiltrating lymphocytes was caused by tumor cells rather than MDSCs. CD3ζ phosphorylation in these T cells was preserved, but linker for activation of T cells and ZAP-70 were not activated. Those signaling defects were transient and involved SHP-1 (4). Thus, defects in TCR signaling in T cells may depend on whether T cells were tolerized in peripheral lymphoid organs or in tumor sites.

Our data present a possibility to resolve conflicting results concerning the extent to which tumors induce CD3ζ dysfunction as a mechanism of T cell inhibition. CD3ζ was proposed to be a major target of tumor-mediated T cell inhibition (29, 30). The central thesis has been that tumor cells and/or tumor-associated myeloid cells induce selective degradation of CD3ζ (31–33). Although many reports corroborate these observations, some notable studies have presented contradictory data. Two groups (34, 35) argued that CD3ζ degradation may not take place in situ, but can occur upon lysing T cells in the presence of contaminating myeloid cells. Others have argued that CD3ζ can undergo oxidative stress-dependent posttranslational modifications that, rather than cause its degradation, can alter the epitopes required for detection by common Abs (36). It was shown that CD3ζ downregulation is not induced by some tumors and that inhibition of T cell function can occur despite normal CD3ζ levels (37). Furthermore, inhibited TCR/CD3ζ signaling has been shown to occur early in T cell responses, although CD3ζ downregulation may only be detectable after many days of in vivo tumor growth and high tumor burden (37, 38). In our system, we demonstrate an MDSC-induced disruption of TCR:CD3ζ interactions, without accompanying alteration in CD3ζ protein levels. Furthermore, this effect was detectable in T cells 2 d post-MDSC exposure, placing it relatively early in the T cell response kinetic. We propose that by subjecting CD3ζ, TCR, and CD8 to nitrosylation and possibly other covalent modifications (36), MDSCs induce early TCR:CD3ζ dissociation, which inhibits Ag-specific T cell responsiveness. Furthermore, it is conceivable that such covalent modifications could ultimately lead to the degradation of separated CD3ζ, consistent with previously reported observations. According to this model, TCR:CD3ζ dissociation would occur early upon MDSC encounter; however, subsequent degradation of unincorporated CD3ζ might represent a consequence, rather than the cause of the crucial inhibitory step.

Acknowledgments

We thank Dr. Blankenstein for providing double Tg mice and Dr. Schonberger for the gift of B3Z cells that were originally made by Dr. Shastri.

Disclosures

The authors have no financial conflicts of interest.

References


