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Cross-Presentation by Osteoclasts Induces FoxP3 in CD8+ T Cells

Jennifer R. Kiesel, Zachary S. Buchwald, and Rajeev Aurora

Bone is remodeled throughout the life of an animal by the action of osteoclasts, which resorb bone, and osteoblasts, which form new bone. It has recently been recognized that T cells regulate osteoclasts by secreting a number of cytokines including type I and II IFNs and receptor activator of NF-κB ligand. In this study, we show that osteoclasts produce chemokines that recruit CD8+ T cells. Using transgenic OT-I mice, we found that in the presence of OVA, osteoclasts induced the secretion of IL-2, IL-6, and IFN-γ as well as the proliferation of CD8+ T cells. CD8+ T cells activated by osteoclasts expressed Foxp3, CTLA4, and receptor activator of NF-κB ligand. The Foxp3+ CD8+ T cells were anergic and suppressed dendritic cell priming of naive responder CD8+ T cells. These results provide two novel observations for osteoimmunology: first, we demonstrate that osteoclasts can cross-present Ags to CD8+ T cells. Second, these data show that osteoclasts are not only regulated by T cells, but they also can regulate T cells forming a feedback control loop. The induction of Foxp3 in T cells through a MHC class I-dependent manner provides a new mechanism to peripherally produce a regulatory T cell. These observations open a new avenue of investigation for the pathogenesis of autoimmune-mediated inflammatory bone diseases. The Journal of Immunology, 2009, 182: 5477–5487.
resorption functions of osteoclasts: they lyse the calcium phosphate mineral layer as well as the bone matrix which contains collagen and other proteins by secreting acid and proteases into a sealed lysosomal compartment. They then take up the breakdown products for further processing and transcytose them from the sealed lysosomal compartment to the cell surface. These functions may also be used for cross-presentation. Therefore, we used splenocytes from a transgenic mouse line OT-I, that primarily express CD8+ T cells specific for OVA, to test whether osteoclasts can process OVA and present to T cells. We assayed for presentation by measuring cytokine release and cell proliferation. To define the phenotype of the primed T cells, we also measured a number of cell markers induced by the interaction with osteoclasts.

Materials and Methods

Animals

Five- to 10-wk-old male C57BL/6 mice (Harlan) were used for generation of osteoclasts. OT-I/Rag1−/− mice were a gift from Prof. M. Buller (Saint Louis University School of Medicine, St. Louis, MO) or were purchased from Taconic Farms. Transgenic OT-II/Rag−/− mice were purchased from Taconic Farms. All animals were maintained in the Department of Comparative Medicine (Saint Louis University School of Medicine) for at least 1 wk before use. The mice were maintained in accordance with institutional and Public Health Service Guidelines.

Antibodies

Abs for fluorescence cytomtery staining were: FITC-conjugated anti-mouse MHC-I H-2Kb/H-2Dd (clone 34-1-2s; eBioscience), FITC-conjugated anti-mouse MHC class II I-Ak (clone AF6-120.1; eBioscience), R-PE- or PerCP-conjugated anti-mouse CD4+ (clone RM4-5; BD Pharmingen), FITC- or allophycocyanin-conjugated anti-mouse CD8a (clone 53-6.7; BD Pharmingen), Alexa Fluor 700-conjugated anti-mouse CD68 (clone HI101; Calbiochem), allophycocyanin-conjugated anti-CD44 (clone IM7; BD Pharmingen), FITC-conjugated anti-CD122 (clone SH4; BD Pharmingen), Pacific Blue-conjugated anti-mouse FoxP3 (clone FJK-16s), PE-conjugated anti-mouse RANKL (clone Ikk2/5; Santa Cruz Biotechnology), PE-Cy7-conjugated anti-mouse CD25 (clone PC61; BD Biosciences), PE-conjugated anti-mouse CTLA4 (clone UC10-4B9; eBioscience), allophycocyanin-conjugated anti-mouse CD80/B7.1 (clone 16-10A1; eBioscience), PE-conjugated anti-mouse CD86/B7.2 (clone P03.1; eBioscience), and PE-conjugated anti-mouse CD54/ICAM-1 (clone YN1/7.4; eBioscience).

Generation of osteoclasts

Osteoclast precursors were isolated as previously described (55, 56). Briefly, the mice were sacrificed by CO2 asphyxiation and the long bones were harvested. The caps of the bones were removed and bone marrow cells were flushed with 0.05% collagenase (Worthington) in 175 μl Hank’s balanced salt solution (HBSS) and incubated in 175 μl Hank’s balanced salt solution (HBSS) with 5 μl G418 (Sigma-Aldrich) at 37°C. Nonspecific binding was blocked with 5% normal goat serum (NGS) in PBS and incubated for 30 min at 4°C with fluorophore-conjugated Ab, washed, fixed with paraformaldehyde, and then analyzed using an LSR instrument with CellQuest (BD Biosciences) software. Analysis was performed using FlowJo software (version 8.73; Tree Star).

Chemotaxis assays

Chemotaxis assays were performed as previously described (57) using the 24-well Corsta Transwell System (5-μm polycarbonate membrane; Corning). Briefly, bone marrow cells were plated at 2 × 10^6 cells/well in 175 μl containing growth medium and treated with 20 ng/ml sRANKL and incubated for 24 h at 37°C/5.5% CO2. Five × 10^6 freshly harvested splenocytes (nonselected) were added in the top insert of the Transwell in 175 μl. Control wells had 100 ng/ml SDF-1α (PeproTech) in 350 μl αMEM growth medium in the bottom well. Cells in the top and bottom wells were collected using Versene (Life Technologies) after 3 h. To obtain cell counts by FACS, cells were stained with anti-CD3, anti-CD8, and anti-CD4 Abs.

T cell priming

Day 4 bone marrow-derived precursors cultured in 20 ng/ml M-CSF, day 4 osteoclasts cultured in 20 ng/ml M-CSF and 20 ng/ml RANKL, or APC were seeded at 5 × 10^6 cells/ml/well in the presence of OVA (A-5503; Sigma-Aldrich), OVA257-264 or control peptide (GenScript) at 10 μM in 48-well tissue culture-treated plates (Corning). The cell line DC2.4 (58) or freshly harvested splenic DC purified by anti-CD11c magnetic beads (Milenyi Biotec) were used as APC. After 14–16 h of incubation, medium was removed and (adherent) cells were washed with prewarmed medium. Freshly harvested splenic T cells purified by negative selection and labeled with CFSE per the manufacturer’s protocol (Invitrogen) were added. All CD8+ T cells were cocultured with osteoclasts or APC for 48 h and CD4+ T cells for 96 h before readout by FACS for proliferation.

Cytokine profiling by multiplexed ELISA

Cytokine quantitation (IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ) was performed using multiplexed ELISA (Millipore/Linco Research) kits as per the vendor’s protocol on a Luminex-100.

CTL assay

CTL activity was measured using the released lactate dehydrogenase (LDH) by the CyTox 96 assay per the manufacturer’s (Promega) protocol using E.G7 target cells. E.G7 cells (American Type Culture Collection) were grown in medium containing G418.
FIGURE 1. Functional assays of in vitro-generated osteoclasts. Osteoclasts (treated with M-CSF plus RANKL labeled as +RANKL) or precursor cells as controls (treated with M-CSF plus PBS labeled as −RANKL) were generated on petri dishes, collected on day 5, and replated on tissue culture-treated dishes. A, TRAP staining: cells stained for TRAP activity, which produces a dark purple color. The average number of TRAP-positive osteoclasts was 190 ± 30/cm² (n = 4) compared with no TRAP-positive cells in the controls. B, Bone resorption assay and calcitonin receptor expression, left panel, the in vitro-generated osteoclasts were capable of pit formation (arrows). Right panel, Osteoclasts were multinucleated (blue, DAPI) and stained for the calcitonin receptor (green, Alexa Fluor 594). The average numbers of calcitonin-positive cells were 88 ± 5% (n = 4) on day 4. No bone resorption activity or calcitonin receptor signals were observed in the control precursor cells. C, Cell surface markers: cell surface markers expressed on osteoclasts. Bone marrow cells from C57BL/6 mice were differentiated to osteoclast precursors with M-CSF and treated with RANKL for 4 days. Osteoclasts were labeled with Fluor-conjugated Abs for flow cytometry. For each characterization, ~100,000 cell counts were collected. The shaded histograms are from isotype controls and the unshaded histograms Ab-specific signals.

**Suppression assay**

DC were isolated from freshly harvested spleen by positive selection using anti-CD11c magnetic microbeads per the manufacturer’s directions (Miltenyi Biotec). Responder CFSE-labeled (5 × 10⁴) CD8⁺ T cells were incubated with 5 × 10⁴ DC pulsed with SINFKEK peptide per well in the presence of graded numbers of FoxP3⁺ CD8⁺ T cells. The number of proliferation cycles of the responders were obtained using the FlowJo software.

**Results**

**Differentiation and phenotype of the osteoclasts**

The differentiation of the osteoclasts from bone marrow-derived cells in cell culture using M-CSF and RANKL has been extensively characterized (6, 30, 56, 59–63). Treatment of bone marrow osteoclast precursors with RANKL produces multinucleated, tartrate-resistant acid phosphatase (TRAP), and calcitonin receptor-positive cells that are capable of resorption (Fig. 1, A and B).

Intact osteoclasts are difficult to harvest once differentiated because they adhere tightly to tissue culture plates. We found that osteoclasts differentiated on nontissue culture plates (e.g., bacterial petri dishes) were easily harvested by treatment with a chelating agent (buffered EDTA/Versene) and gentle scraping. Growing cells on petri dishes allows us to recover both the precursors and the osteoclasts suitable for functional assessment including flow cytometry and resorption assay. Osteoclasts generated on petri dishes were strongly TRAP positive (98. ± 1%) and had mostly binucleated cells; they became multinucleated upon plating on tissue culture plates within 16–20 h. The cells shown in all panels of Fig. 1 were differentiated on petri dishes and then plated on tissue culture dishes or on calcium apatite-coated discs. Such in vitro-differentiated osteoclasts had all of the functional attributes associated with bona fide osteoclasts: they were large, multinucleated, TRAP and calcitonin receptor positive, and capable of bone resorption.

To further phenotype osteoclasts, we used flow cytometry to characterize the cell surface markers. As shown in Fig. 1C, osteoclasts express MHC-I and costimulatory molecule CD80/B7.1 but not CD86/B7.2. Surface expression of MHC class II was not detected on osteoclasts. Finally, they are CD11clow and CD54/ICAM positive. Multicolor staining of the osteoclasts shows that all MHC-I-positive cells by FACS were also CD80⁺, CD11c⁺, CD86⁻, and CD54⁺, indicating that our cell preparation represents a single population. Although the bone marrow contains a complex mixture of cell types, our isolation and differentiation protocol yields osteoclasts that are 97–99% pure at day 4 or later, as assessed by TRAP, calcitonin receptor staining, and cell surface markers.

**Osteoclasts can recruit T cells**

We previously found using whole genome microarrays that differentiating osteoclasts induce mRNA for a number of chemokines (Cc13, Cc14, Cc15, Cxcl5, Cxcl10, and Cxcl11) (55). A number of these, Cc14, Cc15, and Cxcl11 in particular, are known to be chemoattractants for CD8⁺ T cells (64). We quantified the (protein) levels of these chemokines using multiplexed ELISA. The amount of the chemokines ranged from 500 pg/ml for CC15/RANTES to 76,000 pg/ml for CXC110/IP10 (Fig. 2A). We used the Transwell assay to test whether this complex mixture of chemokines produced by the osteoclasts could recruit T cells. The numbers of CD4⁺ and CD8⁺ T cells that migrated through the membrane were assessed by flow cytometry. The data are presented as the percentage of phenotyped input cells that migrated from top to
indicate that the chemokines produced by osteoclasts recruit CD8 T cells in 3 h (Fig. 2B). As a positive control CXCL12/SDF-1α, a chemoattractant for CD8+ cells (57), was used.

No (statistically) significant increase in CD4+ migration was observed when the bottom well contained SDF-1α, only a 1.5-fold increase in the migration of CD4+ cells (p = 0.0765, nonsignificant) was observed at 3 h with osteoclasts in the bottom well. A 2.5-fold increase in the migration of CD8+ T cells was observed in wells that had SDF-1α relative to medium alone (p = 0.0015). In wells that contained osteoclasts, a 3.5-fold increase in the migration of CD8+ cells (p < 0.0042) was observed. These results indicate that the chemokines produced by osteoclasts recruit CD8+ T cells.

Osteoclasts cross-present to CD8+ T cells

Based on our previous analysis of microarray data (55) and on the expression of the cell surface molecules (Fig. 1C), we hypothesized that osteoclasts have the ability to present Ags to CD8+ T cells. To test this hypothesis, we used OT-I/Rag−/− mice (66) as the source of CD8+ T cells. OT-I mice have transgenic TCR that is specific for residues 257–264 (SIINFEKL) of OVA bound to H-2Kb. CD8+ T cells were purified by negative selection using magnetic beads, CFSE labeled, and added to osteoclasts incubated with Ag (either OVA257–264 peptide or intact OVA) or without Ag. Activation of the T cells was assessed by cytokine quantitation and T cell proliferation (Fig. 3, A and B). Wells containing osteoclasts and OT-I splenocytes were observed to release IL-6, IFN-γ, and IL-2 only in the presence of Ag. Of the cytokines we measured, none were detected in wells containing bone marrow-derived precursor cells (i.e., not treated with RANKL) incubated with intact OVA (data not shown). Osteoclasts were also able to induce the proliferation of OT-I splenocytes, as measured by the CFSE dilution assay when pulsed with SIINFEKL peptide or OVA (Fig. 2B). As a positive control, the DC line DC2.4 (58) and splenic CD11c+ DC were used. DC induced expression of IL-2 and TNF-α as expected (66). As negative control, an irrelevant peptide was tested. No cytokine release or proliferation was observed with osteoclasts or DC in the presence of the irrelevant peptide (data not shown), indicating the activation is Ag specific.

To test for osteoclast-CD4+ T cell interactions, OT-II-transgenic mice that have CD4+ T cells with a TCR specific for the OVA sequence that encompass residues 323–339 (67) were also tested. No activation was observed with OT-II cells and osteoclasts as expected, as assayed by detection of cell proliferation or cytokine production, because the osteoclasts do not express MHC class II. In contrast, DC were able to activate the CD4+ cells in an Ag-dependent manner (data not shown). These data show that osteoclasts can activate CD8+ T cells in an Ag-specific manner.

The proteosome inhibitor epoxomicin blocks presentation

To confirm that the activation of CD8+ T cells is by cross-presentation, the proteosome inhibitor epoxomicin was used to block protein processing in osteoclasts. This inhibitor was chosen because it is reported to be specific for the proteosome (68). Epoxomicin treatment of osteoclasts had no effect on peptide presentation, but significantly reduced T cell activation by OVA (Fig. 3C). These results taken together indicate that osteoclasts are able to take up an exogenous protein and to process and cross-present Ags to activate CD8+ T cells.

Osteoclast-T cell interaction induced phosphorylation of ZAP70 in CD8+ T cells

Upon TCR stimulation, ZAP70 is phosphorylated on tyrosine residues and functions in the initial step of TCR-mediated signal transduction. To assess whether osteoclasts bind directly to and activate T cells in a similar manner as DC, an anti-phospho-ZAP70 Ab was used. OVA-treated osteoclasts were incubated with T cells for 90 min, then fixed, permeabilized, and incubated with anti-mouse phospho-ZAP70 Ab. The results, shown in Fig. 3D, clearly demonstrate that CD8+ T cells directly interact with the large multinucleated osteoclasts. The bound T cells have a rimmed pattern of phosphorylated ZAP70 reminiscent of an immunological synapse (69). Confocal microscopy demonstrated direct cell-cell interaction and activation of CD8+ T cells by osteoclasts.

Phenotype of the T cells primed by osteoclasts

APC-activated T cells that produce IFN-γ and IL-2 and proliferate typically form effector CTL (70). To test for CTL activity, we used the E.G7 (71) cell line that expresses OVA as the target cells.
Cytotoxicity was measured by either chromium release from labeled cells or by measuring LDH release. As a positive control, we used DC-primed T cells. When CD8\(^+\) T cells were primed by osteoclasts, no significant (>5%) cytotoxic activity was detected, although the cells proliferated and produced IL-2 and IFN-\(\gamma\). In contrast, the DC-primed CD8\(^+\) T cells were cytotoxic (Fig. 4A). These results suggested that the osteoclast-primed T cells may have a regulatory effector function.

To further define the phenotype of osteoclast-primed T cells, we tested for the expression of a number of intracellular and surface markers using flow cytometry. Fig. 4B (left panel) shows that a small fraction of the freshly isolated, purified CD8\(^+\) T cells express the CD44 marker, and priming by osteoclast induced significant levels of CD44 (Fig. 4B, right panel), as expected, indicating that these T cells were activated. Unexpectedly, the activated T cells (CD44\(^+\) and CD8a\(^+\) subset)
also expressed FoxP3, a marker for T<sub>REG</sub>. This CD44<sup>+</sup> population of osteoclast-primed CD8<sup>+</sup> T cells showed the induction of CD25, CTLA4, CD122, and RANKL (Fig. 4C).

**FoxP3<sup>+</sup> CD8<sup>+</sup> T cells are anergic and suppressive**

T cells that express FoxP3, a transcription factor belonging to the forkhead box family, have the ability to suppress aberrant activation of self-reactive lymphocytes. Based on the induction of FoxP3 expression, we expected that these cells would have the functional phenotype of T<sub>REG</sub>: that they would be anergic and able to suppress DC-induced proliferation and cytokine secretion of responder cells. To test for anergy, we used 5 × 10<sup>5</sup> osteoclast-primed FoxP3<sup>+</sup> CD8<sup>+</sup> T cells (cytokine profile is shown in Fig. 5A, left panel) that were rested for 3 days after priming. The cells were CFSE labeled and 10<sup>5</sup> of the rested cells were challenged with 10<sup>5</sup> DC pulsed with OVA<sub>257–263</sub> (OT-I) peptide. As shown in Fig. 5A (right panel), the production of IL-2, IL-6, and IFN-γ was significantly reduced and proliferation was not detected (data not shown) on challenge. When treated with PMA and Ca<sup>2+</sup> ionophore A23187 for 6 h, the cells proliferated, confirming that the cells were viable (data not shown).

**FIGURE 4.** Phenotype of osteoclast-primed CD8<sup>+</sup> T cells. **A**, CD4<sup>+</sup> T cells primed by osteoclasts lack CTL activity: Purified CD8<sup>+</sup> T cells were primed by osteoclasts (as in Fig. 3) or by purified CD11c DC and then tested for CTL activity. OVA-expressing E.G7 cells were used as targets. Cytotoxicity was measured by assaying for LDH released from apoptotic cells. An average of three experiments is shown. **B**, T cell phenotype. Osteoclasts were incubated with OVA overnight and then used to prime purified CD8<sup>+</sup> T cells from OT-I mice. The T cells were harvested and stained with a panel of fluor-conjugated anti-mouse Abs. As expected, the CD44<sup>+</sup> population increased after priming (right) relative to the unprimed cells (left). **C**, The CD44<sup>+</sup> population (upper right quadrant; boxed) of the osteoclast-primed T cells is shown. The CD8<sup>+</sup> and CD44<sup>+</sup> cells (shaded histograms) stained positive for FoxP3, CD25, CTLA4, RANKL, and CD122. The unprimed cells stained identically are shown as unshaded histograms.
FIGURE 5. Osteoclast-primed CD8⁺ T cells that express FoxP3 are anergic and suppressive. A, Anergy assay. CD8⁺ T cells were primed by osteoclasts for 48 h, then nonadherent cells were transferred to new wells, re-fed with medium, and allowed to rest for 3 days. The rested cells were challenged with DC that had been pulsed with SIINFEKL peptide. Cytokines measured from the priming reaction are shown on the left and from the challenge reaction on the right. B, Suppression assay: to test for suppression by FoxP3⁺ T cells, 50,000 CFSE-labeled CD8⁺ T cells responders were cocultured with 50,000 DC pulsed with SIINFEKL peptide, in the presence of 50,000, 25,000, 12,500, or 6,250 FoxP3⁺ T cells. Proliferation was measured by CFSE dilution. Representative histograms are shown in center section of panel, where the numbers indicate the number of FoxP3⁺CD8⁺ T cells added per well. The percentage of cells that divided in 48 h (obtained using the FlowJo proliferation tool) are plotted as a function of the number of FoxP3⁺ and FoxP3⁻CD8⁺ T cells added. All assays were performed in triplicate wells. Average values from three experiments are plotted.
To test whether these FoxP3-expressing T cells are able to suppress the priming of other CD8+ T cells by APC, 5 x 10^4 CFSE-labeled CD8+ T cells (responders)/well were added to 5 x 10^4 DC/well that had been pulsed with SIINFEKL peptide in the absence or presence of 2-fold serial dilutions of FoxP3+CD8+ T cells starting at 5 x 10^6 cells/well. Indeed, as shown in Fig. 5B, although the naive (FoxP3-) cells had no suppressive effect, the FoxP3+CD8+ T cells behaved as dominant negative regulators to suppress the priming of the responder cells by DC, as measured by CFSE dilution, in a dose-dependent manner.

Discussion

In our previous work, we showed using time course microarray data that RANKL induced a complex mixture of chemokines and up-regulated MHC-I molecules (55). In this study, we show experimentally that these chemokines selectively recruit CD8+ T cells. Upon recruitment, the osteoclasts were able to program CD8+ T cells by cross-presenting Ags and by direct contact to proliferate and induce the secretion of IL-2, IFN-γ, IL-6, and expression of FoxP3, CTLA4, and RANKL. The resulting T cells were rendered anergic and suppressed the activation of CD8+ T cells by DC. This work provides two novel observations. First, we showed that osteoclasts were able to cross-present Ags. Second, we showed that the recruitment, cross-presentation, and priming of CD8+ T cells induced FoxP3 expression. The CD8+ T cells that express FoxP3 have the ability to suppress the activation of responder CD8+ T cells. Therefore, these results also demonstrate that an APC can peripherally induce a TREG through a MHC-I-dependent mechanism.

Osteoclast-T cell interaction forms feedback control circuit

The field of osteoimmunology has previously focused on the regulation of osteoclasts by lymphocytes under inflammatory conditions. The ability of osteoclasts to regulate T cells under non-inflammatory conditions and the induction of RANKL on the T cell surface shows that a feedback loop exists between these two cells. This feedback control provides new insights into regulation of osteoclasts and T cells. For example, substantial evidence indicates that IFN-γ suppresses osteoclastogenesis in vitro (16–18). However, recently, a number of publications have reported positive effects of IFN-γ on osteoclastogenesis in the presence of T cells (16, 72–74). Similarly, contradictory effects of IFN-γ in vivo have been observed. IFN-γ has been reported to be efficacious in the treatment of osteoporosis through activation of osteoclast function in both rodents (75) and humans (76). Since osteoporosis results from loss of osteoclast function, IFN-γ must activate osteoclasts. In contrast, mice that have a knockout of the IFN-γ receptor have an accelerated onset of collagen-induced arthritis and bone resorption. This result was interpreted as the suppression of osteoclasts (77). However, the knowledge of the feedback system explains these results: IFN-γ signaling affects both the T cells and the osteoclasts. Indeed, our results provide a mechanism for the activation of osteoclasts by showing the induction of RANKL on T cells.

Osteoclasts cross-present Ags

Osteoclasts form sealed lysosomal compartments against the bone. They then secrete acid and proteases into this compartment, forming a lacuna as they break down the mineral and protein components of the bone. The fragments are then endocytosed, processed further, and exocytosed out of osteoclasts. Osteoclasts express the machinery required for endocytosis, as well as proteosome subunits, sortins, and other genes needed to process the peptides through the endoplasmic reticulum (in a TAP-dependent manner) (78). Therefore, the ability to endocytose and process exogenous proteins, like OVA, was expected. However, the ability of osteoclasts to present these peptides on MHC-I to recruit and present Ag to CD8+ T cells has not been previously described. These observations have profound implications for osteoimmunology because they show that osteoclasts can direct T cell responses, in addition to being regulated by T cells.

Ninety percent of the protein in bone is type I collagen (79, 80). The amino acid sequence of collagen is rich in the Gly-X-Pro motif and charged residues that would make good MHC Ags. In fact, an established model of autoimmunity-mediated arthritis uses collagen as an Ag (81). Although the model uses type II collagen and IFA to elicit arthritis, the MHC-I peptides derived from both proteins are likely to be similar since the two proteins are similar in their amino acid composition. Characterization of the collagen-induced arthritis model indicates that anticollagen TCR do normally exist in susceptible animals (82). Since nearly all cells express MHC-I, presentation of the secreted peptides may activate the collagen-reactive CD8+ T cell response. Therefore, we hypothesize that the induction of FoxP3 in CD8+ T cells prevents a potential self-reactive response to the neo-Ags produced during bone remodeling.

FoxP3 expression in TREG

T cells that express FoxP3, a transcription factor belonging to the forkhead box family, have the ability to suppress aberrant activation of self-reactive lymphocytes. Loss of FoxP3 function in Scurfy mice results in lethal autoimmunity (83). Mutations in FoxP3 in humans results in the IPEX syndrome (84) (immune dysregulation, polyendocrinopathy, enteropathy, X-linked; OMIM: 304790). Adoptive transfer of T cells expressing FoxP3 in mice with FoxP3 loss-of-function abolishes the autoimmune pathology. FoxP3 is not only a distinctive marker for TREG but it is also required for their development (85–87). Studies of autoimmune suppression have focused on CD4+ T cells that are FoxP3+ (88) and the events that are needed for the induction and the maintenance of CD4+ TREG have been actively investigated (89–94). In contrast, although the CD8+ TREG were discovered in the early 1970s (95), the events that mediate the induction of FoxP3+ in CD8+ T cells to produce TREG are much less defined. The induction of FoxP3 in CD8+ T cells by osteoclasts highlights a novel, inducible means of regulating inappropriate activation of the immune response.

CD8+ TREG

Although CD8+ T cells that express FoxP3 have been observed in mice and in humans, in comparison to FoxP3+CD4+ T cells, this cell type is not well characterized partly because of the inability to date to generate such cells. Furthermore, because FoxP3+CD4+ T cells can suppress the activation of CD8+ T cells (96), the functional role of CD8+ TREG remains unclear (97). Three distinct classes of CD8+ suppressive T cells have been described from humans and mice based on cell surface markers and secreted cytokines (reviewed in Refs. 98–100). One class mediates suppression through IL-10 (101–104). A second class was induced by treatment with IL-2 and GM-CSF for 1 wk in the presence of monocytes (105). Although the expression of FoxP3 in the second class has not been reported, this class appears to mediate its effect by IL-6 and IFN-γ because neutralizing Abs against these two cytokines could block the suppressive effect of these cells. In mice, a third class of suppressive CD8+ T cells has been studied primarily in the context of experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis. Transfer of T cells from mice that have recovered from EAE into susceptible mice renders them resistant to the development of EAE when the
susceptible mice are immunized with myelin basic protein. In contrast, transfer of T cells depleted of CD8+ T cells produces EAE in the susceptible mice upon reimmunization, indicating that a CD8+ T cell subset prevented activation of CD8+ T cells (106, 107). The suppressor CD8+ T cell subset were Ag specific and MHC restricted because inhibition could be blocked by Abs to the nonclassic MHC-Ib molecule Qa-1, but not by Abs to MHC-Ia molecules (108). The (osteoclast-induced) FoxP3+ CD8+ T cells that we have discovered do not fit neatly within the current classification because they do not express IL-10, but secrete IL-2 and IFN-γ during activation and they are CD122 positive, a marker of the natural (thymically produced) CD8+ TREG (109, 110). They also do not produce detectable levels of soluble TGF-β (data not shown). Recently Fan et al. (111) demonstrated induction of FoxP3 expression in CD8+ T cells using TGF-β1 in vitro. They found that in addition to TGF-β the strength of the costimulatory signal, modulated by plate-bound anti-CD3, was critical for the induction of FoxP3. The FoxP3+ CD8+ T cells induced under high costimulatory signals were CD25+ and CTLA4+ and secreted soluble IL-10. In addition, these cells were able to suppress proliferation of responder CD4+ T cells. In addition, they also showed that adoptive transfer of the FoxP3+CD8+ T cells accelerated the growth of a tumor cell line in mice, as assessed by increased number of metastatic foci. These results demonstrate that functional CD8+ TREG can be generated in vitro. How these regulatory cells are similar or different from the osteoclast-induced FoxP3+ CD8+ T cells remains to be determined.

Conclusions

The recognized role of CD8+ T cells in the immune response is protection against viral infections and tumors. They perform this function by cytolytic damage of target cells expressing MHC-I. CD8+ T cells have been demonstrated to play an important role in the pathogenesis of several autoimmune conditions including RA, type 1 diabetes, multiple sclerosis, and lupus. In each of these cases, there is evidence that remodeling of the tissue may play a role in the induction of autoimmunity. A common emerging theme underlying autoimmunity where CD8+ T cells play a dominant role may be through shed neo-epitopes produced during remodeling in combination with the dysfunction of suppressor T cell activity.

A major impediment to studying the FoxP3+ CD8+ T cells has been the inability to isolate significant quantities of such cells. We have demonstrated that osteoclasts are able to recruit and cross-Apps. The CD8+ T cells activated by osteoclasts express FoxP3 and are able to suppress the priming of naive CD8+ T cells. The ability to generate FoxP3+ CD8+ T cells in vitro provides us with a tool to increase our understanding of how an APC can peripherally induce regulatory CD8+ T cells.

The interaction between CD8+ T cells and osteoclasts arose from an inference from time-series microarray data (55). The findings presented in this work are a validation of this inference. The discovery that osteoclasts can act as APC that under noninflammatory conditions induce FoxP3, CTLA4, and RANKL in CD8+ T cells opens several new avenues of investigation. We are currently verifying the induction and the role of FoxP3 in CD8+ T cells by osteoclasts in vivo. In future work, we will use this system to study the role of RANKL and IFN-γ expressed by CD8+ T cells in regulating numbers and activity of osteoclasts, the contribution of CTLA4 and other cell surface proteins in mediating suppression, and the role of FoxP3+CD8+ T cells in suppressing inflammation in models of RA.

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