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Immune Interactions with CD4+ T Cells Promote the Development of Functional Osteoclasts from Murine CD11c+ Dendritic Cells

Mawadda Alnaeeli,*† Josef M. Penninger,‡ and Yen-Tung Andy Teng2*†

Dendritic cells (DC) are innate immune effectors and are critically involved in regulating T cell immunity. Osteoclasts (OC) are bone-resorbing cells derived from the monocyte/macrophage lineage in response to receptor activator of NF-κB ligand (RANKL). DC and T cells form aggregates in the inflammatory infiltrates at active disease sites in human and in experimental rheumatoid arthritis and periodontitis. We investigated whether DC interactions with T cells in the bone environment can support the development of functional OC. In the present study, we demonstrate that upon proper activation by microbial or protein Ags (namely Actinobacillus actinomycetemcomitans, bovine insulin, and outer membrane protein-1) and during immune interactions with CD4+ T cells in vitro, murine BM-derived and splenic CD11c+ DC (CD11b+Ly-6C−CD31−) develop into TRAP+ CT-R+cathespin-k+ functional OC in a RANKL/RANK-dependent manner. Rescue and blocking experiments using CD11c+ DC derived from Csf-1−/−/op/op mice show that M-CSF is required “before” developing such osteoclastogenic potential upstream of RANKL/RANK signaling, suggesting that immature CD11c+ DC can indeed act like OC precursors. In addition, these CD11c+ DC-derived OC are capable of inducing bone loss after adoptive transfer in vivo. These data suggest a direct contribution of DC during immune interactions with CD4+ T cells to inflammation-induced osteoclastogenesis. Therefore, our findings not only provide further evidence for DC plasticity, but also extend the current paradigm of osteoimmunology.

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Inflammatory bone disorders, such as rheumatoid arthritis (RA),1 periodontal disease (PD), and osteomyelitis, manifest imbalanced remodeling processes, resulting in irreversible bone destruction, leading to morbidity, perturbation of life quality, and even potentially life-threatening conditions (1). Bone remodeling is a tightly regulated process by a number of osteogenic cytokines, growth factors, and hormones that exert their effects on bone cells, namely osteoblasts (OB) and osteoclasts (OC). OB and OC function to control bone synthesis and resorption, respectively. OC are derived from the monocytes/macrophages (Mo/MQ) lineage in the presence of receptor activator of NF-κB ligand (RANKL; Refs. 1–3). Functional OC are multinucleated giant cells that express tartrate resistance acid phosphatase (TRAP), calcitonin receptor (CT-R), cathepsin-k, and integrins ανβ3 and are capable of resorbing bone (4). RANKL induces osteoclastogenesis in the presence of M-CSF (or Csf-1; Ref. 5), where M-CSF signals via c-fms up-regulate RANK expression on OC precursors, promoting their survival and differentiation (6, 7). RANK, the receptor of RANKL, and its antagonist, osteoprotegerin (OPG), have been shown to be the key regulators of bone remodeling and are directly involved in the differentiation, activation, and survival of OC and OC precursors (1–3, 8). In addition, RANKL/RANK signaling enhances dendritic cell (DC) survival and is indispensable for lymph node organogenesis (9–11). Genetic mutations of RANKL, RANK and M-CSF demonstrate similar defective phenotypes in OC development with severe osteopetrosis, suggesting that they are essential for osteoclastogenesis and bone remodeling (11–14). OPG transgenic mice are osteopetrotic with defective OC activity, whereas OPG deficient mice are severely osteoporotic (1, 15). Importantly, our studies and others showed that activated T cells express RANKL and mediate osteoclastogenesis (16, 17), and that blocking RANKL activity via OPG injections results in a significant reduction of bone loss in RA, PD, osteoporosis, cancer-related bone metastasis and type 1 diabetes-associated alveolar bone loss (1, 16–20).

DC are bone marrow (BM)-derived leukocytes, with key innate immune functions, including antiviral and antimicrobial properties (21). Their wide distribution allows rapid access to sample the environment for Ags and danger signals. As professional APC, DC capture Ags and efficiently prime naive T cells to initiate adaptive immune effector functions or to induce tolerance (21, 22). The origin and developmental pathways of DC remain controversial (23); however, they are currently classified into conventional (including myeloid and lymphoid) and plasmacytoid DC (21–27). Recent studies reported that: 1) murine DC could be Mo derived (24), suggesting DC and OC may share common progenitors (28).
and that Fli3+ MQ precursors may differentiate sequentially to OC, DC, and microglia (29); 2) immature DC “transdifferentiate” to OC in RA, suggesting that DC may directly contribute to inflammation-induced osteoastagensis (30); and 3) Langerhans cells may give rise to multinucleated TRAP+ OC-like cells in the inflammatory Langerhans cell-histiocytosis lesions (31). Furthermore, DC can activate naïve T cells in the presence of microbial Ags such as Lactobacillus spp. and Helicobacter pylori (32, 33).

DC are present in active disease sites of RA and PD where they aggregate with T cells in the inflammatory foci (34–37) and are thought to contribute indirectly to inflammation-induced bone loss in RA (38). Rivollier et al. (30) showed that human blood Mo-derived DC can “transdifferentiate” into OC in the presence of M-CSF and RANKL in vitro, suggesting that DC might directly contribute to osteoclastogenesis. It remains unclear whether DC/T cell interactions support such development and whether DC can indeed directly contribute to inflammation-induced osteoclastogenesis during immune interactions and activation in the bone environment. In addition, the development of OC from committed DC has not yet been described in the murine system.

In the present study, we hypothesized that murine DC carry osteoclastogenic potential and can develop into functional OC during immune interactions with CD4+ T cells in the presence of Ag stimulation. We pursued this via in vitro coculture studies using CD11c+ DC derived from total BM cultures with GM-CSF and IL-4, followed by MACS sorting to isolate highly purified CD11c+CD11b+ DC subset (thus devoid of classical OC precursors; Refs. 4 and 39). Our results show that 1) through phenotypic and functional analyses, murine CD11c+ DC can act like OC precursors and indeed develop into OC during immune interactions with CD4+ T cells and microbial or protein Ags in bone environment; 2) M-CSF is essential for the development of such osteoclastogenic potential upstream of RANKL/RANK signaling; and 3) DC-derived OC (DDOC) can induce bone resorption after adoptive transfer onto NOD/SCID mice calvarias in vivo. These data indicate that murine CD11c+ DC can develop into bona fide functional OC and that DC/T cells immune interactions can support de novo OC development from local CD11c+ DC subset(s).

Materials and Methods

Mice

Four- to 6-wk-old female BALB/c, B6C3F1 Csf-1−/−, op/op, and wild-type (WT) control mice were purchased from The Jackson Laboratory mice and housed under specific pathogen-free conditions in the Animal Facility of the University of Rochester and the University of Western Ontario. All animal protocols were conducted under each institution’s guidelines and approved by the local ethics and animal experimentation committees.

Cell cultures and reagents

All primary cell cultures were performed in complete RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies), 50 μM 2-ME, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were incubated at 37°C in a humidified 5% CO2 incubator (17). The following reagents were purchased from commercial sources: recombinant mouse (m) GM-CSF (Cedarlane Laboratories), anti-mFrcRII/II (CD11c/32), PE-conjugated, anti-mCD4, FITC-conjugated anti-mCD11c, PerCP/Cy5.5-conjugated anti-mCD4, rat anti-m-α4, baitin anti-mCD31 (ER-MP12), biotin anti-m-ly-6C (ER-MP20), rat anti-mTCR α-chain, rat anti-mCD3e, and goat anti-m-M-CSF (10 μg/ml) Abs. Due to scant DC purity is 99%; see Fig. 1C, CD11c+ DC obtained from Csf-1−/− op/op mouse BM cultures, only 10% Csf-1−/− op/op CD11c+ DC were cocultured with 3 × 106 CD4+ T cells or rmRANKL in HA plates with or without rmM-CSF (25 ng/ml). DC cocultures were incubated for 2–10 days, during which DC were assessed for TRAP and resorptive pits (lacunae) activities, as well as surface phenotype by flow cytometry and immunofluorescent microscopy. DC viability was assessed by trypan blue exclusion during the coculture period. Splenocytes (0.5 × 106) stimulated by Con A for 5 days (43) were used as positive controls for TRAP and resorptive pit assays.

Mixed lymphocyte cultures

Naïve 3 × 105 CD4+ T cells purified from C57BL/6 splenocytes were cocultured in triplicates, with different densities (3 × 103, 3 × 104, 3 × 105, 3 × 106, and 3 × 107) of freshly purified CD11c+ DC derived from BM of BALB/c mice as described above in a flat-bottom 96-well plate in a total volume of 100 μl. Plate-bound anti-CD3 Ab (10 μg/ml)-activated CD4+ T cells were used as positive control for T cell proliferation, whereas untreated CD4+ T cells were used as a negative control. At day 5, 50 μl of WST-1 reagent was added into each well according to the manufacturer’s instructions for colorimetric measurement of cellular proliferation (44). This assay is based on the cleavage of tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenase. OD value was determined 4 h later at 440 nm using Dynatech MR700 Microplate reader (Dynatech).

Quantitation of TRAP+ cells and resorptive pits

Cultured CD11c+ DC were fixed by 2% formaldehyde and 0.2% glutar-
area of (purple-red) TRAP\(^+\) multinucleated cells (more than or equal to three nuclei) were quantified as described previously (20). Briefly, 12–17 randomly chosen fields (covering 33 mm\(^2\) = one-third of the total surface areas of the HA well) were used for automated scanning analysis and quantification. The mean of total number TRAP\(^+\) multinucleated cells per unit area and total surface area of TRAP\(^+\) multinucleated cells were then calculated after subtracting averaged background signals of the negative control. To quantify the total surface area of resorptive pits in the selected fields, cells were stripped with 1 N NaOH for 16 h, after which the images of eroded bone or HA surfaces were captured as described above.

**FACS analysis and scanning IF digital images analysis (SIDIA)**

Cells collected from cocultures at various time points were incubated with 5 μg/ml anti-CD16/32 to block Ig binding, then immunostained with iso-type control IgG or Ab-conjugated to FITC, PE, Cy5, or biotin as follows: cells were stained with FITC-anti-mCD11c, anti-m-Grm-CSF, and anti-mRANK Ab, followed by incubation with 2º biotinylated anti-rabbit-IgG or 3º streptavidin-PE or Cy5; anti-m-CT-R IgG, followed by 2º FITC-conjugated anti-goat IgG; anti-mI-A\(^d\), followed by 2º biotin goat anti-mIgG and 3º streptavidin-Cy5. For intracellular staining of cathepsin-k, cells were fixed for 30 min with fresh 4º formaldehyde and then permeabilized for 10 min with 0.2º Triton X-100, followed by blocking nonspecific binding by 1% BSA and incubation with anti-m-cathepsin k IgG and 2º FITC-conjugated anti-goat IgG on ice. Stained cells were scanned in a FACSCalibur flow cytometer (BD Biosciences). Results were analyzed via CellQuest, and dead cells were excluded by propidium iodide staining. CD\(^+\) T cell activation was assessed by in situ staining of CD25 with 1º biotinylated anti-m-CD25, 2º streptavidin-Cy5, and RANKL with OPG-Fc + FITC-conjugated anti-h-Fc-IgG. To detect F-actin ring formation, DC\(^+\)T/Ag coculture of day 5 was intracellularly stained in situ by Alexa-Fluor 488 phalloidin, according to the manufacturer’s instruction, while 0.1 × 10\(^5\) peritoneal MØ cultured in 25 ng/ml r-CSF and 30 ng/ml rRANKL for 5 days served as a positive control. For quantification of IF signals at the single cell level, 0.25 × 10\(^5\) CD11c\(^+\) cells were labeled by CFSE before cocultures and then at different time points were stained with: 1) anti-mCT-R and 2º Texas red-conjugated anti-goat IgG and anti-mGM-CSF using biotinylated IgG streptavidin-AMCA or 2º streptavidin-Cy5. For intracellular staining of cathepsin-k, cells were fixed for 30 min with fresh 4º formaldehyde and then permeabilized with MACS cell-sorting protocols to produce highly purified CD11c\(^+\) DC. We characterized the Ag-presenting capability of freshly purified CD11c\(^+\) DC and found that they can efficiently induce: 1) proliferation of allogeneic naive CD4\(^+\) T cells from C57BL/6 (H-2\(^b\)) in mixed lymphocytes cultures (Fig. 1A); and 2) activation of naive syngeneic CD4\(^+\) T cells with Ag-Ags as determined by CD25 and RANKL expression (Fig. 1B) and ELISA using culture supernatants (data not shown). Furthermore, freshly purified CD11c\(^+\) DC expressed no detectable CD80/CD86 and low cell surface MHC-II (smHC-II: 1–15%) with the majority of MHC-II localized intracellularly (iMHC-II = 40–60%; day 0 in Fig. 1C, table), indicating their immature phenotype. Based on FACS analyses, day 0 CD11c\(^+\) DC exhibited the following phenotype: GM-CSFR\(^+\)RANK\(^-\)MHC-II\(^+\)CT-R\(^-\)cathepsin-k\(^-\) (Fig. 1C). Importantly, these DC quickly up-regulated surface MHC-II and the costimulatory molecules CD80 and CD86 when activated by sonicated Ag-Ags or E. coli-LPS (Fig. 1C, table). Collectively, these data indicate that CD11c\(^+\) cells used in our study are indeed bona fide DC.

**CD11c\(^+\) DC develop TRAP and bone resorptive activities during cocultures with CD4\(^+\) T cells and foreign Ags in a RANKL-dependent manner**

To explore the osteoclastogenic potential of DC, BALB/c BM-derived MACS-purified CD11c\(^+\) DC were incubated with naive syngeneic CD4\(^+\) T cells and sonicated Ags of Aa, a key human periodontal pathogen (17, 20) on dentine slices, bone discs (46), or HA-coated 48-well plates for 2–10 days. Total splenocytes plus Con A cocultures were used as positive control for TRAP and resorptive activity (43); in addition, freshly prepared TRAP\(^+\) multinucleated BM cells were used to validate the positive control (Fig. 2C). Interestingly, CD11c\(^+\) DC were able to fuse and become TRAP\(^+\) multinucleated giant cells, some of which manifested dendrites and maintained interaction with T cells (Fig. 2G). Since T cells do not express TRAP as determined by TRAP staining of CD4\(^+\) T cells cocultured with and without Aa or Con A, all TRAP\(^+\) cells must be DC derived. TRAP activity on day 5 was assessed by the total number and the total surface area of TRAP\(^+\) cells (Fig. 2A). Ninety to 95% of TRAP\(^+\) cells included in the quantitative analysis were multinucleated (more than or equal to three nuclei). Note that titration of cells to as low as 5 × 10\(^4\) CD11c\(^+\) DC and 5 × 10\(^4\) CD4\(^+\) T cells also yielded significant TRAP and resorptive pit activities, compared with negative controls (data not shown). Furthermore, TRAP expression peaked on day 5 and required stimulation by Aa, as DC cultured alone, DC plus Aa, or DC plus T cells without Aa, did not develop significant TRAP activity (p ≤ 0.03; Fig. 2A). Surprisingly, as early as day 2, total surface area and numbers of TRAP\(^+\) cells were already significantly higher than control samples (data not shown).

Subsequently, cells were stripped from the wells, and captured images of bone surfaces were quantified to determine whether TRAP\(^+\) multinucleated cells formed resorptive pits. The results showed that total surface area of resorptive pits in DC, CD4\(^+\) T cells, and Aa cocultures were significantly higher than control cultures (Fig. 2D; p ≤ 0.03), indicating that these cells can indeed resorb bone in vitro; thus, we called them DDOC. To further assess the significance of DDOC development, we quantified the frequency of TRAP\(^+\) cells per field. On average, 61 ± 2% of CD11c\(^+\) DC per field become TRAP\(^+\) by day 5 on HA-coated wells and bone discs, where >90–95% of TRAP\(^+\) cells are multinucleated (more than or equal to three to five nuclei). This is equivalent to ~2.9 × 10\(^5\) TRAP\(^+\) multinucleated DDOC of 5 × 10\(^5\) CD11c\(^+\) DC cultured in the entire HA-coated wells.

Regardless of the bone substrates used (i.e., HA, bone discs, and dentine slices), we found that DDOC induced significant resorptive
Interestingly, the results of using phalloidin staining (Fig. 2H) formation characteristic of OC activity was detected in DDOC cytosis of apoptotic cells in the cocultures. Furthermore, actin-ring

restored TRAP (Fig. 2A, B) and resorptive activities (with \( p = 0.01 \); Fig. 2; E and G) only in the presence of Aa, when compared with DC + RANKL. Therefore, both RANKL-

RANK signaling and proper Aa-Ag stimulation are critical for CD11c\(^+\) DC to develop TRAP and resorptive activities. Since sRANKL (in the presence of Aa) restored ~75\% of TRAP and

57\% resorptive activities of those developed in DC + T + Aa cocultures (with \( p = 0.5 \); Fig. 2, A and B, D and E), respectively, contributions by T cell signals or molecules other than RANKL cannot be entirely excluded. Thus, we conclude that depending on the signals encountered, CD11c\(^+\) DC have the potential to develop into OC in response to RANKL when stimulated in the bone environment in vitro.

Coculturing CD11c\(^+\) DC with T cells and Con A resulted in significant TRAP and resorptive activity (data not shown). Thus, to further confirm the role of T cell activation in the development of DC into OC, we tested the effects of anti-MHC-II (I-Ad) or -CD3/TCR blocking Abs in these cocultures. Results showed that both Abs were able to abolish TRAP (\( p = 0.008 \) and \( p = 0.02 \); Fig. 2C) as well as resorptive activities (\( p = 0.009 \) and \( p = 0.01 \); Fig. 2F). Furthermore, at least 25–50\% of TRAP\(^+\) multinucleated DDOC/field had direct contact with T cells at the time of analyses (i.e., day 5; data not shown), which is consistent with the above results. Taken together, our data indicate that T cell activation is critically involved in the development of TRAP and resorptive activities by CD11c\(^+\) DC via RANKL production.

**OC development from CD11c\(^+\) DC is not Aa specific**

To assess whether this phenomenon is Aa specific, we cocultured CD11c\(^+\) DC with CD4\(^+\) T cells (primed with BI or OMP-1; Refs. 41 and 47) and protein Ags BI or OMP-1 (20 \( \mu g/ml \)). The results
showed that, although TRAP activity appears to be slightly enhanced in OMP-1 and BI cocultures compared with AA (Fig. 3A), both TRAP and resorptive activities were comparable to those detected in DC+T cells+AA cocultures (Fig. 3A). Moreover, when sonicated Ags of Porphyromonas gingivalis (17) or E. coli were used they induced TRAP and resorative activities comparable to those induced by AA in this coculture system (data not shown). Taken together, our data suggest that this process is not unique to AA but can be induced by different Ags.

We next evaluated any possible presence of Mo/MQ contaminants to further validate our findings. By FACS, we confirmed that MACS-purified CD11c<sup>+</sup> DC were CD11b<sup>-F4/80</sup> ER-MP12<sup>-</sup> ER-MP20<sup>-</sup> (Fig. 3B; note that ER-MP12 = CD31 and ER-MP20 = Ly-6C), excluding the presence of: 1) Mo/MQ contaminants; and 2) CD11b<sup>+</sup> cells (i.e., OC precursors) (4). This is likely due to the use of IL-4 in BM cultures, which in turn inhibit Mo/MQ development (39). To further assess the possible contribution of Mo/MQ to the generation of CD11c<sup>+</sup> DC studied here, mature Mo/MQ were depleted from BM cells, followed by CD11c<sup>+</sup> DC purification by using anti-F4/80, CD11b, ER-MP12, and ER-MP20 mAbs and complements (41, 42). As expected, CD11c<sup>+</sup> DC derived from Mo/MQ-depleted or nondepleted BM cultures expressed similar levels of TRAP and resorative activities by day 5 (Fig. 3, C and D). Thus, CD11c<sup>+</sup> DC are capable of developing into functional OC regardless of whether they are derived from Mo/MQ. Furthermore, the frequency of TRAP<sup>+</sup> bone resorbing DDOC was the same whether DC were generated from Mo/MQ-depleted or nondepleted BM (data not shown). It is unlikely that the purified CD11c<sup>+</sup> DC studied here contain DC/OC precursors or early Mo precursors because: 1) addition of IL-4 and GM-CSF suppresses Mo/MQ and promotes DC development in BM cultures, respectively (39); and 2) CD11c<sup>+</sup> is not expressed on early DC/OC precursors (48, 49). These data indicate that contamination by Mo/MQ cannot be responsible for the reported phenomenon; however, this possibility cannot be entirely excluded.

Activated CD11c<sup>+</sup> DC express functional OC phenotype

The results of TRAP and resorptive pit assays prompted us to further characterize DDOC phenotype. Since CT-R signifies OC function, we used anti-CT-R Ab to detect CT-R expression on DDOC; which has been shown to resemble CT-R detection via radiolabeled CT-R (50). We also assessed the expression of other OC and surface markers on these cells. Our results show that by day 3, a significant percentage of CD11c<sup>+</sup> cells up-regulated the expression of CT-R (<1–58%). In contrast, GM-CSFR expression was down-regulated (93–21%). At day 5 of the coculture, CT-R expression peaked (93–96%), and GM-CSFR expression leveled off to ~25% (Fig. 4A). Thus, during activation and RANKL-RANK signaling, CD11c<sup>+</sup> DC developed into multinucleated TRAP<sup>+</sup>, CT-R<sup>+</sup>, and cathepsin-k<sup>+</sup> OC with resorative activity, whose overall expression profile on days 3 and 5 is as follows: CD11c<sup>+</sup> GMCSF-R<sup>low</sup> to ~"RANK" MHC-II<sup>high</sup>/CT-R<sup>+</sup>, cathepsin-k<sup>+</sup>, and DEC205<sup>low</sup> or ~"CD4<sup>+</sup> CD8<sup>+</sup> or ~"CD11b<sup>high</sup> to ~"F4/80<sup>+</sup> or ~"CD80<sup>low</sup> to ~"CD86<sup>low</sup> to ~" integrins α<sub>i</sub>β<sub>i</sub> (Fig. 4A and data not shown). Importantly, OPG addition at the onset of the coculture significantly abolished CT-R expression (<10% by day 5; Fig. 4A), which correlated with the significantly reduced TRAP and resorptive activities detected (Fig. 2, B, E, and G). Furthermore, analysis of CT-R<sup>+</sup> DDOC revealed that the majority were CD11c<sup>+</sup> RANK<sup>-</sup> MHC-II<sup>low</sup>/GM-CSFR<sup>low</sup> or ~" (Fig. 4B).

Note that a relatively high MHC-II expression by DDOC is interesting and distinct from the classical OC phenotype (4). Whether this unique feature is associated with maintaining their APC function to prime CD4<sup>+</sup> T cells requires further study. On the other hand, GM-CSFR levels were down-regulated regardless of OPG

### Table I. Viability of CD11c<sup>+</sup> DC and CD4<sup>+</sup> T cells at day 5 in the coculture

<table>
<thead>
<tr>
<th>Condition</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC Only</td>
<td>60 ± 0.6%</td>
</tr>
<tr>
<td>DC+AA</td>
<td>97 ± 1.5%</td>
</tr>
<tr>
<td>DC+T+AA</td>
<td>95 ± 2.5% (1:1)</td>
</tr>
<tr>
<td>DC+RANKL+AA</td>
<td>94 ± 3.1%</td>
</tr>
<tr>
<td>DC+T+AA+OPG</td>
<td>82 ± 7%</td>
</tr>
</tbody>
</table>

* Indicates the percentage of T cell viability.

### Figure 2
CD11c<sup>+</sup> DC develop TRAP and resorative activities in a RANKL-dependent manner during cocultures with CD4<sup>+</sup> T cells and AA-Ags in vitro. A total of 0.5 × 10<sup>5</sup> BALB/c BM cells were cultured with AA (10 µg/ml) sonicated Ags with or without 0.5 × 10<sup>5</sup> syngeneic CD4<sup>+</sup> T cells or sRANKL (30 ng/ml). On day 5, cells were fixed and stained for TRAP. Note that 90–95% of TRAP<sup>+</sup> cells included in the quantification are multinucleated (average three to five nuclei; data not shown). A, DC expressed significantly higher levels of TRAP (p < 0.03) after coculture with naive syngeneic CD4<sup>+</sup> T cells and AA when compared with negative controls. B, TRAP expression was abolished by OPG (p < 0.01) and anti-RANK Ab (p < 0.01) compared with DC+T cells+AA and significantly increased in DC+RANKL (30 ng/ml) + AA cultures (p < 0.008) compared with DC+RANKL alone. C, TRAP expression was reduced significantly when DC/T cell interaction was blocked by anti-MHC-II Ab (p < 0.008) or anti-TCR/CD3 Abs (p < 0.02) compared with DC+T cells+AA. To assess OC function, cells were stripped with 1 N NaOH on day 5, and the total surface area of HA resorption was quantified. D, DDOC induced significant bone resorption in T cells+AA coculture compared with negative controls (p < 0.03). E, Both OPG and anti-RANK Ab blocked resorative activity (p < 0.02 and p < 0.007, respectively); RANKL restored this ability in the presence of AA stimulation (p < 0.01) compared with DC+RANKL alone. F, Blocking DC/T cell interaction by anti-MHC-II Ab or anti-TCR/CD3 Abs abolished resorative pits (p < 0.009 and p < 0.01, respectively) compared with DC+T cells+AA. G, Representative images of TRAP activities from DC+T, cells+AA, DC+RANKL+AA, and DC+T cells+AA+OPG cocultures and their resorative pits on HA at day 5 under different magnifications (× 100, × 150, and × 400). Bottom panel, Multinucleated DDOC at day 5 of DC+T+AA coculture (two panels in bottom left corner; × 400) and DDOC in “resorptive tracks” on bone discs (two panels in bottom right corner; × 150), respectively. H, Representative fluorescent images of F-actin ring formation by DDOC at day 5 of DC+T+AA coculture (left) and MQ+M-CSF (25 ng/ml) + RANKL (30 ng/ml) as positive control (right) detected by Alexa-fluor 488 phalloidin (under × 400). I, BM-derived and MACS-purified splenic CD11c<sup>+</sup> DC express comparable levels of TRAP, indicating their osteoclastogenic potential. The results are shown as the total number per unit area and total surface area (mm<sup>2</sup>) of TRAP<sup>+</sup> multinucleated cells or resorative pits from 12 to 17 randomly selected fields per well (mean ± SEM) from five independent experiments using total splenocytes + Con A as the positive control. The results of splenic CD11c<sup>+</sup> DC resorative pits assay mirrored the TRAP (data not shown here).
treatment, suggesting that RANKL-RANK signaling may not influence GM-CSFR expression. These data illustrate OC development from activated CD11c<sup>+</sup>/H11001 DC and further confirm the critical role of RANKL-RANK/OPG in this process.

To evaluate these phenotypic changes at the single cell level, CD11c<sup>+</sup>/H11001 DC were CFSE labeled before their coculture; then, cells were immunostained at different time points. The results of SIDIA showed that CT-R expression was significantly up-regulated and GM-CSFR down-regulated, whereas RANK levels remained relatively unchanged by day 3–5 of the coculture (Fig. 4, C and D), which is consistent with the results of FACS analyses in Fig. 4, A and B. The addition of OPG significantly inhibited CT-R up-regulation (Fig. 4, C and D). Taken together, these results strongly suggest that CD11c<sup>+</sup> DC can develop into functional OC after receiving appropriate activation signals in the bone environment (i.e., Ag and RANKL) and during interaction with activated CD4<sup>+</sup> T cells.

M-CSF is required for OC development from CD11c<sup>+</sup> DC

M-CSF is important for the development of OC precursors as Csf-1<sup>_op/op_</sup> mice manifest severe osteopetrosis (6, 7, 13, 14). Moreover, M-CSF is expressed by activated CD4<sup>+</sup> T cells (50) and can be detected in our DC+T+Aa culture supernatants (data not shown).

FIGURE 3. CD11c<sup>+</sup> DC can develop into TRAP<sup>+</sup> cells with resorptive activity in response to various Ag stimulations. BM-derived CD11c<sup>+</sup> DC (10<sup>5</sup>) were cocultured with syngeneic CD4<sup>+</sup> T cells in the presence of Aa (10 μg/ml), OMP-1, or BI (20 μg/ml). On day 5, TRAP and resorptive activities were quantified. Note that T cells in DC+T+Aa cocultures were purified from naive mice, while those in DC+T+Omp-1 or BI cultures were purified from Omp-1/CFA and BI/CFA-primed BALB/c mice, respectively. A, Aa, OMP-1, and BI induced similar levels of TRAP and resorptive activities; total number and total surface area of TRAP<sup>+</sup> multinucleated cells per unit area (top panel) and resorptive pits (bottom panel) in Aa, OMP-1, and BI cocultures (p > 0.05). B, Freshly isolated CD11c<sup>+</sup> DC were stained for CD11b, F4/80, ER-MP12, and ER-MP20 to exclude Mo/MQ contamination. C and D, The levels of TRAP and resorptive pits activities by DDOC, generated from BM with or without Mo/MQ depletion, in CD4<sup>+</sup> T cell +Aa cocultures (p > 0.5 and p > 0.4, respectively). Representative data from three independent experiments are shown.
shown). It was shown previously that osteoclastogenesis is independent of M-CSF produced by activated T cells in vitro (51). To address whether M-CSF is involved in the process of DDOC development, 10 μg/ml anti-M-CSF Ab was added at the onset of coculture. The results showed that there was no significant change in either TRAP or resorptive activity compared with that of DC+T+Aa (p > 0.5; Fig. 5A), suggesting that M-CSF may not be directly involved in the development of DDOC in the cocultures.

**FIGURE 4.** Activated CD11c+ DC develop functional OC phenotype during coculture with CD4+T cells and Aa. CD11c+ DC were collected after 3 and 5 days cocultures with or without OPG (30 ng/ml) and were stained to characterize their cell surface phenotypes. A, Dot plots depict the percentage of DC expressing surface CD11c, GM-CSFR, RANK, MHC-II, CT-R, and intracellular cathepsin-k on days 3 and 5 ± OPG. B, Summary of the cell surface phenotype of CT-R+ DDOC costained for CD11c, GM-CSFR, RANK, and MHC-II. C, SIDIA of CD11c+ DC (day 0) and DDOC (days 3 and 5) fluorescently stained for CT-R and RANK or CT-R and GM-CSFR surface expression levels are shown as mean fluorescence intensity in pixels ± SEM at the single cell level. D, Representative immunofluorescent images (under ×400) of single DDOC at days 0, 3, and 5 ± OPG of DC+T+Aa and stained for CT-R (Texas red), GM-CSFR (AMCA), and RANK (AMCA). Representative data out of three independent experiments with similar results are shown here.
Meanwhile, we proceeded to investigate whether CD11c+ DC generated from BM of osteopetrotic (Csf-1-op/op) mice could give rise to TRAP and resorptive activities compared with those from control WT mice. To do so, CD11c+ DC were purified from GM-CSF- and IL-4-treated BM cultures of Csf-1-op/op mice as described above. Due to the low yield, BM cells from at least five to seven op/op mice were pooled to generate sufficient numbers of CD11c+ DC, which were then cocultured with WT CD4+ T cells and Aa-Ags with or without 25 ng/ml rmM-CSF. The results showed that Csf-1-op/op CD11c+ DC did not develop TRAP or resorptive pit activity when compared with WT (Fig. 5, B and C). The addition of optimal rM-CSF (25 ng to 25 g/ml titrated and tested) significantly restored both TRAP and resorptive activities (Fig. 5, B and C). Furthermore, replacement of CD4+ T cells with...
sRANKL yielded TRAP and resorptive activities comparable to those seen in the presence of T cells, only when M-CSF was added into the cocultures (Fig. 5, B and C). In parallel, the results of SIDIA further supported the above findings as shown by an expression profile similar to those of WT DDOC (Fig. 5D).

To confirm the contribution of M-CSF during CD11c⁺ DC’s development into OC, we cocultured WT CD11c⁺ DC with Csf-1<sup>-/-</sup> op/op-derived CD4<sup>+</sup> T cells and Aa. Interestingly, WT CD11c⁺ DC were capable of developing into TRAP⁺ functional OC under these conditions (data not shown). Csf-1<sup>-/-</sup> op/op DC, on the other hand, did not develop TRAP activity or resorptive pits unless exogenous M-CSF was added in their coculture with T cells or sRANKL and resorptive pits unless exogenous M-CSF was added in their coculture with T cells or sRANKL and Aa (Fig. 5, B and C). These findings are consistent with the current understanding that RANKL is essential for the differentiation and activation of bona fide OC, whereas M-CSF is required for the development and survival of OC precursors (1–4, 6, 13, 15).

**CD11c⁺ DC-derived OC can induce bone resorption in vivo**

To assess whether DDOC can induce bone resorption in vivo, not just in vitro, CFSE-labeled WT CD11c⁺ DC were cocultured in vitro as described above for 2.5 to 3 days before injection onto the periosteum of NOD/SCID mice calvarias. This protocol allowed the in situ detection of labeled cells postinjection. The results of histological and immunohistochemical analyses revealed that injection of PBS-only, CD4<sup>+</sup> T cells alone or CD11c⁺ DC alone (Aa stimulated or not) did not induce bone resorption in vivo (Fig. 6, A, B, and F). In contrast, CFSE-labeled CD11c⁺ DC from DC+T+Aa expressed TRAP activity postadoptive transfer (Fig. 6, C, D, E, H, G, H, and I) and induced morbidly higher bone resorption than the control groups (see the eroded bone surfaces highlighted on the histological sections of Fig. 6, F and G). Moreover, these cells maintained CD11c expression in vivo. This finding suggests that, after proper stimulation, CD11c⁺ DC can develop into activated OC capable of resoring bone in vivo. However, whether DDOC represent a physiological process of osteoclastogenesis in vivo is unclear and awaits further study.

**Discussion**

DC are a heterogeneous population of leukocytes whose immune functions have been studied extensively (21–27). It is evident that: 1) CD11c⁺ DC are present in the inflamed synovia of RA (34, 35); and 2) DC/T cell interactions play a pivotal role in inflammatory bone disorders such as RA, PD (34–38), and possibly osteomyelitis. In this study, we investigated the ability of murine CD11c⁺ DC to develop into functional OC during immune interactions with CD4<sup>+</sup> T cells and foreign Ags. Our findings indicate a potentially critical contribution of CD11c⁺ DC subset(s) to elevated osteoclastogenesis associated with inflammatory bone disorders where they may not only act as potent APC for the activation and regulation of immunity, but also as OC precursors, directly involved in bone resorption.
We characterized DDOC phenotype by FACS, and the results were consistent with those of IF at single cell level by SIDIA, suggesting that FACS can be applied to study the phenotypic changes of DDOC. The results from M-CSF blocking, coculturing WT DC with csf-1−/− op/op-derived T cells and Aa (data not shown), and the M-CSF rescue studies indicate a stage-specific requirement of M-CSF “before” their transition to OC upstream of RANKL-RANK signaling. Exposure of OC precursors to M-CSF has been shown to be essential for the development of their osteoclastogenic potential possibly by up-regulating RANK expression (1, 6). Alternatively, one may suspect the presence of few contaminating precursors among WT DC that are absent in Cs-f-1−/− op/op DC cultures. The CD11c+ DC subset studied here is highly pure since: 1) it has been shown that CD11c is not expressed on early precursors (49); and 2) the presence of both MQ (CD11b+ and F4/80+) and Mo (ER-MP12+ and ER-MP20+) contaminants were excluded (Fig. 3B). Thus, this alternative possibility is unlikely.

In contrast to the proposed model by Miyamoto et al. (28), where “committed” DC derived from common DC/OC progenitors lack the ability to develop into OC, our data demonstrate CD11c+ DC development into functional multinucleated TRAP+ CT-R+ cathepsin-k+ MHC-II+ OC, whereby they acquire the ability to induce bone resorption not only in vitro but also in vivo (Figs. 2 and 6), suggesting that they posses certain developmental plasticity and may represent an alternative OC differentiation pathway. The molecular mechanisms responsible for such development appear to involve primarily RANKL/RANK signaling in addition to Ag activation and possibly stimulation via TLR. Various effects of LPS on osteoclastogenesis in the presence or absence of OB and stromal cells have been reported, although the precise effects remain unclear (52–54). By using BM-derived Mo, it has been shown recently that LPS stimulation of OC precursors could result in inhibition or enhancement of osteoclastogenesis depending on their differentiation stage (55). In the present study, Aa-Ags and OMP-1, likely containing traces of LPS, and LPS-free BI protein all yield comparable TRAP and resorptive activities (Fig. 3A). Second, there was no detectable TRAP or resorptive activities in CD11c+ DC when cocultured with E. coli-derived LPS in the presence or absence of sRANKL (data not shown). These findings argue against a direct role for LPS in OC development in our system, in agreement with the report by Takami et al. (56), where LPS directly inhibits osteoclastogenesis from OC precursors. Studies are currently underway to examine the role of TLR on osteoclastogenesis using MyD88−/−CD11c+ DC in our coculture system.

The cellular signaling pathways of RANKL/RANK in OC and/or OC precursors involve TNFR-associated factors, p38 MAPK, ERK, and JNK, and downstream transcription factors such as NF-κB and c-Fos (57). However, it remains unclear whether the same pathways are also involved in OC development from CD11c+ DC. Moreover, multiple Ig-like receptors associated with ITAM-harboring adaptors, FcR common γ-chain and DNAX-activating protein 12, have been implicated recently in osteoclastogenesis, which further strengthens the link between the immune system and bone remodeling (58). Whether these factors play any role in the development of DDOC remains to be explored. Genetic analyses are being pursued to study the signaling molecules in CD11c+ DC responsible for the induction of osteoclastogenesis leading to OC development.

Rivollier et al. (30) recently demonstrated that human circulating blood Mo-derived DC were capable of transdifferentiating into bone resorbing OC in the presence of M-CSF and RANKL in vitro. Our study clearly indicates that interactions of murine BM-derived and splenic CD11c+ DC with CD4+ T cells in the bone environment can promote DDOC development in response to microbial or protein Ags and RANKL-RANK signaling (Fig. 2). Although in both studies DDOC develop under different conditions and kinetics, they appear to carry similar phenotypes with some differences (see Table II: murine DDOC are CD86+ CD11b+ with dendrites). Nevertheless, based on the results of both studies and findings by Page et al. (59), it is reasonable to speculate that DDOC may develop not only in the presence of M-CSF and RANKL produced by OB, synovial, and stromal cells (60), but also in response to RANKL produced by inflammatory T cells and stimulatory Ags during immune interactions in the bone environment.

In summary, we report the development of functional OC from murine CD11c+ DC subset(s) capable of inducing bone resorption in vitro and in vivo. At present, it is unclear whether all DC subsets can become OC during interaction with T cells in vivo and whether this development is a function of environmental stimuli such as Ags and/or cytokines in the microenvironment. Nonetheless, our data suggest that T cells can support OC development from DC in the context of immune interactions. The dynamic process of OC differentiation, development, and activation from CD11c+ DC through orchestrated M-CSF and RANKL signaling and immune cell interactions open new avenues for future research. This DC plasticity supports a link between innate immunity and osteoclastogenesis, beyond the current paradigm of osteoimmunology, and further our understanding of the immune interactions potentially involved in bone remodeling under pathological conditions such as RA, PD, osteomyelitis, and other inflammatory bone disorders.

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Disclosures

The University of Rochester has submitted a patent application for CD11c DC development to form OC functional in the bone environment and has listed Y.-T.A. Teng as the inventor.

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