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Perspective

Distinct regulation of myeloid dendritic cell-derived osteoclast precursor (mDDOCp) invokes cytokine milieu-mediated signaling: A new insight into the twist-in-turns of osteoclastogenesis

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Dendritic cells (DCs) bear robust antigen-presenting functions, acting like natural-borne adjuvant to triggering antigen-specific naïve-T-cell responses, besides stimulating and orchestrating the innate-vs.-adaptive immunity and downstream sequelae. To date, contributions of DCs, from their ontogeny to immune-regulations, have involved wide arrays of homeostatic interactions vs. balance or dysregulation of bone remodeling at the skeletal/osteo-immune interface [i.e., Receptor-activator-of-nuclear-factor-kappa-B-ligand (RANKL)-Receptor-activator-of-nuclear-factor-kB (RANK)-Osteoprotegerin (OPG)-triad, *etc.*], implicating in arthritic and osteoporotic conditions such as osteoporosis and periodontitis, *etc.*[1] It is clear that classical osteoclasts (OCs) derive from the myeloid lineage of hematopoietic stem cells in bone marrow (BM; i.e., monocytes/Mo & macrophages/Mϕ); yet, DCs are heterogeneous with plasticity, whose specific subsets can develop into functional OCs and precursors (OCp) both in murine and human, *in-vitro* & *in-vivo*, suggesting the alternative pathway for osteoclastogenesis.[1–3]

It is evident that RANKL/RAMK-OPG-triad is critically responsible for OC development and osteoclastogenesis, whose adaptor called:TRAF6 (Tumor-necrosis-factor-receptor-associated-factor-6), is principally involved in immune-osteotropic signal-cascades, such as: TNF-receptor (TNFR),Toll-like-receptor (TLR)/Interleukin-1-receptor (IL-1R), Transforming-growth-factor-β-receptor-1 (TGF-βR1), T-cell-receptor (TCR) & Toll/IL-1-receptor-domain-containing-adaptor (TRIF) via IL-1R-associated-kinase (IRAK), TGF-β-activated-kinase (TAK), Interferon-regulatory-factor (IRF), Mitogen-activated-protein-kinases (MAPK), Apoptosis-signal-regulating-kinase (ASK), Inhibitory-kB-kinase (IKK)/Mitogen-extra-cellular-activated-protein-kinase-kinase (MEKK), Phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K), CCAAT/enhancer-binding-protein-βδ(C/EBPβδ), NFκB-activator-1(Act1), Myeloid-differentiation-factor-88 (MyD88), *etc.*, intermediate-pathways before triggering transcriptional factors for gene activations.[4] We have reported that murine BM/spleen-derived "immature"-myeloid-DC precursors carrying $CD11c^{+}$-$CD11b^{-}$F4/$80^{-}$$Ly6C^{-}$$CD31^{-}$MHC-$II^{-/or/low}$CD80/$86^{-}$-phenotype are capable of developing to active OCs (termed: mDDOCp) bearing $TRAP^{+}$-CT-R^{+}-Cathepsin-K^{+}-$RANK^{+}$-GM-$CSFR^{-}$-Integrin-$\alpha_v\beta_3^{+}$ hallmarks *in-vitro* & *in-vivo* to resorbing bone in a RANKL/RANK-dependent manner, where their differentiation signals vs. kinetics & morphology carrying multinucleation/>3-nuclei with dendrites are notably unique.[1,3–5] Importantly, once committed to be maturing upon-activation or microbial-stimulation (i.e., TLRs/LPS-signals), mDDOCp loses the osteoclastogenic potential.[6] Further, via genome-wide microarray-screening, confirming the resultant TGF-βRII on differential-signaling and parallel-neutralization assays *in-vitro*-&-*in-vivo*, we pioneered that endogenous TGF-β is critically involved in developing mDDOCp, as OCp, once passing beyond M-CSF/c-FMS-mediated survival-signals.[1,3,5] Herein, to explore and decipher the molecular interactions and role of TGF-β/TGF-βRII signaling in mDDOCp (as OCp without any influence from Mo/Mϕ-derived classical-OCs) for osteoclastogenesis, which remains unclear to date, we employed our established protocols to generate "immature"-$CD11c^{+}$mDDOCp (>98-99%-pure) lacking TRAF6-mediated signaling in BM/splenic-cells (termed: T6KO_BMChi-DC) prepared from >6-wk-old C57BL/6-chimeric mice post-lethal-irradiations and reconstituted with BM-or-fetal-liver cells of 4-to-6-wk-old toothless-$TRAF6^{(-/-)}$-mice *in-vivo* (via genomic-screening of the bred-offspring from $TRAF6^{(+/-)}$-breeding-pairs),[7] then subjected to co-cultures with-or-without syngeneic/splenic-naïve-$CD4^{+}$T-cells (or mRANKL:50—100 ng/ml) and *Aggregatibactor Actinomycetemcomitans*/JP2-strain sonicate-Ag (in-short: *Aa*-Ag), where exogenous mTGF-β vs. anti-TGF-β-neutralizing-Mab or mIL-17 was added individually for 4.5—5 days *in-vitro*, followed by enumerating surface areas of $TRAP^{+}$-$CD11c^{+}$DC/mm^2 in resorptive-pits.[1,3–5] Immature $CD11c^{+}$-mDDOCp prepared from wild-type $TRAF6^{(+/+)}$-mice (termed: WT_BM-DC) were set as the control, and both splenocytes-&-Con-A vs. WT_BM-DC-cells plus mM-CSF-&-mRANKL in co-cultures were employed as positive-control, individually (Fig. 1: upper colored-panel, lower panel of bar-diagrams & Fig-1-legend).

Interestingly, the results showed that: **i)** T6KO_BMChi-DC-cells (as mDDOCp prepared from BM/spleen of chimeras reconstituted with $TRAF6^{(-/-)}$-BM/fetal-cells without Mo/Mϕ-derived-OCs *in-vivo*) co-cultured with mRANKL-&-mM-CSF did not produce or develop any significant $TRAP^{(+)}$>3-multinucleated OC-like activities, compared to that of WT_DC did *in-vitro* [by surface-areas: upper-&-lower panels labeled -3 & -2)], suggesting TRAF6-&-related signaling-cascades were associated with mDDOCp-mediated osteoclastogenesis; **ii)** while WT-DC + T-cells + *Aa*-Ag & WT-DC + RANKL + *Aa*-Ag yielded comparably high-level of robust $TRAP^{(+)}$-mDDOCp/OC activity [Fig. 1: label-6 & -7], compared to those of significantly lowered $TRAP^{(+)}$DC-measured in label-8) when pan-specific anti-TGF-β-neutralizing-Mab was added into co-cultures of label-6/7) [$P = 0.025$], as to that of label-7) when WT_BM-DC cells were replaced with T6KO_BMChi-DC-cells *in-vitro* [Fig. 1: label-9; $P = 0.002$], consistent with our prior findings that TGF-β is critically involved in developing mDDOCp activity at OCp-stage;[1,3,7] **iii)** adding mTGF-β into T6KO_BMChi-DC + RANKL + *Aa*-Ag co-cultures (Fig. 1: label-10) robustly rescued the lowered $TRAP^{(+)}$-DC activity detected in label-9) [$P = 0.006$]; whereas, adding mIL-17 (Fig. 1: label-11; $P = 0.041$) further enhanced such lowered $TRAP^{(+)}$-DC activity measured (in label-9), significantly higher than that of label-10), suggesting that TGF-β and IL-17 individually or synergistically mediated TRAF6-independent rescue-signaling onto mDDOCp development; **iv)** further, addition of anti-TGF-β-neutralizing-Mab (Fig. 1: label-12) in co-cultures of label-11 or replacing mRANKL with naïve-$CD4^{+}$T-cells in the presence of *Aa*-Ag for activation (Fig. 1: label-13) significantly reduced $TRAP^{(+)}$-DC activity detected in co-cultures of lable-11 [$P = 0.008$], suggesting that IL-17-signal for mDDOCp development, as detected in label-11, required TGF-β in its environmental milieu indeed, regardless the presence of $CD4^{+}$T-cells that, post-*Aa*-Ag-activation, expressed RANKL or other osteotropic factors *in-situ*.[1]

The above results are consistent with prior-findings from others, where: i) there is surely alternative routes for OCp differentiation independent of RANKL/RANK-TRAF6-axis,[4,7] ii) TGF-β is required to prime un-committed OCp rendering RANKL-mediated osteoclastogenesis,[8] iii) certain DCs subsets can act like OCp via an alternative pathway of developing into OCs *in-vitro*-&-*in-vivo*.[2,7,8] Intriguingly, our new findings supported that TGF-β/TGF-

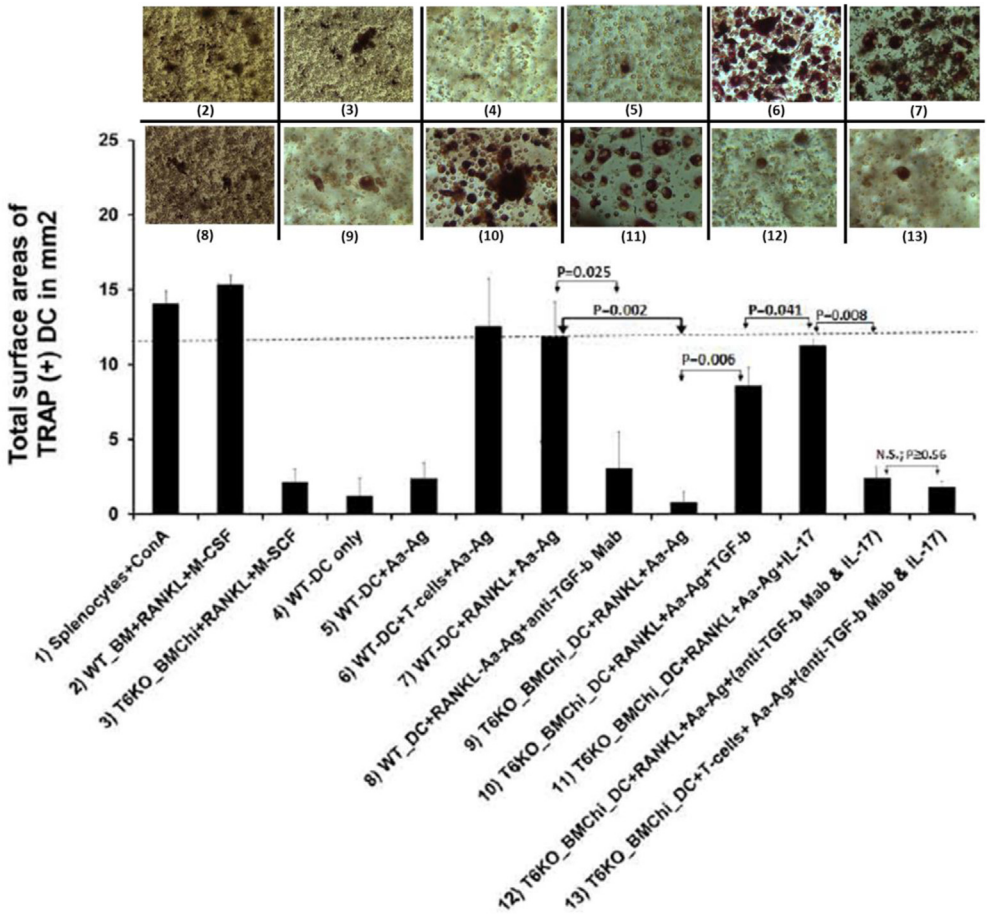

Figure 1 Exogenous TGF-β vs. IL-17 provides rescue-signaling separately or synergistically sufficient to driving mDDOCp-mediated osteolastogenesis, devoid of TRAF6-&-related immune-osteotropic signals (without Mo/Mϕ-derived endogenous OCs *in-vivo*), as shown by $TRAP^{(+)}$-DC staining *in-situ* (deep red-purple color; see the colored top-panel labeled 2—13) and quantitative surface-areas (in mm^2) measured on HA-coated-48/well-plates for 4.5-5-days *in-vitro* (see the lower-panel of bar-diagrams labeled 1—13).[1,3—5] Details of the cells obtained/collected, culture reagents & materials sources on the protocols/ conditions employed in the present study for co-cultures of ~3 or ~4.5—5 days on HA-coated 48/well-plates (OCT-Co., CA, USA) via the automated enumerations [for the signals of $TRAP^{(+)}$-DC-staining & surface-areas of resorptive pits in-mm^2 under 150× magnification; by the mean ± SEM] have all been reported previously.[1,3—5,7] The results presented were from five independent experiments in triplicates/group, where they are briefly outlined in labels of 1-to-13, as follows: **1)** 5×10^5 mouse splenocytes & Con-A (5 μg/ml) co-cultured for 3-days, and **2)** 2×10^5 WT_BM-DC-cells (98—99% pure) plus soluble rmM-CSF (25 ng/ml) & rmRANKL (50—100 ng/ml) co-cultured for 4.5-5-days were set as the positive-controls; **3)** 2×10^5 T6KO_BMChi-DC cells (prepared from 6-wk-old mouse chimeras post lethally-irradiated & reconstituted with BM-or-fetal-liver cells of 4-6-wk-old toothless-TRAF6$^{(-/-)}$-mice[7]) co-cultured with soluble rmRANKL (50—100 ng/ml) & rmM-CSF (25 ng/ml) for 4.5-5-days; **4)** 2×10^5 WT-DC-cells in co-cultures for 4.5-5-days, as background control; **5)** 2×10^5 WT-DC cells alone in co-cultures for 4.5-5-days, as background control; **6)** 2×10^5 WT-DC cells plus 5×10^5 syngeneic splenic-$CD4^+$T-cells (95—97% pure; in 1:2.5 ratio) & *Aa*-Ag (10 μg/ml) were co-cultured for 4.5-5-days, and **7)** 2×10^5 WT-DC cells plus soluble rmRANKL (50—100 ng/ml) & *Aa*-Ag (10 μg/ml) co-cultured for 4.5-5-days, each yielding robust level of $TRAP^{(+)}$-mDDOCp/OC activity developed and set as positive-control for experimental comparison; **8)** 2×10^5 WT-DC cells plus soluble rmRANKL (50—100 ng/ml) & rmM-CSF (25 ng/ml) co-cultured in the presence of pan-specific anti-mTGF-β neutralizing-Mab (100 ng/ml; R&D Systems) for 4.5-5-days; **9)** 2×10^5 T6KO_BMChi-DC cells (same protocols as prepared in label-3 above) co-cultured with soluble rmRANKL (50—100 ng/ml) & *Aa*-Ag (10 μg/ml) for 4.5-5-days; **10)** 2×10^5 T6KO_BMChi-DC cells (same protocols as prepared in label-3/-9 above) co-cultured with soluble rmRANKL (50—100 ng/ml) & *Aa*-Ag (10 μg/ml) in the present of soluble rmTGF-β (50—100 ng/ml) for 4.5-5-days; **11)** 2×10^5 T6KO_BMChi-DC cells (same protocols as in label-3/-9/-10 above) co-cultured with soluble rmRANKL (50—100 ng/ml) & *Aa*-Ag (10 μg/ml) in the presence of mIL-17A (25 ng/ml; R&D Systems) for 4.5-5-days; **12)** 2×10^5 T6KO_BMChi-DC cells (same protocols as in label-3/-9/-10 above) co-cultured with soluble rmRANKL (50—100 ng/ml) & *Aa*-Ag (10 μg/ml) in the presence of soluble rmTGF-β (50—100 ng/ml) & mIL-17A (25 ng/ml) for 4.5-5-days; **13)** 2×10^5 T6KO_BMChi-DC cells (same protocols as in label-3/-9/-10-12 above) were co-cultured with 5×10^5 syngeneic/ splenic-$CD4^+$T-cells (95—97% pure) and *Aa*-Ag (10 μg/ml) in the presence of soluble rmTGF-β (50—100 ng/ml) & mIL-17A (25 ng/ml) for 4.5-5-days. Later, the statistical analyses were employed using two-sided Student t-test via the IBM computing software SPSS-Statistics (SPSS 22, IBM Corp. USA) and the differences between groups were considered significantly different with >95% confidence, when p-value was <0.05. **Note:** The isotypic-control Mab applied (to that of anti-TGF-β neutralizing-Mab tested above) did not affect the resultant $TRAP^{(+)}$-DC staining and quantitative surface-areas (in mm^2) measured *in-situ*, thus were not shown here (data not shown).

βRII, besides IL-17/IL-17Rs, can transduce somewhat rescue-signals in immature-OCp, mDDOCp; where RANKL/RANK-TRAF6 and related-signaling complexes are notably dispensable (Fig. 1: label 9—13), since mDDOCp manifest maturing phenotype(s) post-activation (i.e., being-$CD11b^{+}F4/80^{+}$GM-CSFR^{-}MHC-$II^{+/hi}$, *etc.*),[5] such alternative pathway towards osteoclastogenesis becomes un-permissible.[5,6] Whether TGF-β vs. IL-17-mediated rescue-signaling in mDDOCp play any roles in homeostatic or pathological bone remodeling-&-osteoclastogenesis *in-vivo* will require further study; we already reported comparable $CD11c^{+-}$multinucleated-$TRAP^{+}$OC-like/mDDOCp existed and significantly involved in type-II collagen-induced rheumatoid-arthritis in DBA mice.[1]

TRAF6 is a key-adaptor protein to transducing RANKL/OPG-RANK triad-signals that regulate not only OCs pathophysiology in skeleton, but also wide ranges of immune-vs.-non-immune interactions, including: thymic selection & lymph-organogenesis, B-/Th-cells/T-reg tolerance & memory-responses, myeloid-lineage (Mo-Mϕ & DCs) differentiations, *etc.*[4,7,9] Paradoxically, upon TRAF6-&-related signaling deficiencies, IL-17/IL17Rs vs. TGF-β/TGF-βRII may transduce via other adaptors, including IL-17Rs-TRAF2/5/4-ACT1 by non-canonical signaling vs. TGF-βRII-SMAD2/3 or -RhoA vs. -JAK2/STAT3 or -ALK1/SMAD1/5 intermediates, respectively, for downstream events, where optional actions by direct-positive vs. indirect-negative modes or differential sensitivity to TGF-β through IL-17-inhibitory-signals have been suggested.[8—10] For instance, we showed that JAK/STAT-mediated pro-inflammatory pathway via SOCS3-signaling significantly regulate the development of $CD11c^{+}$-mDCs into TRAP (+)-mDDOCp/OCp for osteoclastogenesis, independent of TRAF6-expressions;[10] yet, potential inter-players, i.e., TNF-α/IL-6, chemokines, *etc.*, for the scenarios described above might be selective and await closer and thorough investigations.

In summary, DCs are borne-heterogeneous, whose certain subsets are immature with plasticity, acting as OCp (e.g., mDDOCp), thereby developing into OCs via an alternative pathway for inflammation-induced osteoclastogenesis.[1,3,8] Importantly, the novel finding presented provide a new insight, where distinct regulation of $CD11c^{+}$-mDDOCp deficient of TRAF6-&-related immune-osteotropic signaling invokes TGF-β-&-IL-17-mediated rescue-signals in the environmental milieu sufficient to driving bona-fide osteoclastogenesis. Such permissive or non-discriminative twist-in-turns pathway(s) will require further study to address its *in-vivo* significance through animal models and human conditions.

Author Contributions

Writing- original draft & revisions, Yen Chun G. Liu and Andy Yen-Tung Teng; Writing-review & editing, Yen Chun Grace Liu and Andy Yen-Tung Teng. YCGL was involved in the study designs, data acquisition, first draft of the manuscript write-up, analyses and revisions of the figures and the manuscript. A Y.-T. T. was involved in all aspects of the study design, protocols, establishments and its modifications along with discussions for analyses, interpretations and the overall issues of the entire project. All authors have read and agreed to the published version of the manuscript.

Approval of the animal use

The present project involving the lab animals was conducted according to the guidelines of animal protection/welfare and use, which was approved for protocol of IACUC #98017 & #98183, by the Institutional Animal Care & Use Committee, Kaohsiung Medical University, Kaohsiung city, Taiwan.

Data availability statement

The data employed in present report is available via the email request to the corresponding author as listed via andytengyt@yahoo.com.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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References

1. Alnaeeli M, Teng AY-T. Dendritic cells: a new player in osteoimmunology. *Curr Mol Med* 2009;9:893—910.
2. Miyamoto T, Ohneda O, Arai F, et al. Bifurcation of osteoclasts and dendritic cells from common progenitors. *Blood* 2001;98:2544—54.
3. Alnaeeli M, Park J, Mahamed D, Penninger JM, Teng AY-T. Dendritic cells at the osteo-immune interface: implications for inflammation-induced bone loss. *J Bone Miner Res* 2007;22:775—80.
4. Walsh MC, Lee JE, Choi YW. Tumor necrosis factor receptor associated factor 6 (TRAF6) regulation of development,

function, and homeostasis of the immune system. *Immunol Rev* 2015;266:72—92.
5. Alnaeeli M, Penninger JM, Teng AY-T. Immune interactions with $CD4^{+}$T cells promote the development of functional osteoclasts from murine CD11c+dendritic cells. *J Immunol* 2006;177:3314—26.
6. Alnaeeli M, Teng AY-T. Dendrtic cells differentiate into osteoclasts in bone marrow microenvironment *in vivo*. *Blood* 2009; 113:264—5.
7. Kim N, Kadono Y, Takami M, et al. Osteoclast differentiation independent of the TRANCE—RANK-TRAF6 axis. *J Exp Med* 2005;202:589—95.
8. Fox SW, Lovibond AC. Current insights into the role of transforming growth factor-beta in bone resorption. *Mol Cell Endocrinol* 2005;243:19—26.
9. Amatya N, Garg AV, Gaffen SL. IL-17 signaling: the yin and the yang. *Trends Immunol* 2017;38:310—22.
10. Zhang X, Alnaeeli M, Singh B, Teng Y-TA. Involvement of SOCS3 in the regulation of $CD11c^{+}$ dendritc cells-derived osteoclastogenesis and severe alveolar bone loss. *Infect Immun* 2009;77: 2000—9.