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Effector memory regulatory T cells were most effective at suppressing RANKL but their frequency was downregulated in tibial fracture patients with delayed union

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ABSTRACT

Delayed union and nonunion occur in a minor subject of bone fractures, presenting ongoing challenges to treatment. RANKL, which promotes the differentiation of bone-resorbing osteoclasts, is thought to negatively impact bone healing. In this study, we recruited patients with isolated closed tibial fracture, who were later categorized into normal healing and delayed healing groups based on their healing progression. The regulatory T cell (Treg) compartment was then investigated in each patient. Based on CD45RA and CD62L expression, we distinguished circulating Treg cells into $CD45RA^+CD62L^+$ naive (N), $CD45RA^-CD62L^+$ central memory (CM), and $CD45RA^-CD62L^-$ effector memory (EM) subsets. Compared to normal patients, delayed patients presented significantly lower EM Treg proportion and significantly higher N Treg proportion. Among the N, EM, and CM Treg cells, the EM Treg cells were the most potent at suppressing RANKL expression in T conventional (Tconv) cells. This functionality of EM Treg cells was present in both normal healing patients and delayed healing patients, and was dependent on IL-10, as neutralization of IL-10 resulted in significantly elevated RANKL expression. EM Treg cells presented the highest IL-10 and TGF-β expression directly ex vivo, as well as after anti-CD3/anti-CD28/IL-2 stimulation. CM Treg cells did not present high expression of inhibitory cytokines ex vivo, but was capable of upregulating cytokine expression upon stimulation. N Treg cells, on the other hand, presented limited capacity to upregulate inhibitory cytokines. In summary, our study identified that, while the EM Treg cells were the most effective at suppressing RANKL, they in delayed union patients were present at lower frequencies with functional impairment, resulting in decreased RANKL suppression. Hence, bone-resorbing osteoclast formation may be favored in these patients thus suggesting a possible mechanism for delayed bone healing.

1. Introduction

Homeostasis of the bone is maintained through balanced actions between the bone-forming osteoblasts and bone-resorbing osteoclasts, in which bone-forming osteoblasts are derived from mesenchymal stromal cells (MSCs) and express RANKL, whereas bone-resorbing osteoclasts are derived from monocytes upon receiving RANKL signaling and ICAM-1-mediated adhesion with osteoblasts [1,2]. Upon bone injury, such as fracture resulting from mechanical stress, actions from osteoblasts are required for remodeling. However, adaptive immune cells are also activated at the site of injury and may hamper bone repair due to their capacity to induce the expression of RANKL and ICAM, which favor osteoclasts [2]. In patients with tibia fracture, enrichment of $CD8^+$ T cells was associated with delayed osteogenesis, attributed to the secretion of TNF-α and IFN-γ [3]. Adoptive transfer of $CD8^+$ T cells impaired hone healing in a murine osteotomy model, while depletion of $CD8^+$ T cells enhanced bone regeneration [3]. T cells isolated from periodontitis patients overexpressed RANKL and TNF-α [4]. In vitro cell culture experiments illustrated that T cells could support the differentiation of osteoclasts from monocytes in a RANKL- and TNF-α-dependent fashion [4–6].

The regulatory T (Treg) cells are a specialized group of $CD4^+$ T cells, whose main functions are to maintain tolerance, regulate innate and adaptive immunity, enhance tissue repair, and inhibit pathogenic

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inflammation [7]. Mounting evidence indicates that Treg cells may promote bone repair. Compared to wild-type mice, Foxp3-transgenic mice developed higher bone mass and presented impaired osteoblast formation [8]. RAG-1$^{-/-}$ mice receiving CD4$^+$CD25$^+$ T cells also presented higher bone mass [8]. These Treg-mediated promotion of osteogenesis may depend the suppression of inflammation by conventional T cells, as human Treg cells suppressed osteoclast differentiation from peripheral mononuclear cells (PBMCs) via suppressive cytokines TGF-β and IL-4 in vitro [9]. Additionally, Treg cells may directly promote bone marrow MSC-based induction of osteoblasts via the inhibition of TNA-α and IFN-γ [10,11].

In human patients with long bone fracture, we demonstrated that the expression of LAG-3, a molecule enriched in Foxp3$^+$ Treg cells, was positively correlated with the restoration of healthy bone function [12]. Conversely, patients with impaired healing presented dysregulated Treg functions, characterized by lower TGF-β, CTLA-4, and LAG-3 expression [13]. Here, we investigated impact of effector memory Treg cells in bone fracture repair.

2. Methods

2.1. Patients

This study received ethical approval from Jinling Hospital Ethics Board and was performed in accordance with the Declaration of Helsinki. Thirty-four patients with single isolated closed tibial fracture due to mechanical stress were recruited at Jinling Hospital. These patients included 10 female subjects and 24 male subjects between 48 and 68 years of age at the time of fracture. All patients were cigarette and alcohol users. Patients with inflammatory arthritis, severe osteoporosis, multiple fractures, liver diseases, kidney diseases, and other diseases characterized by aberrant inflammation, such as diabetes, malignancies and autoimmunity, were excluded. All patients received intramedullary nailing as the main treatment option. Two experienced radiologists performed the X-ray analysis of the healing process, and walking speed test was performed by independent, blinded experimenters. Finally, the experimenters were blinded of the healing status of each sample. Peripheral blood was collected on the day before surgery. PBMCs were harvested using standard Ficoll (Sigma Aldrich) method.

2.2. Flow cytometry

PBMCs were labeled with Fixable Aqua Dead Cell Stain (Life Technologies) and fluorochrome-conjugated anti-human CD3, CD4, CD25, CD45RA, and CD62 L monoclonal antibodies (BioLegend) by incubation for 30 min on ice. PBMCs were washed, and Foxp3 was labeled using Foxp3/Transcription Factor Staining Set (eBioscience). Treg cells were identified via live lymphocyte gating on FCS vs. SSC and Aqua-negative, followed by CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ gating. A minimum of one million PBMCs were acquired for each sample.

2.3. Tconv and Treg fractionation

Untouched CD4 + T cells were isolated from PBMCs using CD4$^+$ T Cell Isolation Kit, human (Miltenyi Biotec). Cells were then labeled with fluorochrome-conjugated anti-human CD25, CD45RA, and CD62 L monoclonal antibodies for 30 min on ice. After washing, Tconv (CD25$^-$) cells, Treg (CD25$^+$) cells, N Treg (CD25$^+$CD45RA$^+$CD62L$^+$) cells, EM Treg (CD25$^+$CD45RA$^-$CD62L$^-$) cells, and CM Treg (CD25$^+$CD45RA$^-$CD62L$^+$) cells were sorted in FACSAria cytometer (BD Biosciences). Categorization of Treg subsets were performed according to the study by Lei et al [14].

2.4. CD4$^+$ T cell incubation

Treg cells and Tconv cells were incubated in unstimulated complete media or media supplemented with 2 μg/mL anti-CD3 antibody OKT3, 2 μg/mL anti-CD28 antibody CD28.2 (BioLegend), and 50 U/mL recombinant human IL-2 (Novus Biologicals). Human TGF-β blocking antibody MM0446-6G14 (Novus Biologicals) and human IL-10 blocking antibody 23738 (R&D Systems) were added when indicated. CD4$^+$ T cell density was at 5×10^4 cells per mL. Incubation condition was 5% CO_2, 100% humidity, and 37 °C. After incubation, the supernatant and the cells were separated via centrifugation at 300 *g* for 5 min. ELISA was performed using the TGF-β1 Human ELISA Kit and the IL-10 Human ELISA Kit (Invitrogen). Three independent replications were performed for each condition.

2.5. mRNA level

Total RNA was collected using RNeasy Mini Kit (QIAGEN), and was used for cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The following TaqMan gene expression assays were used, including TGF-β Hs00998133_m1, IL-10 Hs00961622_m1, RANKL Hs00243522_m1, and β_2M Hs00187842_m1 (Thermo Fisher Scientific). PCR assays were performed in 7500 Fast Real-Time system (Applied Biosystems). Three independent experiments were performed for each condition, and the average result was presented.

2.6. Statistical analysis

All tests were performed using Prism software (GraphPad). The results were considered significantly different if the p value (two-tailed) was smaller than 0.05. The specific tests applied were indicated in the figure legends.

3. Results

3.1. Characteristics of normal and delayed union patients

To investigate Treg cells, we recruited 34 individuals with a single isolated closed tibial fracture at similar severity level, as assessed by experienced surgeons. The PBMCs were collected from each patient on the day before surgery. The healing progress was followed for 18 weeks after surgery. By week 12, patients that demonstrated structure restoration, as confirmed by radiological examination, were classified as the normal healing group, while patients whose healing was delayed or non-progressing were classified as delayed healing group. The delayed group was matched with the normal group in age (Fig. 1A), and received the same type of operation, but demonstrated significantly lower walking speed and higher self-described pain while walking (Fig. 1B and C).

3.2. Effector memory Treg cells were downregulated in delayed union patients

Next, we examined the characteristics of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells from the PBMCs of normal patients and delayed patients. The number of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells per 1×10^6 PBMCs was not significantly different between normal and delayed patients. CD62 L, or L-selectin, enables T cell entry into secondary lymphoid organs via high endothelial venule, and CD45RA is an isoform of CD45 expressed by naive T cells. Using CD45RA and CD62 L expression, circulating Treg cells can be distinguished into CD45RA$^+$CD62L$^+$ naive (N), CD45RA$^-$CD62L$^+$ central memory (CM), and CD45RA$^-$CD62L$^-$ effector memory (EM) subsets (Fig. 2A) [14]. The mean fluorescence intensity (MFI) of CD45RA in N Treg cells from delayed patients was significantly higher than the MFI of CD45RA in N Treg cells from normal patients (Fig. 2B). Also, the frequency of N Treg cells was significantly higher in delayed patients than in normal patients (Fig. 2C). In contrast, the frequency of EM Treg cells was significantly lower in

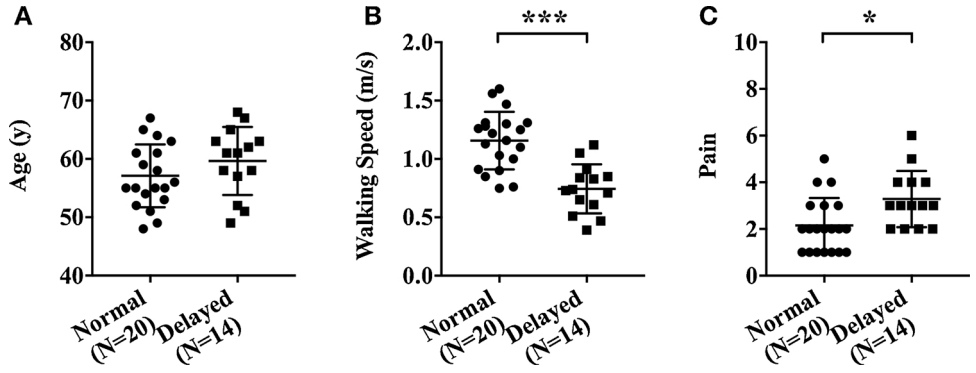

Fig. 1. Age and healing characteristics of patients with normal or delayed healing.
(A) Age of the patients at the time of tibial fracture. (B) Walking speed at week 12 following operation. (C) Self-described pain score (0: none to 10: maximum) during walking at week 12. Unpaired *t*-test *p < 0.05. ***p < 0.001.

delayed patients than in normal patients (Fig. 2D). The frequency of CM Treg cells, on the other hand, was comparable between normal and delayed patients (Fig. 2E).

Subsequently, the numbers of N Treg, EM Treg, and CM Treg cells per 10^6 PBMCs were calculated for each study participant. The numbers of N Treg cells and CM Treg cells were comparable between normal patients and delayed patients (Fig. 2F and 2 H), while the numbers of EM Treg cells were significantly higher in normal patients than in delayed patients (Fig. 2G).

3.3. EM Treg cells were most capable of rapid response and cytokine secretion

To examine the functional characteristics of various subsets of Treg cells, we analyzed the level of TGF-β and IL-10 gene expression by fractionated N-Treg, EM-Treg, and CM-Treg cells. Directly ex vivo, N Treg cells and CM Treg cells presented very little TGF-β gene expression, while the EM Treg cells presented moderate level of TGF-β gene expression (Fig. 3A). The EM Treg cells from delayed patients presented lower TGF-β gene expression than the EM Treg cells from normal patients. IL-10 gene expression directly ex vivo was also mainly found in EM Treg cells, not N Treg or CM Treg cells (Fig. 3B). No significant differences between normal and delayed patients were found.

Subsequently, the expression of TGF-β and IL-10 was investigated in Treg cells stimulated with anti-CD3 and anti-CD28 monoclonal antibodies in the presence of exogenous IL-2. The TGF-β gene expression was investigated. In N Treg cells, stimulation did not significantly increase TGF-β expression, regardless of duration (Fig. 3C). In EM Treg cells, longer stimulation time produced increasingly higher TGF-β gene expression. In CM Treg cells, TGF-β gene expression significantly increased but plateaued between day 3 and day 4. The N Treg cells also failed to upregulate IL-10 gene expression upon stimulation (Fig. 3D). The EM Treg cells and the CM Treg cells significantly upregulated IL-10 gene expression initially but was plateaued after day 3. In both normal patients and delayed patients at the end of stimulation, the EM Treg cells presented significantly higher TGF-β and IL-10 than the CM Treg cells, and the CM Treg cells presented significantly higher TGF-β and IL-10 than the N Treg cells ($p < 0.001$ for all comparisons).

To corroborate the mRNA data, we examined the amount of TGF-β and IL-10 protein secretion in the supernatant of stimulated Treg cells. TGF-β secretion was the highest in EM Treg cells, intermediate in CM Treg cells, and the lowest in N Treg cells (Fig. 3E). The EM Treg cells from delayed patients presented lower TGF-β secretion than the EM Treg cells from normal patients. IL-10 secretion was also the highest in EM Treg cells, intermediate in CM Treg cells, and the lowest in N Treg cells, with no significant difference between normal patients and delayed patients (Fig. 3F).

3.4. EM Treg cells were the most potent at suppressing RANKL expression

To investigate RANKL expression and the effect of Treg cells on RANKL, $CD4^+CD25^-$ T conventional (Tconv) cells and $CD4^+CD25^+$ Treg cells were harvested from normal patients and delayed patients, and were incubated in unstimulated media or with anti-CD3/anti-CD28/IL-2 stimulation. In select experiments, various subsets of Treg cells were added at 1/1 Tconv/Treg ratio. The RANKL transcription was then evaluated. Without stimulation, Tconv cells and Treg cells expressed little RANKL (Fig. 4). With stimulation, RANKL transcription in Tconv was markedly higher, while RANKL transcription in Treg cells remained low. On average, the Tconv cells from delayed patients had higher RANKL expression than the Tconv cells from normal patients, but the difference was not significant. In Tconv/N Treg coculture, the RANKL expression was not significantly different from that in pure Tconv cells. Given that Tconv cells could upregulate CD25 upon stimulation, it was not possible to distinguish Tconv-mediated RANKL from Treg-mediated RANKL after stimulation and coculture. However, it is likely that the majority of RANKL was from the Tconv cells but not the Treg cells, since very little RANKL was found in stimulated pure Treg cells. In Tconv/EM Treg coculture and Tconv/CM Treg coculture, the RANKL transcription was significantly lower than that in pure Tconv cells, indicating that the EM and CM Treg cells, but not N Treg cells, could suppress RANKL expression from Tconv cells. On average, the EM Treg cells suppressed RANKL expression by 49.4% in normal patients and 42.1% in delayed patients, and the CM Treg cells suppressed RANKL expression by 34.6% in normal patients and 32.1% in delayed patients, indicating that the EM Treg cells were the most potent at suppressing RANKL expression. The differences between normal Treg cells and delayed Treg cells were not statistically significant ($p > 0.05$ for all comparisons).

3.5. EM Treg-mediated RANKL suppression was dependent on IL-10

Subsequently, we investigated whether EM Treg-mediated RANKL suppression was dependent on the secretion of TGF-β and/or IL-10, by adding incremental amounts of anti-human TGF-β and anti-human IL-10 neutralizing antibodies in the coculture of Tconv cells and EM Treg cells. In normal patients, anti-IL-10 blocking antibody at 3 and 10 μg/mL and anti-TGF-β blocking antibody at 10 μg/mL significantly increased RANKL expression (Fig. 5A). In delayed patients, anti-IL-10 blocking antibody at 3 and 10 μg/mL, but not anti-TGF-β blocking antibody, significantly increased RANKL expression (Fig. 5B). The EM Treg cells from delayed patients appeared to present higher RANKL expression than the EM Treg cells from normal patients at baseline (blocking antibody concentration = 0); however, the difference was not significant ($p > 0.05$). Also, in the absence of Together, these data demonstrated that EM Treg-mediated RANKL suppression was

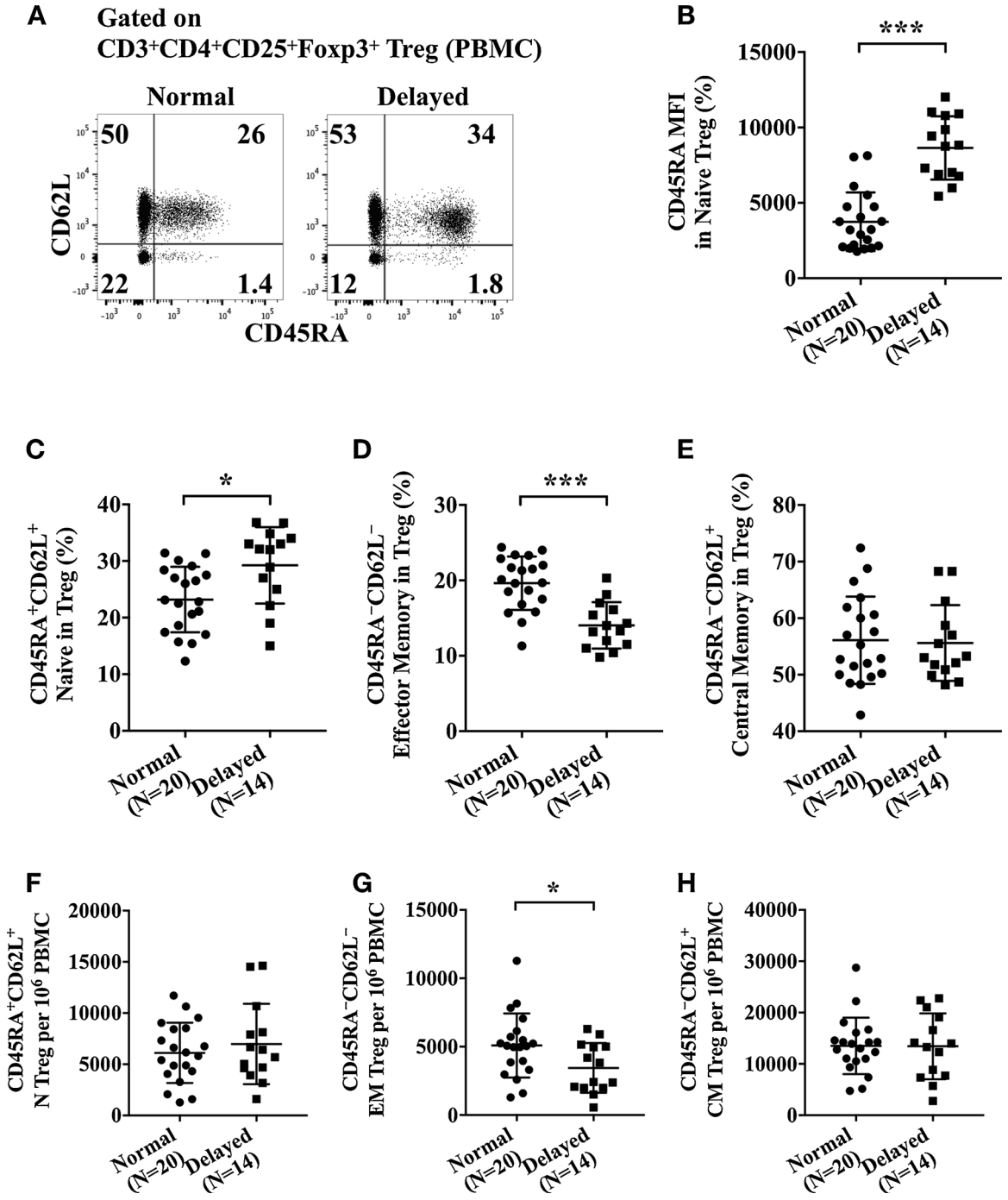

Fig. 2. Circulating Treg proportion in patients with normal or delayed healing.
(A) Expression of CD45RA and CD62 L by circulating Treg cells from one representative patient from the normal group and one representative patient from the delayed group. Numbers represent the percentage of each subset in total Treg cells. (B) The MFI of CD45RA in $CD45RA^+CD62L^+$ N Treg cells. (C) $CD45RA^+CD62L^+$ N Treg frequency. (D) $CD45RA^-CD62L^-$ EM Treg frequency. (E) $CD45RA^-CD62L^+$ CM Treg frequency. (F) $CD45RA^+CD62L^+$ N Treg number per 10^6 PBMCs. (G) $CD45RA^-CD62L^-$ EM Treg number per 10^6 PBMCs. (H) $CD45RA^-CD62L^+$ CM Treg number per 10^6 PBMCs. Unpaired *t*-test *$p < 0.05$. ***$p < 0.001$.

dependent on IL-10 and TGF-β.

4. Discussion

Despite recent advances in fracture management, delayed union and nonunion still occur in a small subset of patients with bone fracture, presenting significant challenges to further treatment [15]. The capacity to identify fracture cases with elevated risk of developing into delayed union or nonunion may allow early interventions and facilitate further management. Epidemiology studies have shown that several genetic, behavioral, and clinical characteristics, such as male gender, smoking, alcoholism, and inflammatory arthritis, are associated with elevated risk of delayed union and nonunion [16]. In this study, we further identified that in blood from patients with delayed healing, the EM Treg cells were reduced in proportion, while the N Treg cells were increased. These characteristics should be validated in a larger cohort of patients to determine whether circulating Treg composition may be used as a predictor of delayed union or nonunion.

Our study identified that, in both normal healing and delayed healing patients, the EM Treg cells were the most potent among N, EM,

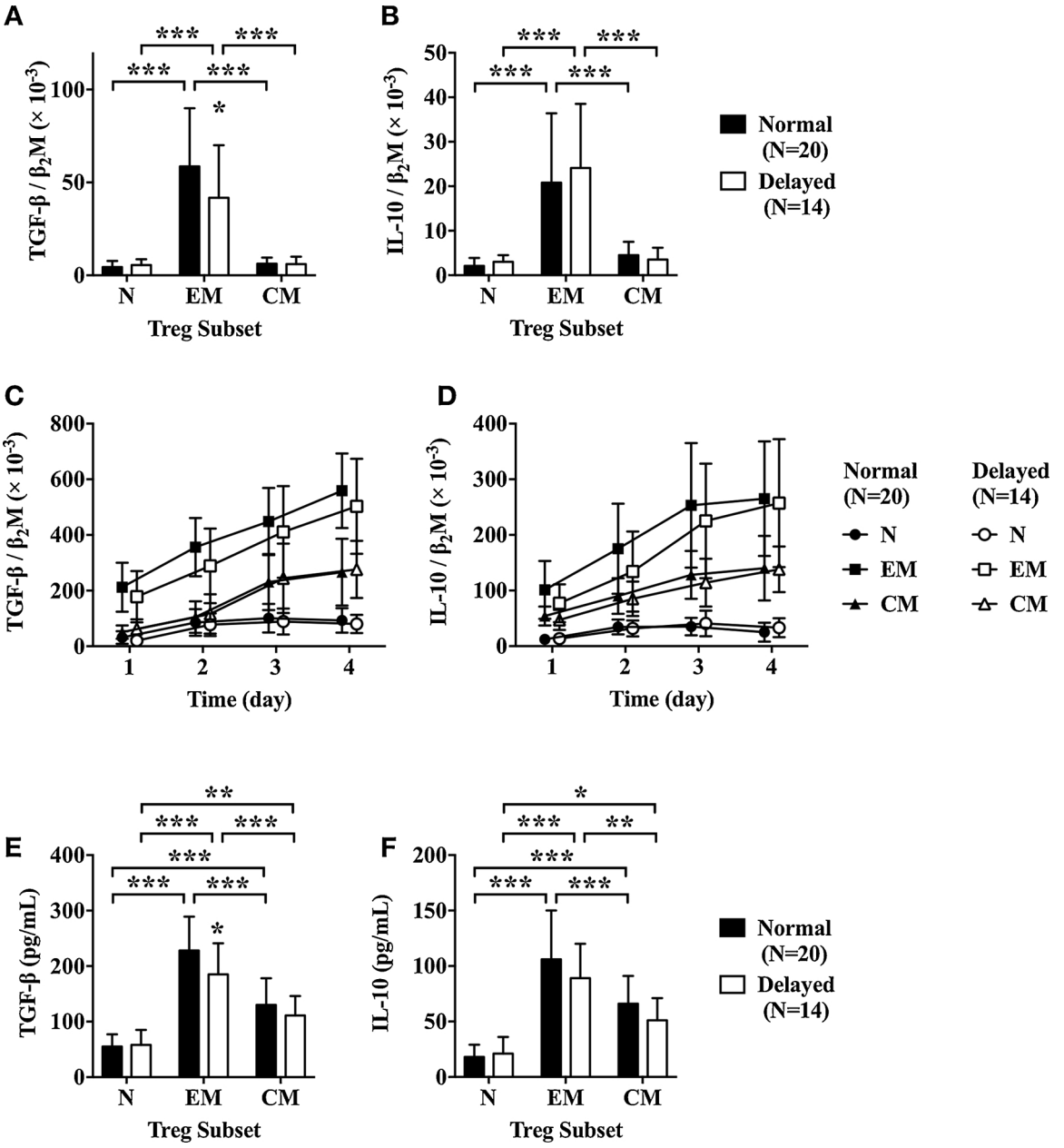

Fig. 3. Expression of cytokines by Treg subsets from normal (n = 20) and delayed (n = 14).
(A) Ex vivo gene expression of TGF-β by N, EM, and CM Treg cells in normal and delayed patients. (B) Ex vivo gene expression of IL-10 by N, EM, and CM Treg cells in normal and delayed patients. (C) N, EM, and CM Treg cells from normal and delayed patients were stimulated by anti-TCR (CD3/CD28) and IL-2, and the TGF-β gene expression was examined every 24 h. (D) N, EM, and CM Treg cells from normal and delayed patients were stimulated by anti-CD3/anti-CD28/IL-2, and the IL-10 gene expression expression was examined every 24 h. (E) TGF-β secretion in supernatant following 4-day stimulation. (F) IL-10 secretion in supernatant following 4-day stimulation. Two-way ANOVA with Sidak's multiple comparison test. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

and CM Treg subsets at suppressing RANKL expression in Tconv cells. However, the frequency of EM Treg cells in delayed healing patients was significantly lower than that in normal patients. In addition, the TGF-β expression was significantly lower in delayed EM Treg cells than in normal EM Treg cells, suggesting functional impairment in EM Treg cells. Inhibition of IL-10 significantly increased RANKL expression in both normal and delayed patients, while inhibition of TGF-β significantly increased RANKL expression in normal patients. EM Treg cells expressed the highest IL-10 directly ex vivo, and after anti-CD3/anti-CD28/IL-2 stimulation. CM Treg cells did not present high IL-10 expression ex vivo, but was capable of upregulating IL-10 expression upon stimulation. N Treg cells, on the other hand, presented limited capacity to upregulate inhibitory cytokines.

RANKL expression is not only found on $CD4^+$ T cells but also present in a variety of tissues and organs [17]. Most notably, RANKL is expressed by bone-forming osteoblasts as a feedback mechanism to stimulate osteoclast formation. The effect of Treg cells on RANKL expression in osteoblasts and other tissues/cells should be investigated in further experiments. In addition, the underlying mechanism of Treg-mediated suppression should be investigated. Whether IL-10 directly suppresses RANKL, or functions by suppressing inflammatory responses that promote RANKL expression, should be distinguished.

Of note, we collected patient samples before surgery. In future studies, the characteristics of Treg cells after surgery, including the proportion, activation status, function, and infiltration at the site of injury, should be further examined. Intra-tissue bias of Treg proportions

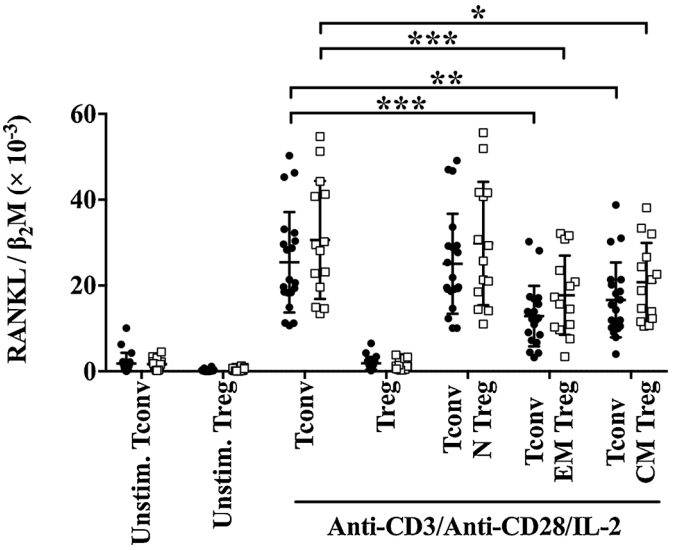

Fig. 4. Expression of RANKL by Tconv cells in the presence of Treg subsets.
$CD4^+CD25^-$ Tconv cells and $CD4^+CD25^+$ Treg cells were incubated without stimulation or with anti-CD3/anti-CD28/IL-2 for 3 days. In addition, Tconv cells were coincubated with N, EM, or CM Treg cells from autologous donors at 1/1 Tconv/Treg ratio, which were also incubated for 3 days in the presence of anti-CD3/anti-CD28/IL-2. RANKL gene expression levels in $CD4^+$ T cells from normal patients (solid circle) and in $CD4^+$ T cells from delayed patients (open square) were presented. Two-way ANOVA with Sidak's multiple comparison test. *p < 0.05. **p < 0.01. ***p < 0.001.

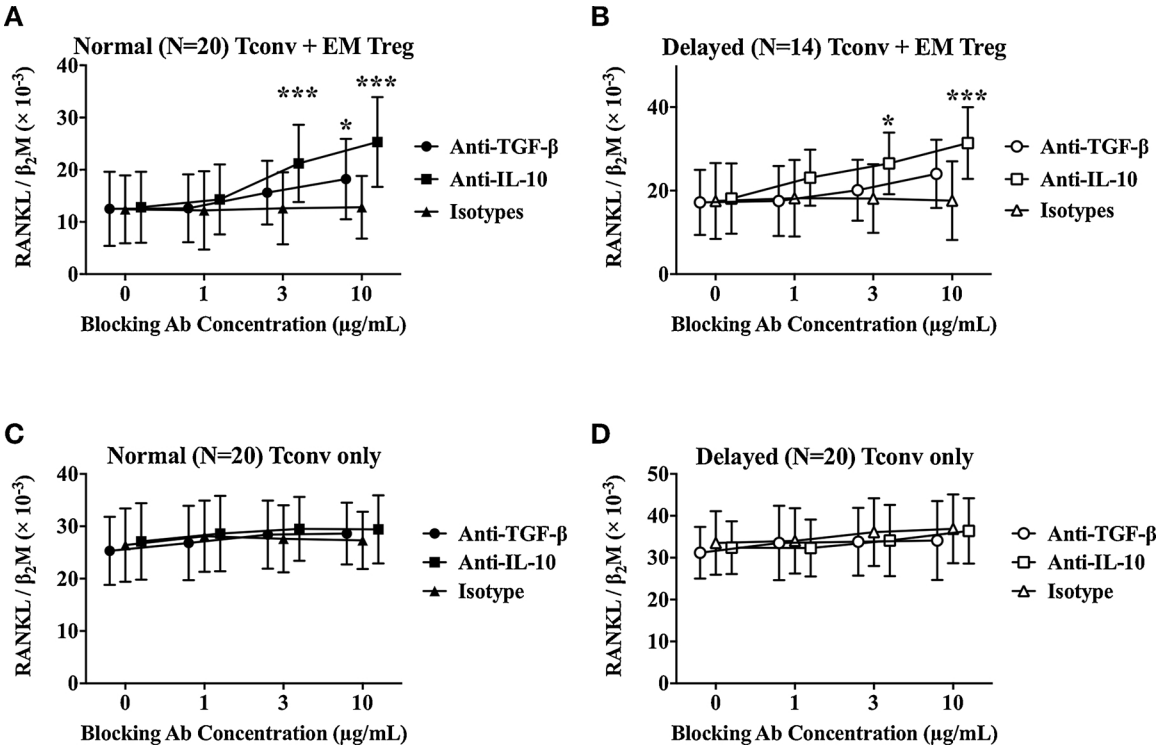

Fig. 5. Effect of TGF-β and IL-10 blocking on RANKL expression.
$CD4^+CD25^-$ Tconv cells were coincubated with $CD4^+CD25^+CD45RA^-CD62L^-$ EM Treg cells at 1/1 Tconv/Treg ratio, with ascending concentrations of anti-human IL-10 blocking antibody, anti-human TGF-β blocking antibody, and isotype controls. (A) RANKL gene expression in Tconv + EM Treg coculture from normal patients. (B) RANKL gene expression in Tconv + EM Treg coculture from delayed patients. (C) RANKL gene expression in Tconv only culture from normal patients. (D) RANKL gene expression in Tconv only culture from delayed patients. Two-way ANOVA with Sidak's multiple comparison test. *p < 0.05. ***p < 0.001.

may occur as the expression of homing receptors could affect Treg infiltration in the inflamed and injured site. Upon receiving tissue samples and by using animal models, Treg proportion may be further investigated in sites of bone fracture. Also, it would be interesting to perform IL-10 and TGF-beta dual blockade in the study. In addition, the impact of EM Treg cells on the formation of osteoclasts and bone healing should be investigated in vivo, most feasibly by adoptive transfer in animal models.

Conflict of interests

None.

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