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Proinflammatory T cells and IL-17 stimulate osteoblast differentiation

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ABSTRACT

The local immune response is important to consider when the aim is to improve bone regeneration. Recently T lymphocytes and their associated cytokines have been identified as regulators in fracture callus formation, but it is not known whether T cells affect bone progenitor cells directly. The goal of this *in vitro* study was to investigate the role of different T cell subsets and their secreted factors on the osteogenic differentiation of human mesenchymal stem cells (MSCs). Significant increases in the alkaline phosphatase activity and the subsequent matrix mineralization by MSCs were found after their exposure to activated T cells or activated T cell-derived conditioned medium. Blocking IFN-γ in the conditioned medium abolished its pro-osteogenic effect, while blocking TGF-β further enhanced osteogenesis. The relative contribution of an anti- or proinflammatory T cell phenotype in MSC osteogenic differentiation was studied next. Enrichment of the fraction of anti-inflammatory regulatory T cells had no beneficial osteogenic effect. In contrast, soluble factors derived from enriched T helper 17 cells upregulated the expression of osteogenic markers by MSCs. IL-17A, and IL-17F, their main proinflammatory cytokines, similarly exhibited strong osteogenic effects when exposed directly to MSCs. IL-17A in particular showed a synergistic action together with bone morphogenetic protein 2. These results indicate that individual T cell subsets, following their activation, affect osteoblast maturation in a different manner through the production of soluble factors. From all T cells, the proinflammatory T cells, including the T helper 17 cells, are most stimulatory for osteogenesis.

1. Introduction

Bone tissue normally has the capacity to fully regenerate in response to injury [1]. Healing can however be impaired under certain conditions such as severe trauma, increased age, metabolic disease, mechanical instability, or the use of nonsteroid anti-inflammatory drugs [2–4]. It has become increasingly clear that a balanced immune response is a prerequisite for successful bone regeneration [5–7]. After injury, proinflammatory cytokines act in concert with bone-promoting factors to initiate repair by regulating angiogenesis and the migration and differentiation of bone progenitor cells [8]. In particular TNF-α, IL-6 and IL-1β show a strong biphasic expression profile following bone injury, coinciding with the inflammatory and remodeling phases of bone regeneration [5,9]. The subsequent upregulation of anti-inflammatory cytokines is suggested to ensure a balanced inflammatory response and optimal regeneration [10]. In a pathological context, unregulated expression of inflammatory cytokines is thought to underlie excessive new bone formation [11–13], suggesting that these factors can stimulate osteoblast maturation of bone progenitor cells.

Although the innate inflammatory response is a key component during early bone repair [14], the adaptive immune system and its associated cytokines have recently also received attention as a regulator in bone regeneration. In the field of osteoimmunology, T lymphocytes are mainly studied for their effects on osteoclast activation and bone resorption in conditions such as inflammatory arthritis and periodontitis [15]. Following the observation that T cells are regulators in soft tissue healing [16], their contribution has also been studied in fracture healing. Studies performed in mice lacking functional lymphocytes, *i.e.* RAG-1-deficient mice, indicate that T cells may indeed play a role in bone regeneration. As such, the fracture calluses of RAG-1-deficient mice exhibit lower levels of bone markers which leads to poor bone quality. This is accompanied by a shift in the local cytokine profile from the expression of proinflammatory cytokines, including IFN-γ and TNF-α, towards the expression of anti-inflammatory cytokines such as IL-10 and IL-4 [17,18].

It is likely that distinct lymphocyte subsets and their associated cytokines have specific roles in osteogenesis [19,20]. Although B cells are more likely involved in the later remodeling phase of bone regeneration

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[21,22], in contrast, T cells may be more important during the acute phase of bone healing [21]. A stimulatory role for the proinflammatory T helper 17 (T_H17) cell subset in osteoblast maturation has been suggested [17], whereas improved bone formation was demonstrated after the delivery of anti-inflammatory $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T (T_{REG}) cells during mesenchymal stem cell (MSC)-based bone regeneration [23]. Finally, a negative correlation has been reported for the number of $CD8^+$ cytotoxic T cells in peripheral blood and the outcome of fracture healing in otherwise healthy patients [24].

The modulation of the adaptive immune response has proven to be a promising approach to stimulating bone regeneration. The local depletion or systemic delivery of different T cell populations has resulted in improved fracture healing or MSC-based bone regeneration [23,24]. It is currently unknown whether T cells are directly involved in osteoblast maturation. The goal of this study was therefore to investigate how different T cell subsets have an effect on the osteogenic differentiation of MSCs. The approach chosen involves coculturing these purified cell populations or exposing MSCs to T cell-secreted factors, followed by monitoring of cell performance and differentiation.

2. Material and methods

2.1. MSC isolation and culture

Bone marrow was obtained from four patients undergoing orthopedic procedures at our institute (University Medical Center Utrecht, Utrecht, The Netherlands). Different bone marrow sources were included to ascertain that the findings were not unique to a specific source (female, 53 yr., iliac crest; male, 62 yr., iliac crest; male, 77 yr., vertebra; adolescent female, calcaneus). All patients gave written informed consent, with approval of the local medical ethical committee. The mononuclear cell fraction was isolated by Ficoll-Paque centrifugation and plated in growth medium [α-MEM (Invitrogen, Carlsbad, CA), 10% (v/v) heat-inactivated fetal bovine serum (Cambrex, East Rutherford, NJ), 100 units/ml penicillin/streptomycin (Invitrogen)] supplemented with 1 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN). Adherent cells were expanded and cryopreserved for use between passage 3 and 6. Cells derived from different donors were never pooled. Culture was performed at 37 °C in a humidified atmosphere containing 5% CO_2. The multipotency of MSCs using this isolation method has been established previously by our group using standard differentiation assays along osteogenic, adipogenic and chondrogenic lineages [25]. Furthermore, cells were characterized for the expression of specific surface antigens defining human MSCs, according to the Mesenchymal and Tissue Stem Cell Committee of the ISCT [26]. Based on FACS analyses, >95% of cells were negative for CD14 and CD45, and >99% of cells were negative for CD19 and CD34. In addition, >95% were positive for CD73, CD105 and CD90 [27].

2.2. T cell isolation and culture

Blood from healthy male and female controls was requested from the blood bank Mini Donor Dienst of the UMC Utrecht after written informed consent and with approval of the local medical ethical committee. The donors were between 25 and 55 years old (mean age 40 yr). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll-Paque centrifugation. $CD3^+$ T cells and $CD4^+$ T helper cells were isolated by positive selection using MicroBeads according to the manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany). To further differentiate the T cells, CD45RO− positive cells were first depleted with MicroBeads. To enrich $CD8^+$ cytotoxic T cells from the PBMCs, CD4 depletion and CD3 positive selection were performed accordingly. The purity of the cell isolates was confirmed by FACS analysis using the following antibodies: CD3 (clone BW264/56, Miltenyi), CD4 (clone VIT4, Miltenyi) and CD45RO (clone UCHL1, BD, Franklin Lakes, NJ). The percentage of $CD3^+$ and $CD4^+$ T cells after selection ranged between 90–99% (Fig. S1A). CD45RO depletion resulted in 80–90% $CD45RO^-$ cells (Fig. S1B). T cells were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO), supplemented with 100 units/ml penicillin/streptomycin, 10% (v/v) heat-inactivated fetal bovine serum and 2 mM L-glutamine (Invitrogen).

2.3. T cell activation and differentiation

For T cell activation, cells were cultured at 1×10^6 cells/ml in plates coated with anti-CD3 mAb (5 μg/ml, clone CLB-T3/4.E, 1XE, Sanquin Reagents, Amsterdam, The Netherlands), and with soluble anti-CD28 mAb (2 μg/ml, clone CLB-CD28/1,15E8, Sanquin Reagents) and rhIL-2 (20 ng/ml, Thermo Fisher Scientific, Carlsbad, CA) for 4 days. As a control, resting T cells were obtained by treatment with IL-2 only. The activation of cells was quantified by FACS analysis for CD25 (clone 4E3, Miltenyi), which was increased from 20% in controls to 60–70% after stimulation (Fig. S1C).

The survival and activation status of the T cells was also studied by FACS analysis. For this purpose, CD4+ T cells were activated as described above and subsequently cultured for 10 days in 24-well plates (750,000 cells/well) on a layer of MSCs (initial density 8500 cells/cm^2). In the control group, the T cells were cultured in the absence of MSCs for 10 days. At different time points, the cells were detached (Accutase, Sigma) and double-stained with CD4 (clone VIT4, Miltenyi) and CD25 (clone 4E3, Miltenyi). To assess the T cell viability, cells were stained with 7-AAD (10 μg/ml in PBS, Sigma) for 30 min.

For T cell differentiation experiments, CD4+ CD45RO− naïve T cells were stimulated with anti-CD3/CD28 mAbs and IL-2. For the enrichment of the Foxp3-expressing regulatory T cell (T_{REG}) fraction, the cultures were supplemented with 20 ng/ml TGF-β1 (R&D) for 5 days. For T helper 17 cell (T_H17) differentiation, cultures were supplemented with TGF-β (10 ng/ml), IL-6 (30 ng/ml, R&D), IL-1β (25 ng/ml, Sigma) and IL-23 (75 ng/ml, eBioscience, San Diego, CA) in the absence of IL-2 for 5 days. T cells were replated after 5 days and cultured for an additional 5 days with these antibodies and IL-2 (20 ng/ml). The enrichment of T_{REG} and T_H17 cell fractions was determined by FACS analysis using anti-human Foxp3 eFluor 450 (eBioscience) and anti-human IL-17A-APC (Miltenyi). T_{REG} polarization resulted in a purity of 40–50% Foxp3-positive cells compared to approximately 20% in the undifferentiated control (Fig. S1D). T_H17 polarization similarly resulted in a doubling of the number of IL-17A-producing cells to about 40% (Fig. S1E).

2.4. Osteogenic differentiation assay

MSCs were seeded at 8500 cells/cm^2. Two types of osteogenic differentiation medium (ODM), containing either dexamethasone or BMP-2, were used for experiments. Although dexamethasone is a strong inducer of the osteogenic differentiation of MSCs *in vitro*, ODM with BMP-2 is thought to better simulate the conditions *in vivo* [28]. As such, growth medium (see Section 2.1) was supplemented with a combination of 10 mM β-glycerophosphate (Sigma) and 10 nM dexamethasone/0.2 mM L-ascorbic acid 2-phosphate (Sigma), or a combination of 10 mM β-glycerophosphate and 500 ng/ml rhBMP-2 (InductOS, Wyeth/Pfizer, NY). Ascorbic acid was not used in cocultures due to their reported effects on T cells [29].

For coculture experiments (Fig. 1), T cell activation/polarization was always performed prior to culture with MSCs. T cells were subsequently added to the MSCs in the different media at different concentrations. Half of the medium was replaced every 3 days. To study the role of cell-to-cell contact, T cells were separated from the MSCs in Transwell supports (6.5 mm, 0.4 μm pores, Sigma).

To obtain conditioned medium (CM), T cells were reseeded at 5×10^5 cells/ml after activation/polarization in medium supplemented with only anti-CD3/CD28 mAbs (Fig. 1). The cell suspensions were harvested after 24 h, centrifuged, and the supernatant was collected for

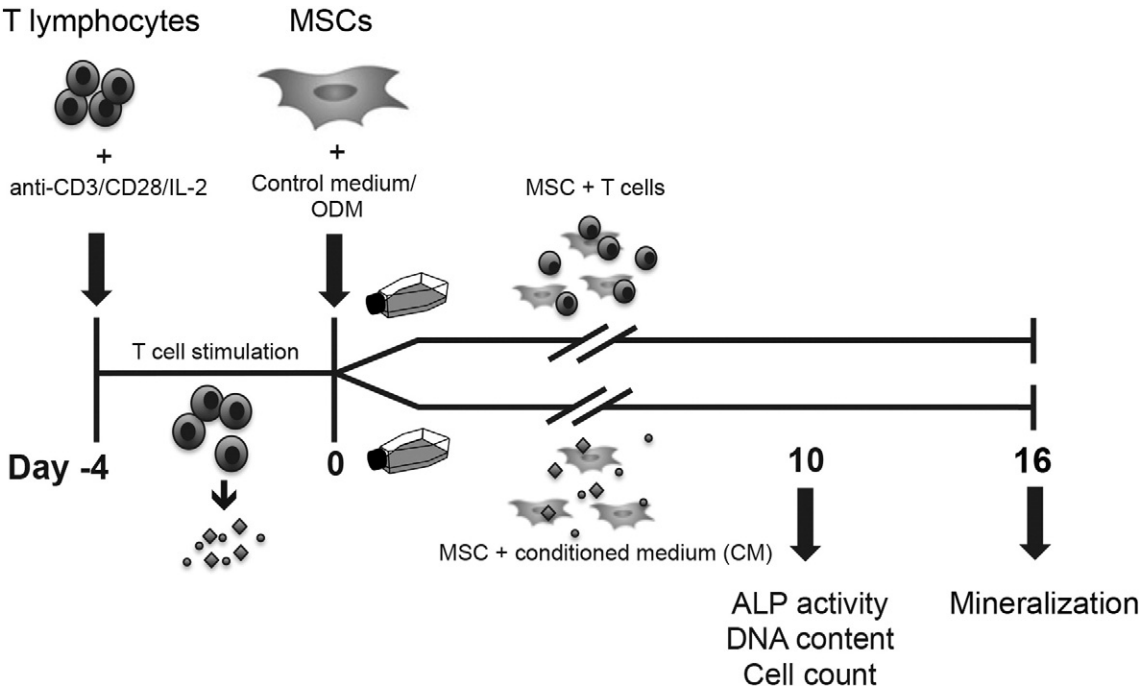

Fig. 1. Experimental design. T cells purified from peripheral blood were activated. MSCs were isolated from bone marrow and cultured in growth medium (control) or osteogenic differentiation media (ODM). Two types of ODM were used, containing either dexamethasone or BMP-2. To study the effect of T cell-secreted factors on osteogenesis, MSCs were cocultured with T cells directly or incubated with T cell-derived conditioned media (CM). Alkaline phosphatase (ALP) activity and MSC proliferation were quantified after 10 days. T cell CM was used to study the effect of T cell cytokines on the matrix mineralization after 16 days.

storage at −80 °C until use. The CM was added to the MSCs at different concentrations (10–50%), and fresh CM was added at each medium change. In ODM culture, the concentration of osteogenic stimuli was corrected for the total volume of medium. To study the effect of T_H17-specific cytokines on osteogenesis, MSCs were cultured with 5–500 ng/ml rhIL-17A or rhIL-17F (R&D). The following mouse anti-human monoclonal antibodies were added to the $CD4^+$ T cell CM to neutralize the activity of these cytokines (all purchased from R&D Systems): IL-17 (IgG_{2B}, 1.75 μg/ml), TNF-α (IgG_1, 1 μg/ml), IFN-γ (IgG_{2B}, 0.85 μg/ml), IL-10 (IgG_{2B}, 0.75 μg/ml) and TGF-β (IgG_1, 0.75 μg/ml). The manufacturer's recommended concentrations were used. Mouse IgG_1 (2 μg/ml) and mouse IgG_{2B} (2 μg/ml) antibodies were used as controls.

The ALP activity in MSCs was measured at day 10, as we and others have shown that the levels generally peak between days 10 and 14 [30]. For ALP determination, cells were lysed in 0.2% (v/v) Triton X-100 in PBS for 30 min. ALP activity was measured by conversion of the p-nitrophenyl phosphate liquid substrate system (Sigma). The absorbance was measured at 405 nm and corrected at 655 nm (Bio-Rad, Hercules, CA). Values were normalized to a standard ALP measurement using serial dilutions of calf intestinal ALP (Sigma) in 0.2% (v/v) Triton X-100 in PBS. The same cell lysate used to measure ALP was stored at −80 °C and used to determine the DNA content with the Quant-It PicoGreen kit (Invitrogen), according to the manufacturer's instructions. In coculture experiments, MSC numbers were quantified by FACS analysis using counting beads (Countbright, Life Technologies), according to the manufacturer.

For the qualitative assessment of matrix mineralization, the cell monolayer was fixed after 16 days culture in 4% (w/v) paraformaldehyde, stained for 10 min with 2% (w/v) Alizarin Red S solution (pH 4.2, Sigma) and examined by light microscopy. For quantification, samples were incubated with 0.2% (w/v) Alizarin Red S for 60 min, washed extensively, and treated with 10% cetylpyridinium to extract the calcium-bound Alizarin. Absorbance was measured at 595 nm and corrected at 655 nm (Bio-Rad).

2.5. Statistical analysis

All data are expressed as the mean ± standard deviation for the different lymphocyte donors. When indicated in the captions, experiments were performed in multiple MSC donors. For statistical analysis, one-way analysis of variance (ANOVA) and Bonferroni post-hoc correction were applied. One-way ANOVA with LSD post-hoc correction was performed to study the effect of the neutralizing antibodies in the conditioned medium experiments.

3. Results

3.1. Activated T cells stimulate the osteogenic differentiation of MSCs

A possible effect of T cells on MSC osteogenesis was first tested in direct cocultures. The MSCs were cultured in growth medium or in medium supplemented with BMP-2, with or without different lymphocyte subsets. Compared to cultures in the absence of any lymphocytes, MSCs exhibited a higher ALP activity when cocultured with PBMCs or with purified $CD4^+$ and $CD8^+$ cells (Fig. 2A). The observation that the ALP activity was also enhanced in MSCs by lymphocytes in cultures without BMP-2 suggests that T cells alone may have the ability to induce osteogenic differentiation.

We furthermore studied the effect of the different T lymphocyte populations on the matrix mineralization by MSCs. Due to the practical limitations of the longer culture time that is required, T cell conditioned media (CM) was used for this purpose. A significantly higher calcium deposition was measured when MSCs were incubated with the CM derived from activated PBMCs or T lymphocytes during their osteogenic differentiation (Fig. 2B). In line with the early ALP activity, the highest matrix mineralization was found after incubation of MSCs with CM collected from the $CD4^+$ T cell population. It appeared that individual T cell subsets, *i.e.* $CD4^+$ and $CD8^+$ T cells, had a higher stimulatory effect compared to the entire T cell population (Fig. 2A and B).

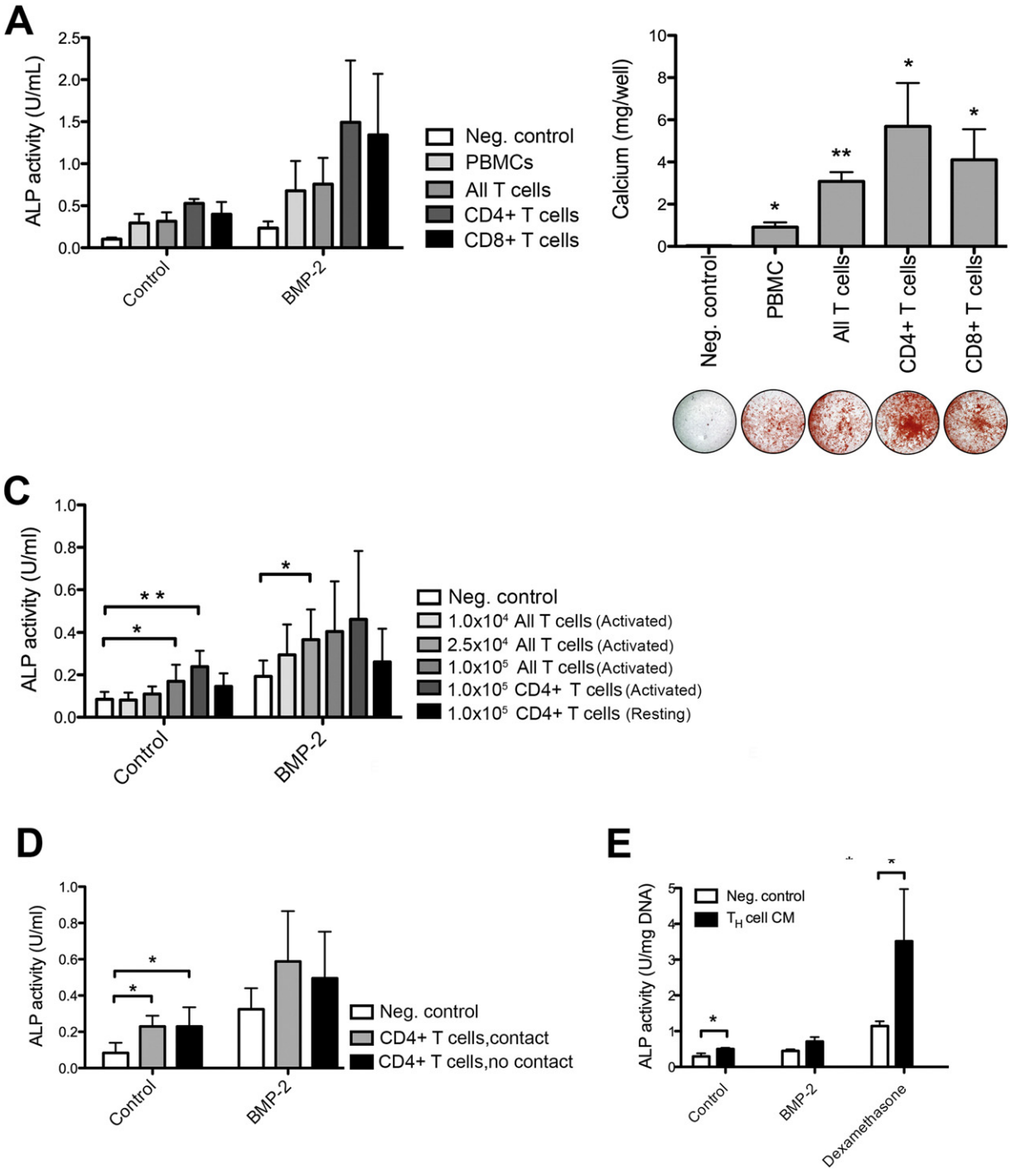

Fig. 2. The effect of T cells and their secreted factors on the osteogenic differentiation of MSCs. (A) Day 10 ALP activity in MSCs cultured in the presence of 1×10^5 activated allogeneic T cells in 96-well plates, $n = 2$ blood donors and one allogeneic MSC donor. (B) Day 16 matrix mineralization by MSCs following osteogenic induction with dexamethasone and treatment with 25% CM from PBMCs and activated lymphocytes. Calcium deposition at day 16 was quantified after Alizarin Red S staining, $n = 3$ blood donors and one MSC donor. * $p < 0.05$/** $p < 0.005$ compared to the negative control. (C) Day 10 ALP activity in MSCs cultured with different amounts of allogeneic T cells in 96-well plates, $n = 5$ blood donors for independent experiments using two MSC donors. (D) Day 10 ALP activity in MSCs cultured in growth medium (control) or BMP-2 osteogenic medium in the presence of 2.5×10^5 activated allogeneic CD4 + T cells in 24-well plates. Transwell inserts were used to inhibit cell-to-cell contact, $n = 4$ blood donors for independent experiments using two MSC donors. (E) Day 10 ALP activity/DNA in MSCs cultured in 25% CM collected from activated CD4 + T cells, $n = 4$ blood donors for independent experiments using two MSC donors. Histograms show the mean ± SD. * $p < 0.05$/** $p < 0.005$ compared to the negative control in the same medium.

3.2. $CD4^+$ T cells secrete factors that promote osteoblast maturation

We studied the $CD4^+$ T cell population in more detail to extend our previous findings. In coculture experiments, we found a dose-dependent significant increase in the ALP activity of MSCs after coculture with the entire T cell pool (Fig. 2C). A higher ALP induction was however found when the fraction of $CD4^+$ T cells was increased by cell sorting, suggesting that the $CD4^+$ population has the most beneficial effects on osteogenesis. No significant changes in the ALP activity were observed when MSCs were cocultured with resting $CD4^+$ T cells, showing that only T cells with an activated phenotype affect MSC osteogenic differentiation.

FACS analysis demonstrated that the CD4$^+$ T cells gradually lost their activation status during the 10-day culture period, as shown by their decrease in CD25 expression (Fig. S2A). The MSCs had an inhibitory effect on the CD25 expression, depending on the donor. In addition, there was a profound loss in T cell viability in the second half of the culture period. The presence of MSCs in the coculture resulted in a significant higher number of non-viable T cells at day 10 compared to T cells which were cultured alone (Fig. S2B). These data together suggest that CD4$^+$ T cells mediate most of their beneficial effects on osteogenesis during initial coculture with the MSCs.

The importance of direct cell contact in the MSC–T cell interactions was explored by culturing T cells separated from MSCs in permeable Transwell inserts. This allowed paracrine signaling between the different cell types in the absence of direct cell-to-cell contact. This did not change the CD4$^+$ T cell-mediated effects on the ALP activity in MSC compared to direct cocultures (Fig. 2D). CM derived from activated CD4$^+$ T cells also significantly enhanced the ALP activity in MSCs (Fig. 2E). This finding suggests that T cell-mediated effects on MSC osteogenic differentiation are largely caused by soluble factors.

To ascertain that the observed T cell-mediated effects on MSCs were not obscured by changes in MSC proliferation, we routinely measured the number of MSCs in parallel to the differentiation assays. The presence of T cells (Fig. 3A) or their soluble factors (Fig. 3B) decreased the proliferation of MSCs modestly. A significant decline in DNA content was observed when MSCs were cultured in high concentrations of T cell CM. These data indicate that changes in ALP activity measured in coculture experiments reflect an increase in osteogenesis and not a change in MSC proliferation.

To identify the factors that contributed to the pro-osteogenic effect of the T cell CM, several cytokines associated with T helper and regulatory T cells were blocked in the cultures (Fig. 4). Two cytokines were found that significantly affected the ALP activity in MSCs when blocked during treatment with CM. Blocking IFN-γ resulted in a 80% decrease in the pro-osteogenic effect of the CM. In contrast, blocking TGF-β further enhanced the effect of the CM on the ALP activity more than two-fold. IFN-γ and TGF-β seemed to have an opposing effect on osteogenesis, as no change in the ALP activity was seen compared to the control when these two cytokines were neutralized simultaneously in the CM.

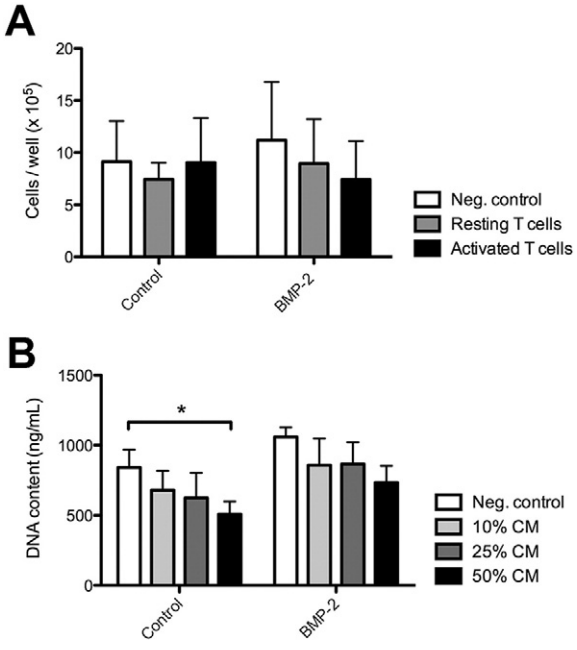

Fig. 3. The effect of T cells on MSC proliferation. (A) MSCs were cultured with 2.5×10^5 activated allogeneic T cells in 24-well plates for 10 days. The MSCs were counted by flow cytometry, $n = 4$ blood donors for independent experiments using two MSC donors. (B) MSCs were cultured in different dilutions of activated T cell CM. DNA levels were measured at day 10, $n = 6$ T cell donors for independent experiments using two MSC donors. Histograms show the mean ± SD. * $p < 0.05$.

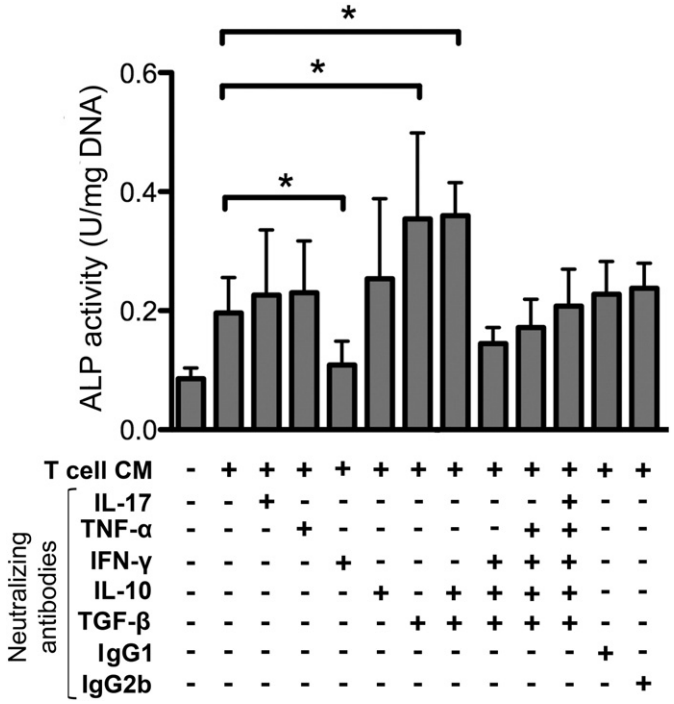

Fig. 4. The contribution of individual cytokines in CD4$^+$ T cell CM-induced osteogenesis. Day 10 ALP activity/DNA in MSCs cultured in growth medium supplemented with 25% CM collected from activated CD4$^+$ T cells. The activity of a number of T cell-derived cytokines was blocked by adding neutralizing antibodies to the medium, either alone or in combinations. The possibility of non-specific binding of the neutralizing antibodies was excluded using isotype-matched control antibodies. The histogram shows the mean ± SD for $n = 4$ blood donors and one MSC donor. * $p < 0.05$ compared to the T cell CM group without antibodies.

3.3. Pro- and anti-inflammatory CD4$^+$ T cell subsets differently affect osteoblast maturation

To further establish the contribution of differentiated CD4$^+$ T cell subpopulations to MSC osteogenesis, naïve CD4$^+$ T cells were differentiated into anti-inflammatory T_{REG} or proinflammatory T_H17 cells. In direct cocultures, an enrichment of the T_{REG} cell fraction had no additional effect on the ALP activity in MSCs compared to the undifferentiated CD4$^+$ T cell population (Fig. 5A). In contrast, enrichment of the T_H17 cell fraction resulted in a loss of the osteogenic effects.

As the MSCs are likely only exposed to a short T cell stimulus in direct cocultures (Fig. S2), we also exposed the MSCs to the CM derived from the enriched T_{REG} or T_H17 subsets. This furthermore allowed us to gain insight in the effects of T_{REG}/T_H17-derived factors alone (Fig. 5B and C). The CM of activated T_{REG} cells exhibited no additional effect on ALP activity in MSCs compared to CM from undifferentiated CD4$^+$ T cells (Fig. 5B). Although MSCs stimulated with BMP-2 and 10% T_{REG} CM showed a tendency towards increased ALP activity, the opposite effect existed for culture in a higher concentration T_{REG} CM. In contrast, MSCs dose-dependently exhibited an increase in ALP activity after treatment with soluble factors from enriched T_H17 cells (Fig. 5C). T_H17-derived CM was more osteogenic than the CM from non-differentiated cells, resulting in a 7-fold increase in the ALP activity in MSCs.

3.4. T_H17-specific cytokines stimulate osteoblast maturation

To evaluate the direct effects of T_H17 cell-specific cytokines on osteoblast differentiation [31], MSCs were treated with recombinant human IL-17A or IL-17F. In ODM, we found significant increases in the ALP

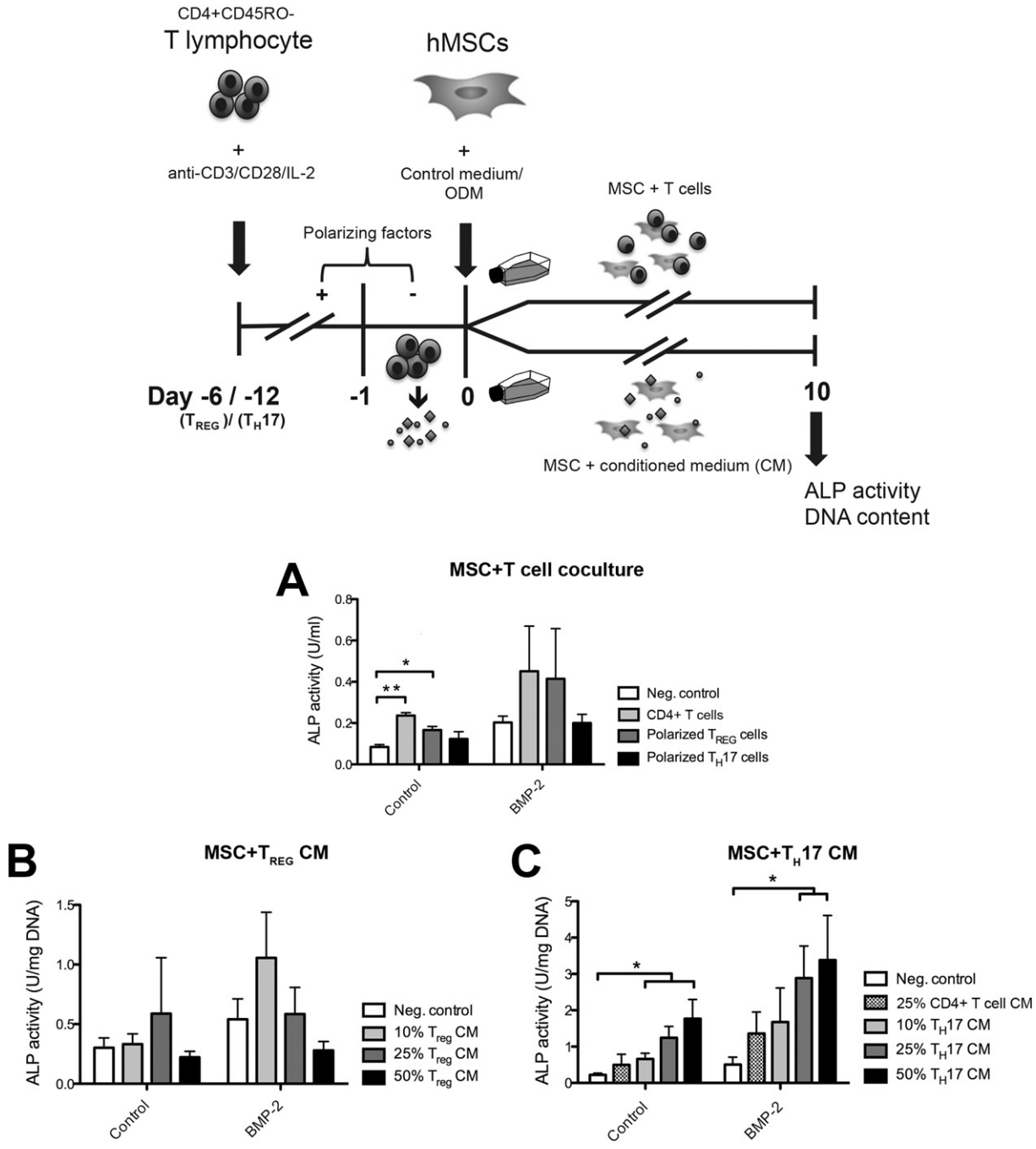

Fig. 5. The effect of individual $CD4^+$ T cell subsets on osteoblast maturation. (A) Naïve $CD4^+$ T cells were activated with anti-CD3/CD28 together with T_{REG}- and T_H17-enriching cytokines. MSCs were cultured together with 1×10^5 enriched allogeneic T cells in 96-well plates and the ALP activity was measured at day 10. (B, C) Naïve T cells were polarized towards T_{REG} (B) or T_H17 (C) cells and MSCs were incubated with their CM. ALP activity was measured at day 10 and normalized for DNA content. Histograms show the mean ± SD for $n = 3$ blood donors combined with one MSC donor. * $p < 0.05$/** $p < 0.005$ compared to the negative control in the same medium.

activity in MSCs after treatment with these proinflammatory factors (Fig. 6A). IL-17A stimulated the ALP activity in MSCs similarly for all concentrations tested, whereas treatment with IL-17F resulted in a dose-dependent increase in the ALP activity. MSCs treated with IL-17 similarly exhibited a marked increase in the mineralized matrix deposition, but only in ODM (Fig. 6B). In particular, BMP-2 and IL-17A had marked synergistic effects on the calcium deposition by MSCs.

4. Discussion

In addition to their role in pathological bone resorption [15,32], T lymphocytes may also exert anabolic effects on bone tissue [21]. The interplay between MSCs and T cells has been investigated extensively to assess the immunomodulatory role of MSCs [33], but little is reported on the effects of T cells on MSCs. Although the interaction between MSCs and T cells partially occurs through macrophages as a third cell type *in vivo* [34–37], we used a simplified human MSC–T cell coculture model to study the direct effect of activated T cells on osteogenesis. Due to the practical and ethical limitations of combining MSCs and freshly-isolated lymphocytes from the same human donor, the MSC–T cell interactions studied here were of allogeneic nature. To eliminate possible confounding effects arising from MSC–T cell HLA mismatching, conditioned media (CM) experiments were performed in parallel. The similar outcomes observed for the CM experiments suggest that the difference

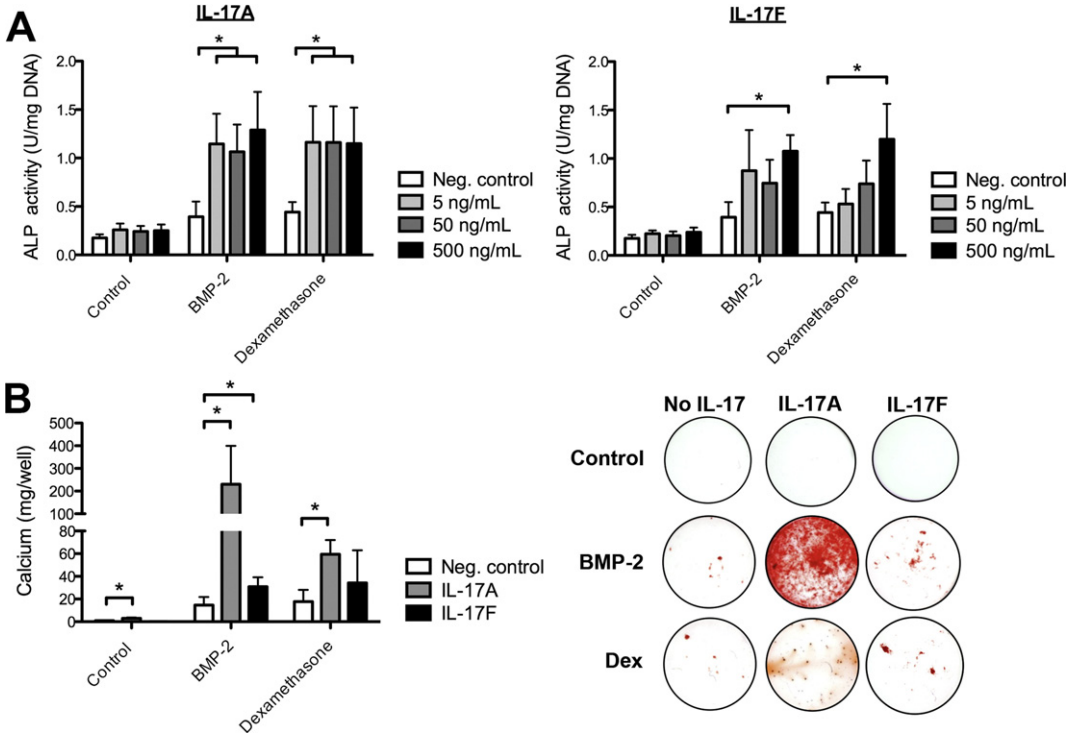

Fig. 6. The effect of T_H17-specific cytokines on the osteogenic differentiation of MSCs. (A) MSCs were cultured with different concentrations of IL-17 A or IL-17F for 10 days in growth medium (control) or ODM (BMP-2 or dexamethasone). ALP activity/DNA was measured at day 10. (B) MSCs were cultured in growth medium (control) or ODM for 16 days during treatment with IL-17 cytokines. Alizarin Red S staining was performed to quantify the calcium deposition (representative images in right panel). Histograms show the mean ± SD for $n = 4$ MSC donors. * $p < 0.05$ compared to the negative control in the same medium.

in donor source did not have a profound effect on the MSC–T cell interactions in the context of osteogenesis. However, the allogeneic MSCs did have an inhibitory effect on the activation status and the viability of the T cells. As a result, the T cells likely only mediated a short-lasting effect on the MSCs in the coculture experiments. In contrast, MSCs were exposed to a continuous stimulus in the CM experiments.

The ALP activity was measured as an early osteogenic marker as it is highly predictive for the *in vivo* bone-forming capacity of human MSCs [38]. After treatment of MSCs with activated T cells or their CM, MSCs exhibited a significantly higher early ALP activity. This was even the case in non-osteogenic medium, which suggests that certain T cell cytokines contain strong osteogenic potency. Although osteocalcin is an important bone-specific marker [39], it was not measured in the current study as its expression does not seem to coincide with the osteoblast differentiation of human MSCs after stimulation with proinflammatory factors [40,41]. In line with the early ALP activity, MSCs treated with soluble factors from activated T cells subsequently exhibited an increase in their ability to mineralize the matrix, as a sign of terminal osteoblast differentiation. Although recipient T cells are thought to inhibit the bone formation induced by allogeneic MSCs [23,42], this may be specifically related to the negative effects of proinflammatory T cell cytokines on the survival of exogenously delivered MSCs. Our results furthermore show that the pro-osteogenic effects of T cells require their activated phenotype. It has previously been shown that after bone injury, lymphocytes quickly migrate towards the site of damage [21]. Based on our data, it can be speculated that bone injury induces the activation of T cells, which then participate in the healing process by stimulating osteoblast development [43]. The activation and recruitment of CD4 + T helper cells during repair has already been established in soft tissue injury [44].

The stimulatory effects of activated T cell-derived CM on osteogenesis were reported before [45]. Here, we further discriminated between T cell subpopulations and established that $CD4^+$ T cells were more stimulatory for osteogenesis than $CD8^+$ cytotoxic (T_{CTX}) T cells. This is in line with the finding that $CD4^+$ T cells may be more beneficial for bone regeneration than their $CD8^+$ T_{CTX} cell counterpart [24,46]. When studying the secreted factors of the $CD4^+$ T cell population more closely [47,48], IFN-γ was found to be an important proinflammatory cytokine contributing to the pro-osteogenic nature of the CM. While the high levels of IFN-γ trigger bone loss under chronic inflammatory conditions [49], it is also suggested that IFN-γ plays a role in the commitment of MSCs into the osteoblastic lineage both *in vitro* and *in vivo* [50]. Furthermore, a loss in IFN-γ signaling may be one of the reasons for the impaired bone healing observed in mice lacking functional T cells [17]. We also demonstrated a negative association between the activity of the anti-inflammatory cytokine TGF-β and osteoblast differentiation. Although similar inhibitory effects *in vitro* have also been reported by others [17], it contradicts the general assumption that TGF-β signaling is beneficial for osteogenesis in an early stage of differentiation [51, 52]. There are data to support that the contradicting roles of IFN-γ and TGF-β may be related to their different actions in endogenous BMP-2 signaling in MSCs [45,53].

The opposite effects observed for IFN-γ and TGF-β in T cell-induced osteogenesis led to the hypothesis that pro- and anti-inflammatory T cell subsets within the $CD4^+$ T population have different effects on osteoblast maturation. We studied the T_H17 and T_{REG} subsets more extensively, because they are recognized as the archetypal proinflammatory and anti-inflammatory $CD4^+$ T cell phenotypes, respectively. Considering the low occurrence of these cells in peripheral blood [54,55], we enriched these cells *in vitro* following polarization protocols. Although the enriched fractions never contained more than 50% T_{REG} or T_H17 cells, the increase in differentiated cells was sufficient to cause significant changes in the ALP activity of MSCs compared to unpolarized cells. T_{REG} cells are known inhibitors of osteoclast formation *in vitro* and *in vivo* [32,56,57], but little is known about their role in osteoblast maturation. The results of our T_{REG} experiments showed that the enriched cells and their soluble factors are likely not important regulators in osteoblast maturation. Although this contradicts the observation

that the delivery of T_{REG} cells enhances MSC-based bone regeneration in mice, this effect is possibly indirect [23]. In line with our current findings for TGF-β, mostly inhibitory effects have been demonstrated when studying the main T_{REG} anti-inflammatory cytokines directly for their role on osteoblast maturation [17,58].

In contrast to our observations for T_{REG} cells, our results show that proinflammatory T cell cytokines have profound stimulatory effects on the osteogenic differentiation of bone progenitor cells. First, the CM derived from enriched T_H17 cells induced osteogenesis significantly more than that derived from undifferentiated T cells. Second, T_H17 cell-derived cytokines showed significant pro-osteogenic effects when tested directly on MSCs. As IFN-γ-producing T_H17 cells can only be found under rare conditions [59], it is unlikely that IFN-γ contributed to the beneficial effects of the T_H17 cell-derived CM in our experiments [60]. Therefore, we studied the IL-17 family of cytokines directly for their role in osteoblast maturation. During fracture healing, BMP-2 is one of the growth factors that is up-regulated together with proinflammatory factors [9,61]. To mimic a more physiological environment for the MSCs, BMP-2 was therefore used as an alternative to dexamethasone in the osteogenic differentiation medium. IL-17A and IL-17F, the two predominant isoforms produced by T_H17 cells, were both strong pro-osteogenic stimuli for MSCs together with BMP-2. IL-17A in particular, showed a synergistic effect together with BMP-2. This novel finding requires more investigation in a preclinical setting, but is in line with the observation that IL-17A protects against bone loss in mice [62]. In contrast to IL-17A [63,64], little is yet known about the effects of IL-17F on osteoblast maturation. Nam et al. only recently proposed an important role for IL-17F in bone repair. Our data obtained with IL-17F in human MSCs agree with the finding from these same authors that IL-17F treatment increases the expression of a panel of bone markers in mouse MSCs [17]. In discordance to the direct effects found for IL-17 and TNF-α on MSCs [40], blocking these cytokines did not reduce the pro-osteogenic effect of the $CD4^+$ T cell CM on the ALP activity. A first explanation is the missing second osteogenic stimulus required for their efficacy [40]. Alternatively, TNF-α and IL-17 may induce different effects on the MSCs in the context of the cocktail of cytokines present in the CM. Based on these results, we hypothesize that the exogenous delivery of IL-17 cytokines, in combination with well-known osteoinductive growth factors, may create a physiological stimulus for bone regeneration. Importantly, IL-17 signaling is well-known for its detrimental effects on bone tissue through the stimulation of osteoclastogenesis [65]. Therefore, a bone anabolic therapy using IL-17 should also rely on methods to directly [66] or indirectly [67,68] suppress the action of osteoclasts.

A contradictory finding was that T_H17 cells did not show an effect on osteoblast differentiation in direct coculture with MSCs. MSCs are known to suppress T_H17 cell differentiation while driving T_{REG} differentiation in coculture models [69,70], which might influence the studied MSC–T cell interaction. However, other studies show that these immunomodulatory effects are less profound in our experimental design, *i.e.* the scenario where the T_H17 cells are exposed to the MSCs after their initial activation and differentiation [71,72]. It is thus more likely that the different outcomes observed for the T_H17 cells between the CM and coculture experiments are related to the duration of the stimulus to which the MSCs are exposed. In the CM experiments, the MSCs are exposed to a continuous stimulus during the entire culture period, while the T cells likely only induce a short-lasting effect on the MSCs in the cocultures (Fig. S2).

Our results indicate that activated T lymphocytes can contribute to osteoblast maturation through the production of soluble factors. Although the $CD4^+$ T cell population demonstrates the highest osteogenic effects as a whole, individual $CD4^+$ T cell subsets can still contribute to MSC osteogenesis in different ways. The beneficial role of IFN-γ in osteoblast differentiation suggests that the T helper 1 subset of the $CD4^+$ population should be further elucidated. Our current polarization experiments show that proinflammatory T cell populations, including the T_H17 cells, are most stimulatory on bone progenitor cells. Our current *in vitro* findings are in agreement with the *in vivo* results of Nam et al., who proposed that the local balance between anti-inflammatory T_{REG} and proinflammatory T_H17 cells affects the outcome of fracture healing [17]. In agreement with this hypothesis, we show that different T cell subsets and their secreted factors may differently affect the outcome of bone regeneration. For instance, the relative occurrence of different $CD4^+$ T cell subsets may be predictive for the success of bone healing. This is in line with data showing that the systemic presence of a subset of $CD8^+$ effector memory T cells is associated with decreased fracture healing [24]. Alternatively, bone-stimulating T cell cytokines may be harnessed to promote bone regeneration in tissue engineering strategies [73].

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