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T Cell-Mediated Increased Osteoclast Formation From Peripheral Blood as a Mechanism for Crohn's Disease-Associated Bone Loss

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ABSTRACT
The pathophysiology of osteoporosis in patients with Crohn's disease (CD) is still not completely elucidated. In this study, we evaluated osteoclastogenesis from peripheral blood cells of CD patients and studied the role of lymphocytes and inflammatory cytokines in this process. Peripheral blood mononuclear cells from seven patients with quiescent CD and matched healthy controls were isolated, and separated into T cells, B cells, and a T- and B-cell depleted fraction. In various culture combinations, osteoclast formation in the absence of the osteoclastogenic factors RANKL and M-CSF was assessed by scoring the number of tartrate-resistant acid phosphatase (TRACP) positive multinucleated cells (MNCs). Cytokine levels in culture supernatants were measured. Formation of heterogeneous cell clusters in culture was noticed; a process that was inhibited by anti-LFA-1. In CD cultures, mean cluster area was up to threefold higher than in control cultures, and shown to be induced by T cells. Over tenfold higher numbers of TRACP$^+$ MNCs were found in CD cultures, but exclusively in cultures containing T cells. Formation of cell clusters correlated strongly with formation of TRACP$^+$ MNCs. Both cell cluster formation and osteoclast formation were related to IL-17 levels in vitro. In conclusion, osteoclastogenesis, preceded by cell cluster formation, is T cell-mediated and increased in patients with quiescent CD. Our findings suggest heterotypic interactions between osteoclast precursors and T cells to be a triggering step in osteoclast formation in CD. Furthermore, our results propose a possible role for IL-17 in osteoclastogenesis in CD patients, and as such in CD-associated bone loss. J. Cell. Biochem. 113: 260–268, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: INFLAMMATORY BOWEL DISEASE; OSTEOCLASTOGENESIS; PERIPHERAL BLOOD; T CELL; IL-17

A common complication in Crohn's disease (CD) is an altered bone metabolism inducing the development of osteoporosis [Compston et al., 1987; Schulte, 2004]. Bone, being a dynamic tissue, is continuously renewed through a complex process of bone resorption by osteoclasts and subsequent bone formation by osteoblasts. An imbalance in this process can result in bone loss, as shown in a previous study on bone histomorphometry in patients with quiescent CD [Oostlander et al., 2011]. For bone degradation to occur, osteoclast progenitor cells differentiate into mature, multinucleated osteoclasts. The precursors of osteoclasts are derived from cells of the monocyte/macrophage lineage. In bone loss-associated diseases, it is thought that osteoclast precursors are recruited from peripheral blood mononuclear cells (PBMCs) [de Vries and Everts, 2009].

Osteoclast formation from PBMCs has been shown to occur when PBMCs are stimulated by the osteoclastogenic factors receptor activator of NFκB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). PBMCs from patients with rheumatic diseases [Ritchlin et al., 2003; Colucci et al., 2007], osteoporosis [D'Amelio et al., 2005; Nose et al., 2009], and periodontitis (Brunetti

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et al., 2005; Tjoa et al., 2008] exhibit a significant increase in osteoclast formation in vitro when compared to PBMCs from healthy controls. Interestingly, increased osteoclast formation particularly occurs when PBMCs from patients with inflammatory diseases are cultured without the addition of osteoclastogenic cytokines (reviewed by de Vries and Everts [2009]). This implies that cytokines necessary for osteoclast formation are produced by PBMCs themselves. In patients with CD, PBMCs are highly activated and are characterized by an altered production of inflammatory cytokines [Mazlam and Hodgson, 1992; Elsasser-Beile et al., 1994]. Therefore, PBMCs from CD patients might have an increased capacity to induce osteoclast formation as well.

Recently, the role of monocytes, T cells, and B cells in osteoclastogenesis has been addressed, as these cells are the primary cell subsets of PBMCs. Spontaneous osteoclastogenesis was reported to occur only in unfractionated PBMC cultures, or in cultures in which monocytes were cultured in the presence of T cells [Brunetti et al., 2005; Colucci et al., 2007; D'Amelio et al., 2008]. Based on these findings, T-cell cytokines have been suggested to be essential drivers of osteoclastogenesis. In CD, markedly increased levels of cytokines associated with both Th1 and Th17 cells have been reported in the inflamed mucosa [Reimund et al., 1996; Liu et al., 2009]. In addition, disturbances at the T-cell level have been observed in peripheral blood of CD patients, as reflected by decreased numbers of regulatory T cells and increased levels of Th17 cells [Eastaff-Leung et al., 2010]. Therefore, both altered cytokine production by T cells and changes in T-cell number and/or T-cell activation in CD patients are likely to be important in the regulation of osteoclastogenesis in this patient group.

To improve our understanding of osteoclastogenesis in patients with CD, we aimed to analyze the process of spontaneous osteoclast formation by peripheral blood from patients with quiescent CD. To clarify the role of lymphocytes in osteoclast formation, we studied the effect of T and B cells on osteoclastogenesis from autologous T- and B-cell-depleted PBMCs.

MATERIALS AND METHODS

STUDY POPULATION

Patients diagnosed with CD according to Lennard-Jones criteria were included [Lennard-Jones, 1989]. Since therapy with bisphosphonates, corticosteroids, and antibodies to TNF-α is known to influence osteoclastogenesis, use of these treatments <6 months prior to inclusion belonged to the exclusion criteria. Consequently, patients included were in a quiescent state of disease (i.e., C-reactive protein < 15 mg/L). Other exclusion criteria were metabolic bone diseases and hypo- and hyperthyroidism. The patient group consisted of three male and four female CD patients with a mean age of 33.6 ± 3.5 years (mean ± SEM). Each patient was matched for gender and age with a healthy control. The control group consisted of three male and four female subjects with a mean age of 32.4 ± 3.4 years (mean ± SEM). The study was approved by the Institutional Review Board of the VU University Medical Center, and all individuals gave written informed consent.

SERUM MEASUREMENTS

Serum samples from all individuals were collected and stored at −80°C. Serum concentrations of a bone resorption marker (CTx) and a bone formation marker (P1NP) were assessed using, respectively, the β-CrossLaps serum assay (Roche Diagnostics, Mannheim, Germany) and the UniQ P1NP radioimmunoassay (Orion Diagnostica, Espoo, Finland). Measurements were performed according to manufacturer's instructions. Enzyme-linked immunosorbent assays (ELISAs) were performed to quantify the serum levels of the following cytokines (detection ranges in parentheses): IL-1α (RD Systems, Minneapolis, MN; 8–1,000 pg/ml), IL-1β (Sanquin, Amsterdam, The Netherlands; 5–600 pg/ml), IL-6 (R&D; 10–1,200 pg/ml), IL-13 (Sanquin; 4–500 pg/ml), IL-17 (eBioscience, San Diego, CA; 3–2,000 pg/ml), TNF-α (Sanquin; 3–400 pg/ml), IFN-γ (Sanquin; 6–800 pg/ml), VEGF (R&D; 16–2,000 pg/ml), HGF (R&D; 63–8,000 pg/ml), and MCP-1 (R&D; 16–2,000 pg/ml).

CELL ISOLATION

Peripheral blood samples from patients and matched controls were obtained on the same day and time, and were processed within 2 h. Two milliliter of blood was kept apart to analyze blood cell composition by flow cytometry. Peripheral blood mononuclear cells were isolated from 25 ml whole blood using Ficoll density gradient centrifugation as described previously [Tjoa et al., 2008]. T cells were isolated from PBMCs by positive selection using anti-CD3-conjugated microbeads and the PosselD protocol of an autoMACS cell sorter following the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Subsequently, the negative selection was incubated with anti-CD19-conjugated microbeads (Miltenyi Biotech) to isolate B cells. Thus, in total three cell fractions were obtained. First, a $CD3^-/CD19^-$ fraction, from now on designated as the negative fraction, containing monocytes and NK cells as confirmed by mRNA expression levels of CD14 and CD56. Second, a $CD3^+$ T-cell fraction as confirmed by CD3 mRNA expression. And third, a $CD19^+$ B-cell fraction as confirmed by CD19 mRNA expression.

BLOOD CELL COMPOSITION

Four-color flow cytometry was performed to determine the whole blood cell composition of peripheral blood as described previously [Tjoa et al., 2008].

CELL CULTURE

The negative fraction was cultured separately (1.2×10^5 cells/well), and together with autologous T cells (2×10^5 cells/well) and/or autologous B cells (4×10^4 cells/well). Monocultures consisting of B cells and T cells, thus without putative osteoclast precursor cells, were performed as well. The choice of cell density in culture was based on the average prevalence of monocytes, B cells and T cells in whole blood.

Cells were seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) on plastic and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Fetal Clone 1, HyClone) and 1% antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml Amphotericin B; Sigma). Cultures were grown without the addition

of osteoclastogenic factors M-CSF and RANKL. For resorption experiments, cells were cultured on 500-μm-thick elephant teeth dentin slices in the absence and presence of 25 ng/ml M-CSF and 40 ng/ml RANKL (optimal concentrations according to Olivier et al. [2008]). All cultures were run in duplicate, and maintained at 37°C in a humidified atmosphere with 5% CO_2. Medium was replenished twice weekly. Culture supernatants were harvested after 7, 14, and 21 days of culture and stored at −20°C.

CELL CLUSTER ANALYSIS

From all culture conditions, after 7 and 14 days of culture three micrographs per well were taken from pre-determined positions (center of the well and an adjacent field to the left and to the right) at a ×40 magnification, using a digital camera mounted on an inverted light microscope (both from Leica, Wetzlar, Germany). Formation of cell clusters, starting from day 1 of culture, was noticed. Cell cluster number and size were quantified using Image-Pro Plus software (Media Cybernetics, Silver Spring). Subsequently, cell cluster area was calculated as the product of cell cluster number and size.

In order to establish the contribution of the various cell fractions to cluster formation, PBMCs were reconstituted from the negative fraction with T cells and B cells. For each possible combination, a single cell population was labeled with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Invitrogen). In short, after cell separation cells were washed with PBS and incubated with a final concentration of 1.5 μM CFSE solution for 15 min at 37°C. After washing repeatedly with PBS and culture medium, cells were seeded in 96-well plates and allowed to settle overnight. Cultures were analyzed for their cell content after 1–2 days of culture.

To determine whether cluster formation was facilitated by leukocyte function-associated antigen-1 (LFA-1) dependent cell–cell interactions, blocking experiments with an antibody to LFA-1 were performed. In short, the negative fraction alone and with added T cells and/or B cells was cultured in the presence of vehicle (PBS), 20 μg/ml anti-LFA-1α [van Kooyk et al., 1994] or 20 μg/ml IgG2a isotype control (Sanquin) for 7 days. After 2, 7, and 14 days of culture micrographs were taken to determine cell cluster formation. After 21 days of culture the formation of multinucleated cells (MNCs) was assessed.

MULTINUCLEATED CELL ANALYSIS

The formation of tartrate-resistant acid phosphatase (TRACP) positive multinucleated cells ($TRACP^+$ MNCs) from the various culture combinations was assessed after 3 weeks of culture, as described previously [Tjoa et al., 2008]. The number of $TRACP^+$ MNCs per well was determined at a ×200 magnification. Only cells with three or more nuclei were considered as MNC.

OSTEOCLAST RESORPTION ASSAY

In order to evaluate osteoclast activity, after culturing cells on dentin slices for 4 weeks resorption was analyzed as described previously [Tjoa et al., 2008]. In addition, scanning electron microscopy (Philips XL20; Eindhoven, The Netherlands) was performed to visualize formation of resorption pits. Three days after the last medium refreshment, concentrations of CTx were measured in culture supernatants.

RNA ISOLATION AND QUANTITATIVE PCR

RNA was isolated from all cell types at baseline (before the start of culture) using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA concentration was measured with the Nanodrop spectrophotometer (Nanodrop Technologies). cDNA preparation, primer designation, and the PCR reaction were performed as described previously [Tjoa et al., 2008]. PCR primers included: CD3, CD14, CD19, CD56, RANK, RANKL, OPG, M-CSF, c-FMS, ICAM-1, LFA-1, TNF-α, TNF-R1, TNF-R2, IFN-γ, TGF-β, IL-1β, IL-4, IL-6, IL-7, IL-13, and IL-17 (for detailed information see Supplementary data). For the PCR analysis, samples were normalized for the expression of PBGD by calculating the ΔCt ($Ct_{\text{gene of interest}} - Ct_{PBGD}$). Expression of the different genes is expressed as $2^{\wedge-(\Delta Ct)}$.

CYTOKINE MEASUREMENT IN CULTURE MEDIUM

A custom Milliplex Human Cytokine/Chemokine kit (Millipore) was used to simultaneously quantify levels of 10 cytokines in conditioned media obtained after 7 and 14 days of culture. Culture supernatants from the negative fraction, the T-cell fraction, and the negative fraction plus T cells, were assayed for their content of IL-1α, IL-1β, IL-6, IL-7, IL-13, IL-17, TNF-α, IFN-γ, VEGF, and MCP-1. Assays were run in duplicate and performed according to the manufacturer's recommendations.

STATISTICAL ANALYSIS

Data showing a normal distribution are expressed as mean ± standard error of the mean (SEM), data not showing a normal distribution are expressed as median [inter-quartile range]. Differences between various cell fractions were tested using a repeated measures ANOVA and Dunnett's post hoc test, or when appropriate a non-parametric Friedman and Dunn's post hoc test. Differences between control and patient cultures were analyzed by a paired Student's *t*-test, or when appropriate a non-parametric Wilcoxon signed-rank test. Correlations were calculated using Spearman's rank correlation coefficient. Analyses were performed using SPSS for Windows version 16.0 (SPSS. Inc., Chicago, IL) and GraphPad Prism 4 (GraphPad Software, San, Diego, CA). A *P*-value of <0.05 was considered statistically significant.

RESULTS

BASAL CHARACTERISTICS

Patient characteristics are summarized in Table I. The leukocyte composition of whole blood was unchanged in patients with CD when compared to healthy controls (Table II). CTx and P1NP levels in serum were comparable between healthy controls and CD patients (451 ± 132 vs. 356 ± 124 ng/L; and 60 ± 8 vs. 56 ± 14 μg/L, respectively). Serum cytokine levels were generally low or undetectable in both healthy controls and quiescent CD patients, and did not differ between patients and healthy controls (data not shown).

Basal RNA expression levels of genes possibly contributing to osteoclast formation were determined in the negative fraction, T- and B-cell fraction isolated from PBMCs of healthy controls and CD patients (Table III). Relative expression levels of IL-6 tended to be

TABLE I. Patient Characteristics

	CD patients
Disease activity [CRP (mg/L)]	2.5 [2.5–3.6]
Location of disease (n)	
Terminal ileum	3
Colon/rectum	4
Bowel resection, yes/no (n)	4/3
Disease duration (years)	10 [8–15]
Age at diagnosis (years)	19 [15–28]
Corticosteroid-free (years)	3 [2–8]
Current medication use (n)	
5-ASA derivatives	2
No medication	5
25(OH)D (nmol/L)	49 [31–74]
ALP (U/L)	63 [57–72]
BMD lumbar spine (g/cm^2)	0.96 [0.86–1.04]
T-score (SD)	−1.2 [−1.7 to −0.25]
BMD total hip (g/cm^2)	0.95 [0.91–1.10]
T-score (SD)	0 [−0.35 to 0.50]

Data are expressed as median [25th–75th quartile] (n = 7).

higher in both the negative and T-cell fraction of CD patients when compared to healthy controls (both $P = 0.0625$). Relative expression levels of IL-17 tended to be higher in T cells from CD patients when compared to healthy control cells ($P = 0.0625$).

CELL CLUSTER FORMATION IS INCREASED IN CD CULTURES AND HIGHEST IN CULTURES WITH T CELLS

Typical for all culture conditions containing cells from the negative fraction was the presence of cell clusters formed early in culture in both cultures from controls and CD patients (Fig. 1A,B). When comparing control and CD cultures, it became apparent that in CD patient-derived cultures where T cells were present, mean cluster area was up to threefold higher (Fig. 1C; data on monocultures of B and T cells not shown). Moreover, comparison of the various culture combinations within CD cultures revealed that the mean cluster area was 2.5-fold higher in the presence of T cells in comparison to cultures without T cells. In control cultures, the presence of T cells did not affect formation of cell clusters. The addition of B cells to the negative fraction, with or without T cells, had no apparent effect on cluster formation, both in healthy control and CD cultures.

CELL CLUSTERS ARE HETEROGENEOUS AND CONSIST OF B- AND T-CELL DEPLETED PBMCS AND T CELLS

To evaluate which cells contributed to cluster formation, healthy control co-cultures of the negative fraction plus T and B cells were plated in threefold, where in each plating a different cell type was labeled. These experiments revealed that cell clusters were heterogeneous and that nearly all clusters contained cells from the negative fraction and T cells (Fig. 2A,B). B cells generally remained solitary. In those clusters containing B cells, B cells were present only at a low number per cluster.

TABLE II. Whole Blood Cell Composition From Healthy Controls and CD Patients

	Controls	CD patients	*P*-value
Granulocytes	52.6 ± 4.9	57.0 ± 3.5	0.3706
Monocytes	5.6 ± 0.5	5.2 ± 0.2	0.5954
NK cells	6.0 ± 1.2	3.6 ± 0.8	0.1149
T cells	26.6 ± 4.7	23.6 ± 3.0	0.4047
CD4+ T cells	55.4 ± 3.8	64.4 ± 4.4	0.1282
CD8+ T cells	32.9 ± 3.3	25.6 ± 4.2	0.1240
CD4+/CD8+ T-cell ratio	1.9 ± 0.3	3.0 ± 0.5	0.0737
B cells	3.0 ± 0.2	3.2 ± 0.5	0.7432

Data are expressed as mean percentage ± SEM of whole blood cell count (n = 7).

CELL CLUSTER FORMATION IS LFA-1 MEDIATED

LFA-1 is an integrin that coordinates interaction between monocytes and lymphocytes via its adhesion partner intercellular adhesion molecule-1 (ICAM-1) [Marlin and Springer, 1987]. To determine whether cell–cell contact through LFA-1 facilitated heterotypic cluster formation, healthy control cells were cultured in the presence of an antibody against LFA-1. Representative figures of LFA-1 blocking experiments are shown in Figure 2C. In cultures with control IgG, cluster area did not differ from vehicle treated cultures (data not shown). Addition of anti-LFA-1 decreased cluster formation by over tenfold when compared to IgG control cultures, but the difference reached statistical significance only in cultures where T cells were present (Fig. 2D). During subsequent culturing in the absence of anti-LFA-1, the inhibitory effect of anti-LFA-1 on cell cluster formation diminished. The presence of anti-LFA-1 during the first week of culture affected the formation of TRACP$^+$ MNCs after 3 weeks of culture in a similar way as the formation of cell clusters (Fig. 2E).

SPONTANEOUS FORMATION OF TRACP$^+$ MNCS IS INCREASED IN CD CULTURES AND EXCLUSIVELY OBSERVED IN CULTURES WITH T CELLS

Spontaneous formation of TRACP$^+$ MNCs (Fig. 3A,B) was observed exclusively in co-cultures containing the negative fraction and T cells (Fig. 3C; data on monocultures of B cells and T cells not shown). In CD cultures, but not in healthy control cultures, the formation of TRACP$^+$ MNCs was statistically significant higher in the presence of T cells in comparison to cultures without T cells. The addition of B cells to the negative fraction, alone or together with T cells, did not induce the formation of TRACP$^+$ MNCs. An overall comparison of all culture conditions between controls and CD patients revealed over tenfold higher numbers of TRACP$^+$ MNCs in cultures from CD patients ($P = 0.0354$).

Correlation analysis showed a strong positive correlation between the formation of TRACP$^+$ MNCs after 3 weeks of culture and cell cluster formation analyzed at both week 1 and 2 of culture ($r = 0.830$, $P < 0.0001$; and $r = 0.929$, $P < 0.0001$, respectively; Fig. 3D).

In parallel, cells from controls and patients were also cultured in the presence of M-CSF and RANKL. Here, in line with previous reports for other diseases, similar mean numbers of TRACP$^+$ MNCs were observed in control and CD cultures (267 [35–536] and 250 [89–391], respectively) [D'Amelio et al., 2008; Olivier et al., 2008; Tjoa et al., 2008]. Thus, the increased osteoclastogenesis induced by T cells in the CD group could be attributed to their own osteoclastogenic cytokine production.

BONE RESORPTION

To confirm the osteoclastic potential of the MNCs generated from peripheral blood of patients and controls, cells were cultured on

TABLE III. Relative RNA Expression Levels in Cells From Healthy Controls and CD Patients

	Negative fraction		T-cell fraction		B-cell fraction	
	Controls	CD patients	Controls	CD patients	Controls	CD patients
RANKL	0.04 ± 0.009	0.04 ± 0.009	0.04 ± 0.008	0.03 ± 0.012	0.01 ± 0.003	0.02 ± 0.008
RANK	0.12 ± 0.040	0.09 ± 0.038	0.04 ± 0.021	0.04 ± 0.024	0.01 ± 0.010	0.02 ± 0.006
OPG	0.001 ± 0.0005	0.002 ± 0.0006	n.d.	n.d.	n.d.	n.d.
M-CSF	16 ± 2.0	16 ± 2.6	0.4 ± 0.18	0.3 ± 0.08	0.4 ± 0.17	0.6 ± 0.25
C-FMS	0.1 ± 0.01	0.1 ± 0.02	0.6 ± 0.16	0.4 ± 0.09	0.2 ± 0.09	0.1 ± 0.06
ICAM-1	2.5 ± 0.40	3.0 ± 0.75	0.9 ± 0.24	0.8 ± 0.20	0.7 ± 0.10	1.1 ± 0.23
LFA-1	16 ± 2.9	13 ± 1.5	18 ± 1.2	17 ± 5.1	9.6 ± 1.3	18 ± 3.5
TNF-RI	5.3 ± 0.50	5.0 ± 0.78	2.4 ± 0.21	2.4 ± 0.49	1.6 ± 0.14	1.0 ± 0.26
TNF-RII	35 ± 5.0	32 ± 4.2	17 ± 2.2	13 ± 3.2	8.5 ± 1.6	15 ± 0.2
TNF-α	3.6 ± 0.54	3.2 ± 0.39	2.1 ± 0.49	2.0 ± 0.33	2.6 ± 0.38	3.7 ± 0.93
TGF-β	41 ± 3.7	39 ± 4.5	41 ± 5.1	42 ± 8.8	34 ± 5.7	38 ± 10
IFN-γ	0.2 ± 0.03	0.2 ± 0.03	0.7 ± 0.10	0.7 ± 0.21	0.6 ± 0.14	0.5 ± 0.20
IL-1β	5.3 ± 1.73	8.0 ± 2.98	0.2 ± 0.08	0.1 ± 0.05	0.6 ± 0.34	0.3 ± 0.15
IL-4	0.25 ± 0.027	0.30 ± 0.113	0.02 ± 0.006	0.03 ± 0.009	0.02 ± 0.001	0.04 ± 0.008
IL-6	0.02 ± 0.005	0.04 ± 0.010*	0.01 ± 0.004	0.02 ± 0.004*	0.4 ± 0.11	1.0 ± 0.25
IL-7	0.08 ± 0.008	0.12 ± 0.020	0.15 ± 0.034	0.14 ± 0.034	1.6 ± 0.59	1.9 ± 0.37
IL-13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IL-17	n.d.	n.d.	0.002 ± 0.002	0.005 ± 0.002*	n.d.	n.d.

Neg, negative fraction; T, T-cell fraction; B, B-cell fraction; n.d., not detected.
Data are expressed as mean ± SEM (n = 6).
*$P = 0.0625$ versus healthy control.

dentin slices both in the presence and absence of M-CSF and RANKL. Similar to previous experience, very few TRACP$^+$ MNCs formed under these conditions (data not shown) [Olivier et al., 2008; Tjoa et al., 2008]. Limited bone resorption, as assessed with pit assays (Coomassie brilliant blue staining and scanning electron microscopy) and CTx measurements in conditioned culture medium did not reveal statistically significant differences between CD and control cultures (data not shown).

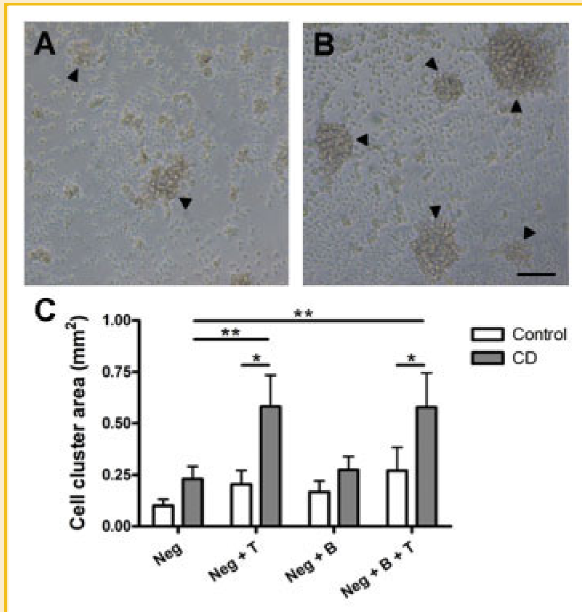

Fig. 1. Cell cluster formation is increased in cultures from CD patients and is highest in cultures containing T cells. A,B: Representative micrographs of cell clusters formed after 7 days of culture in cultures of the negative fraction + added T cells (Neg + T) from a healthy control and CD patient, respectively. Arrows depict cell clusters. Scale bar = 100 μm. C: Mean cell cluster area was higher in cultures from CD patients in comparison to healthy control cultures. Moreover, in CD cultures the presence of T cells increased cell cluster area, whereas presence of T cells in control cultures did not affect the formation of cell clusters. Data are depicted as mean ± SEM (n = 6). *$P < 0.05$, **$P < 0.01$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

INCREASED IL-17 LEVELS IN CULTURE SUPERNATANTS ARE RELATED TO CLUSTER FORMATION AND THE FORMATION OF TRACP$^+$ MNCS

The above findings show that co-culturing of the negative fraction and T cells induces the formation of cell clusters, and appears to be a prerequisite for the formation of TRACP$^+$ MNCs. To determine whether co-culturing influenced levels of cytokines that stimulate the formation of cell clusters and TRACP$^+$ MNCs, cytokine levels were measured in conditioned media from the negative fraction, the T-cell fraction, and from co-cultures of the negative fraction with T cells of both healthy control and CD cultures.

Cytokine levels in conditioned medium obtained after 1 week of culture are shown in Table IV. In monocultures of the negative fraction, cytokine levels were generally higher in cultures from CD patients than in those of healthy controls. In healthy control cultures, co-culture of the negative fraction with T cells resulted in increased levels of IL-1α, IL-1β, IL-13, IL-17, and TNF-α compared to levels in monocultures. In contrast, in CD cultures only IL-17 levels were increased by co-culturing. Comparison between control and CD co-cultures revealed higher IL-17 levels in CD patient-derived culture supernatants.

In culture supernatants obtained after 2 weeks of culture, hardly any detectable levels of the cytokines were observed (data not shown). Nevertheless, IL-17 and TNF-α levels persisted and tended to be higher in CD co-cultures than in control co-cultures (IL-17: 6.2 [0.5–40] vs. 0.3 [0.1–4.1] pg/ml, $P = 0.0625$; and TNF-α: 4.8 [1.5–176] vs. 2.9 [0.1–9.4] pg/ml, $P = 0.0313$).

Of all cytokines tested, correlation analysis with data obtained at week 1 of culture showed a positive correlation between cell cluster area and cytokine levels in conditioned medium only for

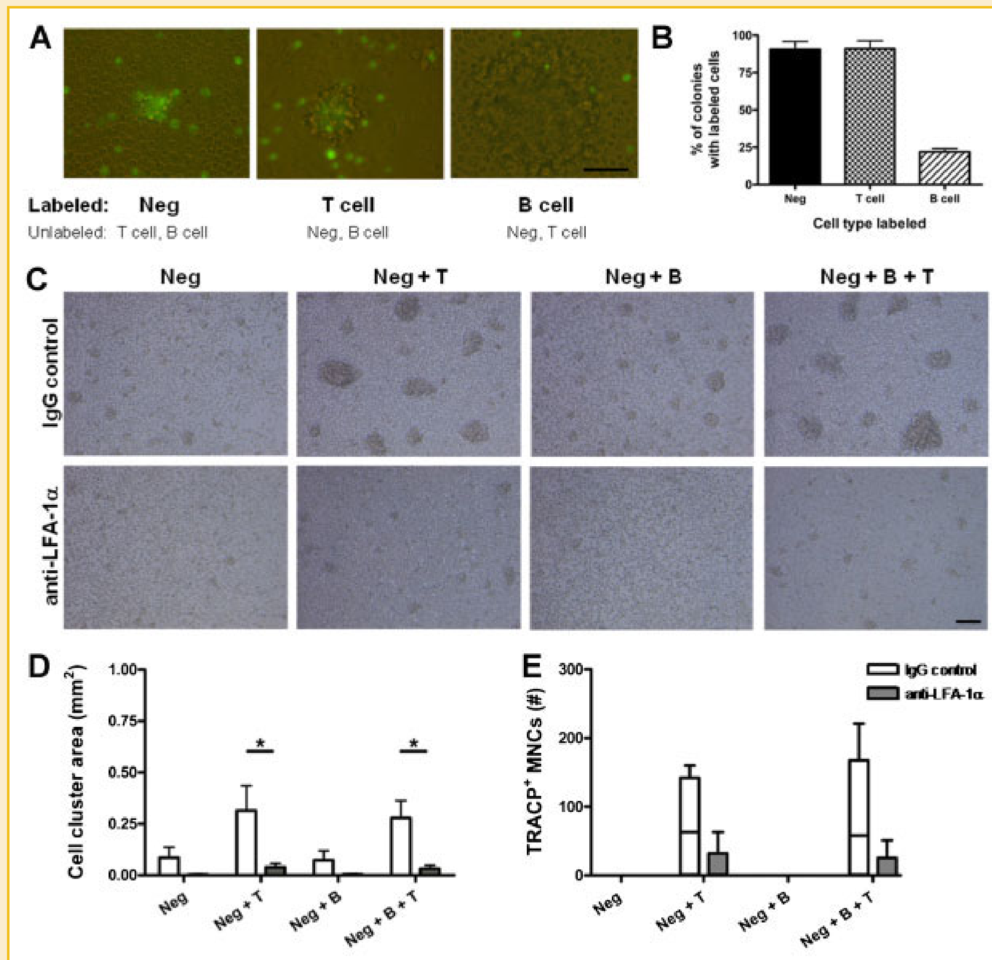

Fig. 2. Cell clusters are heterogeneous and formation can be inhibited by anti-LFA-1. A: Representative micrographs of a healthy control culture containing the negative fraction plus T cells plus B cells (Neg + B + T), in which in each case a single cell fraction is labeled with CFSE. Scale bar = 100 μm. B: Clusters are heterogeneous and contain cells from the negative fraction and T cells. The presence of B cells in cell clusters is relatively uncommon. Data are depicted as mean ± SEM (n = 4). C: Representative micrographs of addition of an antibody against LFA-1 to a healthy control culture. Scale bar = 100 μm. D: Blocking of LFA-1α reduced cell cluster area, but only in cultures where T cells were present. Data are depicted as mean ± SEM (n = 4). E: Blocking of LFA-1α tended to reduce the formation of TRACP$^+$ MNCs. Data are depicted as median [25th–75th quartile] and minimum and maximum value (n = 4). $^*P < 0.05$.

IL-17 and VEGF ($r = 0.548$, $P = 0.0005$; and $r = 0.642$, $P < 0.0001$, respectively). Analysis of data obtained at week 2 of culture, showed cultures with the highest levels of IL-6, IL-13, IL-17, TNF-α, and IFN-γ in conditioned medium to present the highest cell cluster area as well as the highest number of TRACP$^+$ MNCs in culture.

DISCUSSION

In the present study, we demonstrated that in the absence of the osteoclastogenesis inducing cytokines M-CSF and RANKL, the spontaneous formation of TRACP$^+$ MNCs from peripheral blood precursor cells was increased in patients with quiescent CD when compared to cells obtained from healthy controls. We observed T cells to be critical for spontaneous osteoclastogenesis, as osteoclast formation was completely abolished in T-cell-depleted PBMC cultures of CD patients. Furthermore, our data suggest that B cells do not play a role in spontaneous osteoclastogenesis in CD patients.

A novel and intriguing pathogenic finding in this study is that osteoclast formation was preceded by the formation of cell clusters in cultures containing osteoclast precursor cells. Although cell fusion is known as a common process in osteoclastogenesis, we are the first to report formation of heterotypic clusters in cultures of peripheral blood cells in vitro. Here, we showed cell cluster formation to be increased in cultures from CD patients. Moreover, cluster formation was induced by the presence of T cells, but exclusively in CD cultures. These data suggest a functional difference between T cells from CD patients and T cells from healthy controls. Formation of cell clusters followed by the formation of multinucleated giant cells has been previously observed in cultures of blood cells obtained from HIV patients [Kazazi et al., 1994]. The cell cluster formation reported by Kazazi et al. was mediated by interactions of the cell adhesion molecules

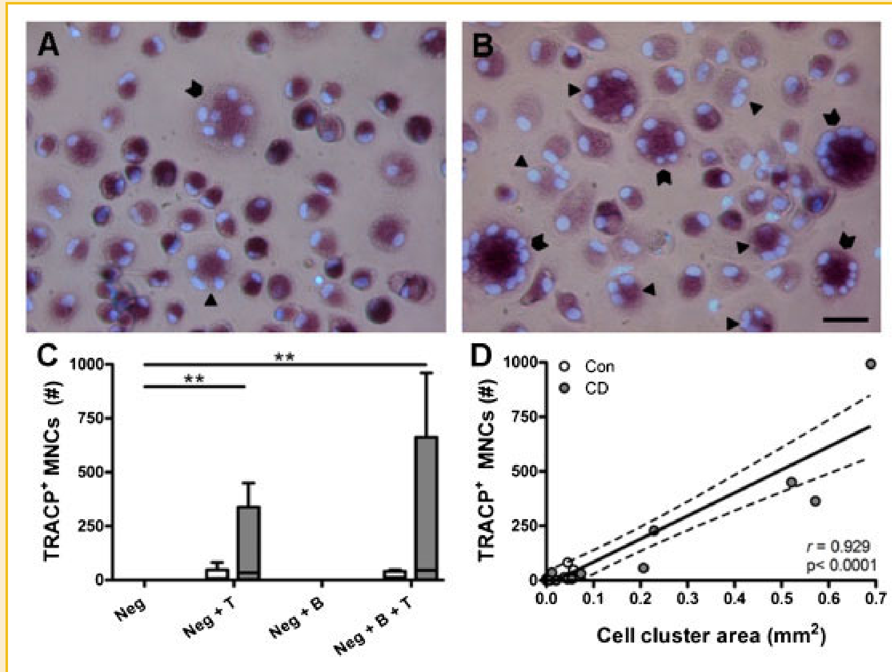

Fig. 3. Formation of TRACP$^+$ MNCs is increased in cultures from CD patients and is exclusively observed in cultures containing T cells. A,B: Representative micrographs of TRACP$^+$ MNCs in cultures of the negative fraction + added T cells (Neg + T) from a healthy control and CD patient, respectively. Osteoclasts are stained red (TRACP) and their nuclei are stained blue (DAPI). Arrowheads depict TRACP$^+$ MNCs with three to five nuclei, double arrowheads depict TRACP$^+$ MNCs with 6–10 nuclei. Scale bar = 50 μm. C: Formation of TRACP$^+$ MNCs was observed exclusively in cultures where T cells were present, and was higher in an overall comparison between cultures from CD patients (grey boxplots) and control cultures (white boxplots) ($P = 0.0354$). Data are depicted as median [25th–75th quartile] and minimum and maximum value (n = 6). D: Correlation analysis of all culture conditions from control and CD cultures combined showed a strong relation between mean cluster area and the number of TRACP$^+$ MNCs formed. $^{**}P < 0.01$.

LFA-1 and ICAM-1. Interestingly, interactions between LFA-1 and ICAM-1 are thought to be involved in osteoclastogenesis as well [Kurachi et al., 1993; Harada et al., 1998]. In the current study, we showed that heterotypic cluster formation was strongly reduced in cultures in which LFA-1 was blocked. Similarly, the presence of anti-LFA-1 during the first week of culture tended to decrease the formation of TRACP$^+$ MNCs after 3 weeks of culture.

The increased formation of cell clusters and the spontaneous osteoclastogenesis in quiescent CD patients appeared not to be the result of an altered composition of peripheral blood. Therefore, differences in the activity rather than in the number of blood cells from CD patients is likely to have caused the increased cluster formation and osteoclast formation.

Analysis of basal RNA expression levels in the different PBMC cell fractions from quiescent CD patients and healthy controls revealed potentially higher IL-6 expression in B- and T-cell-depleted PBMCs from CD patients. Moreover, T cells from CD patients tended to have increased expression levels of IL-6 and

TABLE IV. Cytokine Levels in Conditioned Medium From Healthy Control and CD Cultures

	Negative fraction		Co-culture of Neg + T		T-cell fraction	
	Controls	CD patients	Controls	CD patients	Controls	CD patients
IL-1α	3.0 [1.2–4.7]	5.5 [2.5–146][a]	44 [12–86][b]	26 [17–111]	n.d.	n.d.
IL-1β	0.3 [0.1–1.2]	1.1 [0.1–66][a]	1.7 [0.8–3.9][b]	2.2 [0.9–3.0]	n.d.	n.d.
IL-6	75 [27–149]	120 [69–1,906]	67 [23–149]	99 [26–133]	n.d.	n.d.
IL-7	n.d.	n.d.	1.5 [0.1–3.0]	0.5 [0.1–1.9]	n.d.	n.d.
IL-13	0.1 [0.1–0.6]	0.7 [0.1–27]	38 [10–58][b]	39 [6–199]	0.3 [0.1–0.7]	1.6 [0.7–2.9][*]
IL-17	0.4 [0.2–2.7]	2.5 [0.1–9.4]	8.7 [4.3–29][b]	42 [19–56][a,b]	0.2 [0.1–0.4]	0.7 [0.2–2.3][*]
TNF-α	14 [8–33]	30 [10–137]	102 [46–205][b]	83 [47–183]	32 [17–47]	38 [31–47]
IFN-γ	0.4 [0.1–3.6]	7.1 [0.1–303]	61 [4–3,332]	33 [8–1,397]	1.2 [0.6–5.3]	1.6 [0.8–7.1]
MCP-1	>2,000	>2,000	>2,000	>2,000	10 [5–32]	10 [8–26]
VEGF	17 [12–25]	33 [11–39]	15 [2–23]	15 [4–21]	4.2 [0.1–9.4]	0.1 [0.1–6.9]

Neg, negative fraction; T, T-cell fraction; n.d., not detected.
Data are expressed as median [25th–75th quartile] in pg/ml (n = 6).
[a]Control versus CD, $P < 0.05$.
[b]Neg versus Neg + T, $P < 0.05$.
[*]Control versus CD, $P = 0.0625$.

IL-17. The elevated expression levels of these pro-inflammatory cytokines reflect a higher activation state of particularly T cells from CD patients, even when clinically quiescent. This might have triggered a partial differentiation of osteoclast precursor cells from CD patients already in the circulation, and may explain the increased capability of peripheral blood cells from CD patients to spontaneously form osteoclasts.

A finding relevant for a possible mechanistic insight in the difference in the formation of $TRACP^+$ MNCs between clinically quiescent CD patients and controls, is the elevated production of cytokines in vitro. Unstimulated B- and T-cell-depleted PBMC cultures from CD patients produced increased levels of IL-1α and IL-1β. In addition, monocultures of T cells from CD patients showed increased levels of IL-13 and IL-17. This is in line with our RNA expression data, as well as with previous studies on cytokine production by peripheral blood cells of IBD patients [Mazlam and Hodgson, 1992; Nakamura et al., 1992; Elsasser-Beile et al., 1994], and is consistent with a higher activation level of peripheral blood cells from CD patients. Co-culturing of B- and T-cell-depleted PBMC cultures with T cells resulted in induced IL-17 levels when compared to monocultures, a phenomenon most pronounced in CD cultures. Prolonged culturing of peripheral blood cells in the absence of exogenous cytokines resulted in low or undetectable cytokine levels in cultures from healthy controls. However, some cultures from CD patients still produced IL-6, IL-17, and TNF-α. Strikingly, the highest numbers of osteoclasts were observed in those cultures with the highest levels of these cytokines present, which underlines the importance of these pro-inflammatory cytokines in osteoclast formation in CD patients. Besides, these findings fit in with the alleged association between an increased pro-inflammatory potential and induced bone loss in these patients, and provide support for the beneficial effects of anti-TNF-α therapy on bone mineral density in CD patients.

Our data suggest a particular role for IL-17 in osteoclastogenesis in CD patients; T cells from CD patients had elevated basal expression levels of IL-17, cultures from CD patients secreted higher levels of IL-17, and levels of IL-17 were related to both cluster formation and the formation of $TRACP^+$ MNCs. Recently, Th17 cells have been identified as an osteoclastogenic Th cell subset that links T-cell activation and bone resorption [Sato et al., 2006]. An IL-17-dependent pathway for dendritic cell fusion leading to the formation of giant cells expressing TRACP has been reported in Langerhans cell histiocytosis patients [Coury et al., 2008]. Moreover, IL-17 has been shown to induce osteoclastogenesis from peripheral blood cells of rheumatoid arthritis patients [Miranda-Carús et al., 2006; Yago et al., 2009]. Therefore, IL-17 seems to have an important role in inflammation-mediated osteoclast formation.

We are aware that some of the outcomes in this study might be limited due to the small number of patients studied and the high individual variability within the patient group. This variability, observed throughout many parameters in this study, is typical for patients with (quiescent) CD as these patients by definition are part of a heterogeneous population. Nonetheless, even with the presented sample size, we observed substantial differences in the process of osteoclastogenesis and the role of T cells. The increased formation of osteoclasts in this population of quiescent CD patients reflects a disease-specific alteration, since drug use was excluded. Therefore, our results provide new insight in the mechanism for CD-associated bone loss.

In conclusion, spontaneous osteoclastogenesis is increased in patients with quiescent CD through elevated activation levels of peripheral blood cells. T cells from CD patients induced a LFA-1-mediated formation of large cell clusters which preceded the formation of $TRACP^+$ MNCs. Our findings suggest heterotypic interactions between osteoclast precursors and T cells to be a decisive step in osteoclast formation in CD.

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