Adaptive Immune Response Inhibits Ectopic Mature Bone Formation Induced by BMSCs/BCP/Plasma Composite in Immune-Competent Mice

Sébastien Bouvet-Gerbettaz, DDS, PhD,1,2 Florian Boukhechba, PhD,1,3,4 Thierry Balaguer, MD, PhD,1,3-6 Heidy Schmid-Antomarchi, PhD,1,3,4 Jean-François Michiels, MD, PhD,1,6 Jean-Claude Scimeca, PhD,1,3,4 and Nathalie Rochet, MD, PhD1,3,4

A combination of autologous bone marrow stromal cells (BMSCs) and biomaterials is a strategy largely developed in bone tissue engineering, and subcutaneous implantation in rodents or large animals is often a first step to evaluate the potential of new biomaterials. This study aimed at investigating the influence of the immune status of the recipient animal on BMSCs-induced bone formation. BMSCs prepared from C57BL/6 mice, composed of a mixture of mesenchymal stromal and monocytic cells, were combined with a biomaterial that consisted of biphasic calcium phosphate (BCP) particles and plasma clot. This composite was implanted subcutaneously either in syngenic C57BL/6 immune-competent mice or in T-lymphocyte-deficient Nude (Nude) mice. Using histology, immunohistochemistry, and histomorphometry, we show here that this BMSC/BCP/plasma clot composite implanted in Nude mice induces the formation of mature lamellar bone associated to hematopoietic areas and numerous vessels. Comparatively, implantation in C57BL/6 results in the formation of woven bone without hematopoietic tissue, a lower number of new vessels, and numerous multinucleated giant cells (MNGCs). In situ hybridization, which enabled to follow the fate of the BMSCs, revealed that BMSCs implanted in Nude mice survived longer than BMSCs implanted in C57BL/6 mice. Quantitative expression analysis of 280 genes in the implants indicated that the differences between C57BL/6 and Nude implants corresponded almost exclusively to genes related to the immune response. Gene expression profile in C57BL/6 implants was consistent with a mild chronic inflammation reaction characterized by Th1, Th2, and cytotoxic T-lymphocyte activation. In the implants retrieved from T-deficient Nude mice, Mmp14, Il6st, and Tgfb3 genes were over-expressed, suggesting their putative role in bone regeneration and hematopoiesis. In conclusion, we show here that the T-mediated inflammatory microenvironment is detrimental to BMSCs-induced bone formation and shortens the survival of implanted cells. Conversely, the lack of T-lymphocyte reaction in T-deficient animals is beneficial to BMSCs-induced mature bone formation. This should be taken into account when evaluating cell/biomaterial composites in these models.

Introduction

The development of new bone substitutes based on the combination of osteoconductive scaffolds and osteogenic cells such as bone marrow or adipose tissue stromal cells has led to promising results in animal models. Subcutaneous (SC) implantation in mice is frequently used as a first step to evaluate the bone formation induced by these composites. Many authors have described that murine or human bone marrow stromal cells (BMSCs)/calcium phosphate (CaP) composites implanted subcutaneously in immune T-cell-compromised mice result in mature bone formation associated to bone marrow.1-10 Other authors have shown that BMSCs/CaP implanted subcutaneously in immune-competent mice result in the formation of immature woven bone and no hematopoietic tissue.10-12 These results strongly suggest that BMSCs/CaP-mediated bone depends, at least in part, on the immunological status of the recipient animal and that recipient T cells inhibit BMSCs/CaP-mediated mature bone formation. In line with this, Liu et al.

1UFR Médecine F-06107, Université Nice Sophia Antipolis, Nice, France.
2UFR Odontologie F-06357, Université Nice Sophia Antipolis, Nice, France.
3CNRS, UMR7277, F-06108 Nice, France.
4Inserm U1091, F-06108 Nice, France.
5Service de Chirurgie Plastique, Centre Hospitalier Universitaire de Nice, Nice, France.
6Service d’Anatomopathologie, Centre Hospitalier Universitaire de Nice, Nice, France.
demonstrated that mature bone formation in T-deficient Nude mice was correlated to the decrease of IFN-γ and TNF-α secretion in the implants.10

The aim of the present study was to contribute data to the effect of the host immune response on SC bone formation induced by BMSCs combined with a biomaterial that we had previously described.12,13 This consisted of calibrated bi-phasic calcium phosphate (BCP) particles and plasma clot. A comparison of the local microenvironments in the implants retrieved from immune-competent or immune-deficient mice focused on quantitative and qualitative colonization; gene expression of cytokines, chemokines, and receptors; and survival of implanted cells.

Materials and Methods

Preparation and characterization of BMSCs

All the animal experiments were conducted at the central animal facility of the faculty of medicine according to the guidelines of the Direction Départementale des Services Vétérinaires and received the approval of the local committee for animal care (no. NCA/2007/12-07). BMSCs from male C57BL/6 mice were prepared as previously described.12 Briefly, 10-week-old C57BL/6 mice (JANVIER LABS) were anesthetized by an intraperitoneal injection of ketamine 90 mg/kg (Virbac) and xylazine 4.5 mg/kg (Ceva). Blood was withdrawn from C57BL/6 mice with sodium citrate as an anticoagulant by intracardiac puncture and plasma, obtained after centrifugation for 15 min at 3000 g, was immediately frozen at −20°C until implantation time. The mice were then sacrificed by cervical dislocation, and long bones (humerus, femur, and tibia) were dissected to flush the bone marrow. Bone marrow cell suspensions were seeded at a concentration of 4 × 10⁵ mononuclear cells/cm² in complete medium consisting of minimum essential medium (MEM) alpha (Lonza) containing 10% fetal calf serum (FCS) Hyclone (Perbio), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Lonza). The medium was replaced after 4 days and then, every 3 days. After 14 days, the cells were confluent and ready for implantation. The total adherent cell population was detached using trypsin-ethylenediaminetetracetic acid (EDTA; Lonza), washed in FCS-free medium, and used for immediate implant preparation.

To assess the osteogenic phenotype of the implanted BMSCs, alkaline phosphatase (ALP) enzymatic activity and red alizarin staining of the mineralized matrix were analyzed after, respectively, 14 and 25 days of culture following the manufacturer’s instructions (Sigma). Moreover, after 7, 14, and 25 days of culture, total RNA was isolated directly from plated cells using 1 mL RNAble kit and following the manufacturer’s instructions (Eurobio); then, quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) of a set of osteoblastic markers, namely Cbfal, collagen I (Coll1), bone morphogenetic protein 2 (Bmp2), bone sialoprotein (Bsp2), osteocalcin (Oc), and Alp, was analyzed as previously described.14 Finally, flow cytometry analysis of CD45 cell marker expression was performed after 14 days of culture using an FITC-conjugated antibody against CD45 (BD Biosciences) or a control FITC-conjugated antibody (BD Biosciences). All these analyses were performed in three independent preparations.

Biomaterial, implant preparation, and SC implantation

Calibrated (80–200 μm) BCP particles supplied by Graftys SA (Aix-En-Provence) are composed of 60% hydroxyapatite [Ca₁₀(PO₄)₆(OH)₂] and 40% beta-tricalcium phosphate [Ca₃(PO₄)₂]. The particles were sterilized by heating to 180°C for 2 h. The final biomaterial consisted of plasma clotted around the BCP particles as previously described.12,13

The implants were prepared peroperatively in 1 mL syringes by mixing 50 mg of BCP particles with 100 μL of plasma containing 1.5 × 10⁵ BMSCs cells that were obtained after 14 days of culture. Plasma coagulation was triggered by adding 10 μL of a 2% CaCl₂·2H₂O solution. The syringes were rolled on a wheel during the coagulation process to obtain homogeneous repartition of the particles and cells into the clot. The implantation was performed on 10-week-old mice (JANVIER LABS) that were anesthetized as described earlier. The implants were positioned symmetrically on both sides of the vertebral axis in SC pockets beneath the dorsal skin and close to the root of the tale. The skin was sutured, and mice were placed under a heat lamp during the recovery period. Each animal received two implants.

In all the experiments but one, BMSCs were prepared from male C57BL/6 mice and implanted in male mice that were either immune-competent inbred C57BL/6 or immune-deficient outbred Nude (or NMRI-nu) corresponding to the Foxn1nu gene mutation on Naval Medical Research Institute (NMRI) background. Only in the experiments dedicated to analyze the fate of the implanted BMSCs (Fig. 7), male BMSCs from C57BL/6 mice were implanted in female recipients, which were either C57BL/6 or Nude mice. When indicated, BMSCs were prepared from wild-type NMRI mice and implanted in parallel in immune-competent NMRI and in immune-deficient Nude mice.

Histological analysis of the implants and evaluation of bone tissue formation

The implants were retrieved at two different timepoints corresponding to 2 and 4 weeks; the mice were anesthetized, the implants were retrieved, and the animals were sacrificed by cervical dislocation. The implants were sectioned in the middle following their longest axis; half part was fixed in 10% buffered formalin, and the other half was fixed in an alcoholic-based FineFix solution (Milestone) for 24 h. Implants were then partially decalcified in 10% (w/v) EDTA (ICN Biomedica) solution for 48 h at room temperature and embedded in paraffin. Decalcified serial sections of 4 μm were prepared for hematoxylin, erythrosyne, and saffron (HES) staining and sections of 5 μm were prepared for immunohistochemical experiments. Histomorphometry was performed on six independent implant mid-sections for each time and condition from HES pictures at 20× of whole implant surface using ICS Framework software (Trivbn). Measurements of mature bone corresponding to lamellar bone associated to bone marrow, immature woven bone, and non-colonized areas were expressed as a percentage of total implant mid-section surfaces and represented by box plots. Cell density was determined using the same software by counting the number of dark blue nuclei per mm² of tissue area after hematoxylin staining and represented by box plots. MNGCs and blood vessels were counted by two different observers on six independent implant HES-stained mid-sections for each time and condition, under
light microscopy (20×), expressed as number per mm² of tissue area, and represented by box plots.

**Analysis of circulating blood leucocytes**

After anesthesia, whole blood was withdrawn on a heparin anticoagulant from six C57BL/6 and six Nude mice aged 12 weeks (Janvier Labs) by intracardiac puncture. Blood cell populations were counted from a 200 μL blood sample by a routine hematological laboratory analyzer (XE-2100) using fluorescence analysis cytometry and hydrodynamic focusing technology. The remaining blood was treated with red blood cell lysis buffer and washed twice in MEM alpha supplemented by 5% FCS. Fluorescent monoclonal antibodies anti-B220 FITC, anti-CD8 PE-Cy7, and anti-CD4 PE-Cy5 (BD Biosciences) were selected to characterize circulating B and T lymphocyte populations, respectively, by a flow cytometry analyzer FACSCanto (BD Biosciences). Cell number was calculated based on lymphocyte number/mm² obtained from a routine hematological laboratory analyzer, and the results were expressed as median of cell count ± standard deviation (SD).

**Immunohistochemistry**

The anti-F4/80 and anti-CD3 polyclonal antibodies (Abcam) that were specific, respectively, for mouse macrophages and pan T lymphocytes were used for an immunohistochemistry experiment on six independent implant mid-sections for each time and condition. Briefly, 5-μm-thick formalin-fixed implant mid-sections were deparaffinized and hydrated in decreasing concentrations of alcohol. Antigen retrieval was performed in pH 6 sodium citrate buffer, and a neutralization of endogenous peroxide enzyme activity was obtained by incubation in 0.3% H₂O₂ for 10 min. After two washes in phosphate-buffered saline (PBS), the slides were incubated in 1.5% normal goat serum (blocking buffer) for 1 h and then with primary antibody at 1/100 dilution in blocking serum for 30 min. After two washes in PBS, incubation with biotinylated secondary antibody and immunoperoxidase staining procedure were performed using the rat Vectastain Elite ABC system (Vector Labs). Sections were washed in 2x saline sodium citrate (SSC) and then incubated in 0.2 M HCl for 12 min, washed, incubated in 1 M sodium thiocyanate (NaSCN) at 76°C for 20 min, washed again before being dehydrated, and dried. They were denatured at 76°C for 5 min in a solution containing 70% formamide in 2× SSC, then incubated in cold ethanol, and dried at 37°C. The probe was added to each section that was covered with a cover slip and sealed, and then incubated at 37°C overnight. The next day, the cover slips were removed and sections were washed at 55°C for 3 min in a solution containing 0.3% Nonidet P40 in 0.4× SSC and then incubated for 30 min at 37°C in a solution containing 0.1% Tween 20, 3% bovine serum in 4× SSC. An anti-digoxigenin rhodamine antibody (Roche Diagnostics) was added to the sections for 45 min at 37°C with a solution containing 0.1% Tween 20, 1% bovine serum albumin in 4× SSC. Finally, the sections were washed, mounted in the presence of DAPI for nuclei staining, and observed under a fluorescent microscope (Zeiss). Y-chromosome positive cells were counted on 20× pictures of whole implant mid-sections, and their density was presented using box plots.

**Isolation of RNA from BMSCs implants and RT-qPCR analysis**

Gene expression in BMSCs implants was explored after 14 days of implantation through a selection of 280 markers using RT-qPCR amplification. Total RNA from three independent BMSCs implants after 14 days was isolated directly after implant retrieval using an RNAplus kit following the manufacturer’s instructions (Bioprobe Systems). One microgram of each preparation of total RNA was used as a template for cDNA synthesis using random primers and superscript reverse transcriptase (Invitrogen) using the PTC 100 thermal cycler (MJ Research). Total cDNAs were diluted 1/10, and 5 μL of dilution was used for RT-qPCR assay (ABI PRISM 7900ht system; Applied Biosystems). The reactions were performed in 20 μL of final volume using the qPCR Mastermix Plus for SYBR Green I (Eurogentec). Amplification conditions were as follows: 50°C, 2 min; 95°C, 10 min (95°C, 15 s; 60°C, 1 min), cycled 40 times. The expression of selected genes was normalized to a mean of Actb, Gapdh, Hprt, and Ubiquitin gene expression, and quantified using the comparative 2⁻ΔΔCt method. Primers were designed and validated at the Plateforme d’Etude du Transcriptome (CHU de Nice; Cancéropole PACA).

**Fluorescent in situ hybridization of the Y chromosome**

To evaluate and compare the survival of implanted cells, BMSCs from C57BL/6 male mice were implanted in the back of C57BL/6 or Nude female mice as previously described. After 2, 4, and 8 weeks, the implants were retrieved, fixed in alcoholic-based solution Finefix, decalcified in EDTA 10% solution, dehydrated, and embedded in paraffin. Three independent implants for each time and condition were cut in 7 μm sections on poly-lysine slides. Living BMSCs were labeled using fluorescent in situ hybridization (FISH) of the Y chromosome as previously described. The probe was prepared by PCR using a degenerate primer (5'-3': CGC ACT CGA GNN NNN NTA CAC C and a DNA template derived from mouse Y chromosome (generous gift of Dr. Diane S Krause) under the following amplification conditions: 95°C, 10 min. (94°C, 45 s, 15°C, 45 s; 37°C, 12 min) for 2 cycles, (94°C, 40 s, 37°C, 45 s, 66°C, 4 min) for 5 cycles, and (94°C, 40 s, 54°C, 45 s, 66°C, 4 min) for 24 cycles, 66°C, 10 min. The probe was then labeled with digoxigenin (DIG-Nick Translation Mix; Roche Diagnostics) and prepared for FISH. Briefly, this was achieved by adding DNA from salmon sperm, mouse Cot-1 DNA, and sodium acetate followed by precipitation, incubation in formamide for 10 min at 37°C, addition of dextran sulfate at 20%, denaturation at 75°C for 7 min, and annealing at 37°C for 60 min. Each implant mid-section was deparaffinized, hydrated, and incubated for 30 min in Target Retrieval Solution (DakoCytomation) at 95°C. After 20 min at room temperature, the sections were washed in 2× saline sodium citrate (SSC) and then incubated in 0.2 M HCl for 12 min, washed, incubated in 1 M sodium thiocyanate (NaSCN) at 76°C for 20 min, washed again before being dehydrated, and dried. They were denatured at 76°C for 5 min in a solution containing 70% formamide in 2× SSC, then incubated in cold ethanol, and dried at 37°C. The probe was added to each section that was covered with a cover slip and sealed, and then incubated at 37°C overnight. The next day, the cover slips were removed and sections were washed at 55°C for 3 min in a solution containing 0.3% Nonidet P40 in 0.4× SSC and then incubated for 30 min at 37°C in a solution containing 0.1% Tween 20, 3% bovine serum in 4× SSC. An anti-digoxigenin rhodamine antibody (Roche Diagnostics) was added to the sections for 45 min at 37°C with a solution containing 0.1% Tween 20, 1% bovine serum albumin in 4× SSC. Finally, the sections were washed, mounted in the presence of DAPI for nuclei staining, and observed under a fluorescent microscope (Zeiss). Y-chromosome positive cells were counted on 20× pictures of whole implant mid-sections, and their density was presented using box plots.
Statistical analyses

Histomorphometrical values were expressed by box plots (maximum, upper quartile, median, lower quartile, and minimum) and were obtained from mid-sections of six independent implants for each time and condition (n = 6). The density of living BMSCs was evaluated on mid-sections from three independent implants for each time and condition (n = 3) and was expressed by box plots. Flow cytometry analysis data were expressed as median ± SD values and were obtained from blood samples of six independent mice from both strains (n = 6). The data were statistically analyzed using the Mann–Whitney–Wilcoxon test, and differences were considered statistically significant at *p < 0.05 and **p < 0.01. The RT-qPCR was performed in duplicate from 3 independent mRNA preparations for each condition (n = 3), and gene expression ratios were represented in a histogram considering a significant threshold of 3.

Results

Characterization of the BMSC preparation

As shown in Figure 1A, bone marrow cells cultured for 14 days resulted in the formation of colonies of fibroblast-like cells exhibiting high ALP activity. After 25 days, the spontaneous mineralization of the extracellular matrix was assessed by red alizarin staining as shown in Figure 1B. RT-qPCR experiments (Fig. 1D) showed that gene expression of all the osteoblastic markers tested markedly increased over the 25 day culture, namely Cbfa1, Coll1, Bmp2, Bsp2, Oc, and Alp. Flow cytometry analysis (Fig. 1C) of detached cells at day 14 showed that 42% were CD45 positive, indicating their hematopoietic origin. These adherent CD45^+ cells are considered mostly monocytes/macrophages cells because of their long survival, their morphology under light microscopy, and their capacity to differentiate into osteoclast-like cells when stimulated by a receptor activator of nuclear factor kappa-B ligand (RANK-L) (data not shown). Altogether, these experiments indicated that BMSCs used for implantation and that we obtained after 14 days in the absence of differentiating agents were composed of ~60% mesenchymal stromal cells that spontaneously differentiated into osteogenic cells producing a mineralized collagen matrix, and 40% CD45^+ hematopoietic cells exhibiting monocyte/macrophage characteristics.

Circulating white blood cell counts

To document and compare the immune status of Nude and C57BL/6 mice, circulating white blood cell (WBC) profiles were analyzed in both strains. Total WBC, granulocytes (GC), B220^+ lymphocytes (B cells), CD4^+ Th lymphocytes (Th cells), CD8^+ cytotoxic T lymphocytes (CTL), and monocytes (MO) were enumerated. As shown in Figure 2, WBC and B...
cells were higher in Nude mice but not significantly different from C57BL/6 mice. GC and MO were significantly higher in Nude mice. As expected, Th cells and CTL were normally present in C57BL/6 mice and were found at a very low level in Nude mice. These results confirmed the deficiency of the Nude mice regarding Th cells and CTL and revealed the significant increase of circulating GC and MO.

Influence of the recipient on bone tissue formation and maturation

BCP particles and plasma clot combined with BMSCs prepared from C57BL/6 mice were implanted subcutaneously either in syngenic immune-competent C57BL/6 mice or in Nude immune-compromised mice and analyzed after 2 and 4 weeks. After 2 weeks (Fig. 3A, B), histomorphometry revealed that colonization was 57% in C57BL/6 mice (Fig. 3A) and 100% in NMRI-nu mice (Fig. 3B). At this time, woven bone, characterized by low cohesive matrix, MNGCs, and capillaries, was observed in the inter space of

FIG. 2. Circulating white blood cells (WBC) in C57BL/6 and Naval Medical Research Institute (NMRI)-nu mice. Total WBC, granulocytes (GC), B220+ lymphocytes (B cells), CD4+ T lymphocytes (Th cells), CD8+ cytotoxic T lymphocytes (CTL), and monocytes (MO) were counted. The results are expressed as median ± standard value and (*) indicate statistically significant differences with *p<0.05 and **p<0.01.

FIG. 3. Histological analysis of subcutaneous implants retrieved from C57BL/6 (left) or in NMRI-nu mice (right). Representative hematoxylin, erythrosyne, and saffron (HES) cross-sections of 2 week implants showing that mean colonization was 57% in C57BL/6 (A) and 100% in NMRI-nu mice (B). Representative HES cross-sections of 4 week implants showing (C) C57BL/6 implants colonized by woven bone and (D) NMRI-nu implants with large areas of lamellar mature bone. (E) Higher magnification of the insert in (C) showing woven bone characterized by a low cohesive collagen matrix, multinucleated giant cells (MNGCs) (white arrows) in the particle (p) inter space; (F) Higher magnification of the insert in (D) showing lamellar bone characterized by an organized collagen matrix, osteocytes (white arrows), hematopoietic bone marrow areas (black arrow) with adipocytes (black star), erythroblasts, and megakaryocytic cells (white circle). Scale bars (A–D)=200 μm; (E, F)=100 μm. Color images available online at www.liebertpub.com/tea
the BCP particles except for one implant retrieved from Nude mice in which a small islet of mature bone was observed. After 4 weeks, all the implants were fully colonized (Fig. 3C, D). Mature bone was observed in all the implants retrieved from Nude mice but only in one implant retrieved from the C57BL/6 mice (Fig. 4). Mature bone was characterized by lamellar mineralized collagen matrix, many osteocytes, very few MNGCs, and active bone marrow areas assessed by the presence of adipocytes, megakaryocytic cells, and erythroblasts as shown in Figure 3F. In Nude mice, the amount of mature bone corresponded to a median percentage area of 26% of the implant mid-section (Fig. 4). These results indicated that mature bone and hematopoiesis formation after SC implantation occurred in immune-compromised mice but very rarely in immune-competent mice.

Owing to the differences of bone formation that we observed between the immune-competent C57BL/6 and the Nude recipients, we asked whether this discrepancy might be related to the different genetic background of these two different recipients, namely C57BL/6 and NMRI. To answer this question, in parallel to the previous experiments, BMSCs were prepared from wild-type NMRI mice, combined with plasma and BCP particles, and implanted either in wild-type NMRI or in immune-compromised NMRI nude mice. As shown in Table 1, histomorphometry analysis of bone formation in the implants after 4 weeks revealed that mature bone developed almost only in the implants of immune-deficient Nude mice, similar to what was observed with C57BL/6 mice. The amount of mature bone measured in Nude mice was similar, irrespective of the BMSCs origin with median values of 7.07% and 7.56% with BMSCs prepared from C57BL/6 and NMRI mice, respectively. This experiment, thus, demonstrated that the differences of bone formation between C57BL/6 and Nude recipients were not related to the genetic background of the Nude mice but rather to their immune status.

### Influence of the recipient on the characteristics of the newly formed bone in the implants

A comparison of bone tissue developed in the implants retrieved from C57BL/6 or Nude mice was further documented by analyzing the density of cell invasion, blood vessels, CD3⁺ lymphocytes, MNGCs, and macrophages. As shown in Figure 5A, whole cell number into the implants after 2 and 4 weeks was significantly lower in Nude mice compared with C57BL/6 mice. Conversely, the number of blood vessels was significantly higher in Nude mice and for both recipients, blood vessel density decreased with time (Fig. 5B). CD3 labeling (Fig. 5C) after 4 weeks showed the presence of T lymphocytes with a density of 10 cell/mm² in C57BL/6 mice and 1 cell/mm² in Nude mice (Fig. 5D). At all time points, the number of MNGCs enumerated from HES sections was lower in Nude mice compared with C57BL/6 mice. This number decreased with time in Nude mice, whereas it increased significantly in C57BL/6 mice (Fig. 6A). Moreover, the number of nuclei per MNGC was much lower in Nude mice than in C57BL/6 recipients (not shown). The number of macrophages estimated from F4/80 labeling (Fig. 6B–D) was not significantly different in the implants retrieved from C57BL/6 and Nude mice, and this number decreased with time in both mouse strains. Nevertheless, in C57BL/6, macrophages were grouped around the BCP particles and fused to form F4/80⁺ MNGCs (Fig. 6C); whereas individual F4/80⁺ cells were homogenously distributed in particle interspaces in Nude mice (Fig. 6D).

### Influence of the recipient on BMSC survival in the implants

BMSCs survival was assessed after implantation of male C57BL/6 BMSCs in female recipient mice, either syngenic C57BL/6 or Nude mice. The number of living male cells was evaluated after various time points in the implants using FISH with a specific probe targeting the Y chromosome (Fig. 7A–C) as was described in a previous study. As shown in Figure 7A, 2 weeks after implantation, ~10% of the male cells initially grafted (1150 ± 365 cells/mm²) were detected in the implants, with no significant
FIG. 5. Histomorphometric analysis of HES cross-sections of implants retrieved from C57BL/6 or NMRI-nu mice, after 2 and 4 weeks. (A) Total cell numbers and (B) blood vessel numbers expressed per mm²; (C) Representative picture of the immunohistochemistry of the CD3 cell surface marker on formalin-fixed implant sections retrieved from C57BL/6 mice after 4 weeks. The CD3⁺ cells appear in blue/green (black arrow). Scale bar: 50 μm; (D) quantification of CD3⁺ T lymphocytes per mm² of colonized area. Box plots indicate the sample maximum, upper quartile, median, lower quartile, and minimum. (*) indicates statistically significant differences. Color images available online at www.liebertpub.com/tea

FIG. 6. Histomorphometric analysis of HES cross-sections of implants retrieved from C57BL/6 or NMRI-nu mice after 2 and 4 weeks. (A) MNGCs with more than five nuclei were counted from HES stained sections and represented per mm²; (B–D) Immunohistochemistry of the macrophage surface marker F4/80 on formalin-fixed implant sections. (B) F4/80⁺ cells was counted using the histomorphometry software TRIBVN and expressed per mm². Box plots represent numbers/mm² of the total implant section and indicate the sample maximum, upper quartile, median, lower quartile, and sample minimum for seven implants (n = 7). Statistical analysis was carried out using the Wilcoxon test and *p < 0.05; Representative picture of F4/80⁺ cells stained blue/green on implant sections retrieved (C) from C57BL/6 mice showing macrophages stacked on biphasic calcium phosphate (BCP) particles and fused in multinucleated cells (black arrow), and (D) from NMRI-nu mice showing macrophages homogeneously distributed in the BCP particle inter space. Scale bars: 100 μm. Color images available online at www.liebertpub.com/tea
difference between recipients (respectively 86 ± 61 cells/mm² and 100 ± 73 cells/mm² for C57BL/6 and Nude mice). After 4 weeks, this number had decreased in both recipients but remained higher in Nude than in C57BL/6 mice, with, respectively, 43 ± 38 cells/mm² and 7 ± 28 cells/mm². After 8 weeks, no more implanted male cells were detected in C57BL/6 mice (1 ± 9 cells/mm²), while 14 ± 23 cells/mm² were counted in the implants retrieved from Nude mice. These data indicate that, although implanted cells rapidly and continuously disappear from the implants, a small number of living BMSCs were present for at least 4 more weeks in immune-deficient Nude mice compared with immune-competent C57BL/6 mice.

Influence of the recipient on cytokine, cytokine receptor, chemokine, chemokine receptor, and inflammation marker gene expression in BMSC implants

To compare the microenvironment of the implants retrieved from C57BL/6 and Nude mice, the expression of 280 genes was analyzed.

Table 2. Listing of the 280 Genes Analyzed in the BMSCs Implants Retrieved After 2 Weeks from C57BL/6 or NMRI-nu Mice

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<tr>
<th>Group 1: Genes over-expressed in C57BL/6 mice</th>
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<tr>
<td>Il13, H2-Eb1, Ccl17, Il2ra, Il2, Ccl22, Il5, Ccl1, Cld8b, Ccr8, Il4, Klrg1 (Mafa), Il9r, H2-Ab1, Cd209b, Il18r1, Il13ra2, Ccr7, Il3, Cita, Cd6, Il12b, Cd8A, Ltb, Ccl5, Ifng, Tcrb, Il9, Il2rb, Ccr5, Cdh1, Ccr4, Ltb4r1, Ccr3, Il11ra, Cxcl9, Tgbr1, Itgae, Ccl8, Ccr6, Il21r, Il22, Tbet, Tcra, Cd22.</td>
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<th>Group 2: Genes with similar expression in C57BL/6 and Nude mice</th>
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<tbody>
<tr>
<td>Ccl6, Ccr2, Il7, Tcrd, Ccl12, Il1d, Ccl11, Xcl1, Ccl21, Iggb6, Ccl19, B3gnt3, C5, Il1a, Mmp9, Ccl28, Tnfb, Il12rb2, Ccl7, Ccl9, Lgsq3 (Cd223), Mmp12, Lirs, Cxcl10, Mmp7, Mrc1, Itgb7, Csf2ra (Gmcsfr), Xcr1, Il15, Ccl2, Cxcr3, Ccl4, Itgb2, Tgfb1, Ccl3, Ltf, Selp, Ifna2, Ccl24, Ptgs1 (Cox1), Ccl20, Itgax, Klrb1c (Cd161), Il3ra, Il23, Gop9, Tnfrsf1a, Kitl, Cxcl16, Selplg, Il1r11, Hrh1, Il10ra, Cx3c11, Gm2a, Cxcr4, Itgax1, Il2rg, Itgam (Cd11b), Mmp16, Cds17, Itgal (Cd11a), Cxcr6, Jnfr1, Il10rb, Cxcr5, Il12a, Pecam1, Gata3, Jnfr2, Timp3, Itgax (Cd11c), Csf2 (Gmcsfr), Il6r, Cd63, Tgfb3, Madcam1, Csf1 (Mraf), Ccr10, Hprt, Cxcr1, Ifnb1, Iggb5, Cd4, Cs3fr (Gcsfr), Itgby4 (Cd104), Csf1r (Mraf), Cxcl25, Gapdh, Actb, Il12rb1, Lta4h, Cyba, Igsh, Gpr44 (Ctrh2), Itgax6, Vcam1, Tnfrsf18, Cxcl4, Itgax4 (Cd49), Lif, Icam2 (Cd102), Cd151, Cd19, Osm, Il6, Pecam1 (Cd31), Ubiquitin, Fcer2 (Cd23), Sph (Cd43), Itgax7, Timp2, Edn1, Il5ra, Il24, Lbr, Mmp11, Adam17, Cds18, Ccr11, Ly75, Vnn1, Cr2 (Cd21), It14, Cld19, Itga2b (Cds1), Igga3a, Il1b, Cd47, Ccr1, Hrh1, Lamp2, Iggb1 (Cd29), Tnfa, Cxcl5, Cd5, Cd69, Il11r, Ltbp2, Il12, Itgax5 (Cd49e), Mcam (Cd146), Itgax8, Il4ra, Cd65 (Cd144), Cxcl12, Mf, Ifnar1, Itgav, Icam1, Elane, Mrc2, Fn1, Tgfb2, Eng, Il17r, Il10, Ighe, Bsg (Cd147), Timp1, Tgfb2r, Slt3, Cs3f (Gcsf), Spp1, Hyal1, Cecamm-psl, Itgax3b, Mmp8, Mme, Syt1, Itgax2 (Cd49b), Cxcl11, Mmp10, Mmp23, Chst4, Ncam1 (Cd56), Sele, Il7ra, Il20rb, Il1e, Il11, Gcn1, Il21, Itgb3 (Cd61), Iggh1, Il18, Darc, Alcam (Cd16), Dpt, Iggh2, Mpo, Llcm1 (Cd171), Mmp15, Il1ra, Ccl27, Mmp24, Ptgs2 (Cox2), Cx3cr1, Il22r, Prg2, Il15ra, Mmp5, Cxcl13, Glycam1, Il17, Osmr.</td>
</tr>
</tbody>
</table>

Housekeeping genes: Actb, Gapdh, Hprt, Ubiquitin

<table>
<thead>
<tr>
<th>Group 3: Genes over-expressed in Nude mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmp14, Il6st, Tgbr3, Ltb1, Il13ra1, Ifnar2, Il20, Ltb3p, Cxcl14, Il1rap, Il16, Cxcl1, Il19, Il20ra, Cd44, Kit.</td>
</tr>
</tbody>
</table>

The genes were sorted into three groups. Group 1 corresponding to genes of which the expression ratio C57BL/6/NMRI-nu was superior to 3; Group 2 corresponding to genes of which the expression ratio C57BL/6/NMRI-nu was inferior to 3; and Group 3 corresponding to genes of which the expression ratio NMRI-nu/C57BL/6 was superior to 3. In each group, the genes are listed from the highest to the lowest ratio.
(listed in Table 2) was measured by RT-qPCR after 14 days and the ratios between the values obtained from C57BL/6 and those obtained from Nude recipients were calculated. Considering a significant threshold ratio of 3, the genes were sorted in three groups (Table 2). Group 1 corresponded to the 45 genes (16% of all the genes tested) with higher expression in C57BL/6 than in Nude mice. All of these genes were related to the immune response. Group 2 was the largest and included 219 genes (78% of the genes tested) similarly expressed in both recipients. Group 3 corresponded to 16 genes (5.7%) with higher expression in Nude mice than in C57BL/6 mice.

The 45 genes of the first group were further sorted in three functional clusters (Fig. 8A–C). The first one (Fig. 8A) corresponded to T-lymphocyte activation markers, including Tcra (×3.1) and Tcra (×4.7) genes encoding the T-cell receptor subunits and the Cdb (×5.5) gene encoding the costimulator glycoprotein CD6. T-cell activation was further assessed by the over-expression of Il2 (×22.7), Il2ra (×4.5), and Il2rb (×24.1), and Il2rb (×4.5) genes encoding the main T-cell activation cytokine and its receptor subunits, and by the over-expression of Ccl1 (×16.1) and Ccl5 (×5.3) genes encoding chemokines mostly secreted by activated T cells. The second cluster (Fig. 8B) corresponded to Th1 lymphocyte response markers such as Tbx21/Tbet (×3.1) gene encoding the T-box transcription factor responsible for Th1 cell differentiation. Th1-related cytokine genes such as Il12b (×5.4), Ifng (×5.3), or Cxc5 (×3.5) and Th1 lymphocyte surface markers such as Ccr5 (×4.6) or H18r (×6.4) were also over-expressed in the implants retrieved from C57BL/6. Our findings further revealed the presence of immune cells usually associated to the Th1 lymphocyte reaction, namely CTL, on the one hand as shown by the over-expression of Cd8a (×5.4), Cd8b (×10.4), and Klrg1 (×7.6) genes, and M1-activated macrophages or dendritic cells, on the other hand, as shown by the over-expression of class II MHC-related genes such as H2-ab1 (×6.9), H2-eb1 (×64.9), and Ciita (×5.6) encoding the class II MHC transactivator. The third cluster (Fig. 8C) was related to the Th2 lymphocyte response such as genes encoding for cytokines secreted by Th2 lymphocytes, namely Il3 (×5.6), Il4

**FIG. 8.** Relative gene expression in implants retrieved from C57BL/6 or NMRI-nu mice. From the panel of genes described in Table 1, only the genes with an expression ratio superior to 3 were sorted. (A–C) Genes of which expression were higher in C57BL/6 than in NMRI-nu mice. These genes were sorted as representative of (A) T-cell activation; (B) Th1 differentiation; and (C) Th2 differentiation. (D) Genes of which expression were higher in NMRI-nu than in C57BL/6 mice.
Discussion

This study was designed to investigate the influence of the immune system of the recipient animals on the ectopic bone formation induced by implants composed of cultured BMSCs combined to a biomaterial that consisted of BCP particles and plasma clot. Other authors have described that mature bone and hematopoiesis appear in immune-deficient mutant rodents after ectopic grafting of human or murine BMSCs associated to BCP particles.1,2,5,10,17,18 Nevertheless, a comparison of these results with syngenic models of grafting has been more rarely documented. We have compared a set of cellular and molecular events occurring during ectopic bone formation in immune-competent or in immune-deficient recipient animals. Using a model that was previously described,12 BMSCs were prepared from C57BL/6 mice, combined with BCP particles and plasma clot, and implanted subcutaneously either in C57BL/6 or in Nude mutant mice. We show here that SC implantation of BMSCs/biomaterial composite in Nude mice results in the formation of lamellar bone associated to hematopoietic areas, whereas implantation of the same composite in syngenic C57BL/6 mice results in woven bone without hematopoiesis. Our results also indicate that this difference of bone maturation is not related to the genetic background of the recipient animal, that is, C57BL/6 versus NMRI, but to their immune status, as similar differences of bone formation were obtained after SC implantation of wild-type NMRI BMSCs/BCP implants either in wild-type NMRI or in Nude mice.

Owing to the discrepancy between ectopic bone maturation in Nude and C57BL/6 mice, we aimed at further elucidating the mechanisms involved in this phenomenon by comparing a panel of cellular and molecular markers associated to bone formation in the implants. All these markers were, thus, analyzed in parallel in implants retrieved from C57BL/6 or Nude mice. This revealed that the implants retrieved from C57BL/6 evidenced the presence of CD3+ T cells, a higher number of F4/80+ macrophages, and a significantly higher number of MNGCs at all time points, along with a lower number of blood vessels. In parallel, expression analysis of a large set of genes revealed that almost all the genes over-expressed in C57BL/6 compared with Nude mice reflected the activation of the acquired immune system, namely T-cell activation and Th1/Th2 commitments.

The over-production of IFN-γ that we observed in the implants retrieved from C57BL/6 mice is consistent with that of Liu et al., who reported that the increase of IFN-γ production in the implants was correlated to the decrease of bone formation.10 These authors recently showed that mature bone and hematopoiesis develop in immune-deficient mice and decrease on infusion of CD8+ CTLs (partial decrease) or CD4+CD25+ Th cells (total decrease), suggesting that LT immune reaction is deleterious to the development of BMSCs-mediated mature bone and hematopoiesis, at least in an ectopic site.10 IFN-γ has been reported in vitro to inhibit the main transcription factor of osteoblastic differentiation Runx2 and could directly participate in the inhibition of bone maturation. IFN-γ secreted by Th1 lymphocytes may bind to IFN-γ receptors on macrophages, resulting in their M1 classical activation and the production of hydrolytic enzymes, nitric oxide, and toxic oxygen radicals. In parallel to Th1 commitment, the over-expression of genes encoding for the Ccl17 and Ccl22 chemokines and for the receptors Ccr3, Ccr4, Ccr7, and Ccr8 suggests a strong chemotactic activity toward Th2 lymphocytes classically described as responsible for the foreign body reaction triggered by biomaterial implantation.19,20 In C57BL/6 implants, the marked over-expression of Il13 (×423) and Il4 (×8.3) encoding for cytokines known to trigger and promote the fusion of biomaterial-adherent macrophages21,22 is consistent with the higher number of F4/80+ MNGCs that we found in the proximity of the BCP particles.8,23,24 One can hypothesize that the presence of these foreign body MNGCs, which can express both classically M1- and alternatively M2-activated macrophage phenotypes, can impair bone maturation in C57BL/6 implants through the secretion of cytokines, maintaining a chronic inflammatory microenvironment. This is consistent with previous results showing that CaP biomaterials by themselves initiate an inflammatory reaction both in vitro25–29 and in vivo.12,30,31 We have previously shown that the biomaterial used in the present study, consisting of BCP particles and plasma clot, induces a granular polymorphic reaction, including inflammatory cells, many capillaries, and fibroblastic proliferation, and that this reaction is similar in the absence or in the presence of the BMSCs.12 We show here that this reaction is downregulated in Nude mice and that this is likely beneficial to bone maturation.

In Nude recipients, the presence of lamellar bone and hematopoiesis was associated with the downregulation of many inflammatory signals such as a lower number of total cells, F4/80+ macrophages, and MNGCs, a very low expression level of inflammation markers, as well as a higher expression of the homeostatic chemokine Cxcl14 gene.32 This was associated to a larger number of capillaries, which...
is a key factor in the long-term bone regeneration process already described.\textsuperscript{35} The higher number of blood vessels that we observed in Nude mice after 2 weeks is consistent with the higher expression of the proangiogenic chemokine \textit{Cxcl1} gene and, conversely, to the higher expression of the angiostatic chemokine \textit{Cxcl9} in C57BL/6 mice. This was paralleled in Nude mice by a higher expression level of genes related to bone development and hematopoiesis such as \textit{Ltbp1}, \textit{Ltbp3}, \textit{Tgfbr3}, and \textit{Mmp14}.\textsuperscript{34-37} The marked over-expression of the \textit{Mmp14} gene that we identified here for the first time in an ectopic model of bone formation could play a central role in angiogenesis and bone maturation within Nude implants. The \textit{Mmp14} gene encodes the MT1-MMP membrane-bound matrix metalloproteinase, which is considered as playing an indispensable role in driving neo vessel formation,\textsuperscript{38} modeling of skeletal and extraskeletal connective tissues,\textsuperscript{34,35,39,40} and in postnatal hematopoiesis.\textsuperscript{37,41} Besides MT1-MMP, bone maturation could be also mediated by the over-expression observed for \textit{Il6st} gene whose expression participates in the maintenance of bone homeostasis and hematopoiesis.\textsuperscript{42-44} Finally, the higher number of circulating MO and GC that we observe here in Nude mice, consistent with a previous study,\textsuperscript{45} may also play a role in the faster colonization and vascularization of the implants, through metalloproteinase and growth factor secretion previously suggested.\textsuperscript{46}

A comparison of BMSCs survival between both recipients reveals that the survival rate of the implanted cells was higher in Nude than in C57BL/6 implants. These findings resulted from sex-mismatch implantations where male BMSCs combined with plasma and BCP particles were implanted on female mice and were followed by \textit{in situ} hybridization and qPCR of the Y chromosome. We have previously demonstrated that bone formation was similar when male BMSCs were implanted in female recipients compared with female cells implanted in male mice.\textsuperscript{12} From these experiments showing that both sex-mismatch implantation led to similar bone formation, we concluded that male cells bearing the H-Y antigen do not induce any significant host immune response in female mice in this model and that cell survival which we measured here for male cells implanted in female mice should be equivalent after implantation of male cells in male recipients. Altogether, these results support the idea that the experiments shown here in Figure 7 can be extrapolated to the other experiments where male BMSCs/plasma/BCP were implanted in male mice. The longer cell survival that we observed here in immune-deficient mice might result from the lower level of inflammatory reaction occurring in the Nude mice compared with C57BL/6 implants in which Th1/Th2 and CD8+ CTL cell activation likely accelerates BMSCs death as demonstrated \textit{in vitro} by Liu \textit{et al.}\textsuperscript{10} One can also hypothesize that the longer survival of BMSCs in Nude mice might participate in the downregulation of the inflammation signals and might be involved in bone maturation either through the production of bone tissue or through the secretion of growth factors attracting endothelial and stem cells from the neighborhood microenvironment.\textsuperscript{47,48}

All the current results obtained in an ectopic model of bone formation are consistent with the observation of Toben \textit{et al.} in the recombination activating gene 1 knock out (\textit{Rag1} \textsuperscript{-/-}) mice.\textsuperscript{49} In these mice, which completely lack mature T and B-lymphocytes, these authors have shown that bone fracture healing was accelerated. Moreover, they also demonstrated that over-expression of \textit{Il2} in bone callus was related to an impaired and delayed bone repair in a fracture model, suggesting detrimental functions of lymphocytes on fracture healing. In line with these findings, Schmidt-Bleek \textit{et al.} have found in a model of delayed healing of bone osteotomy that significantly higher T-cell percentage and longer pro-inflammatory processes were present in the bone hematoma and the adjacent bone marrow compared with the normal healing bone osteotomy.\textsuperscript{50}

As a whole, we show here \textit{in vivo} that a chronic inflammatory microenvironment, including both Th1 and Th2 reactions, which occurs in immune-competent mice after BMSC/BCP implantation, is correlated to the absence of mature bone formation and hematopoiesis. This T-lymphocyte response may shorten the BMSCs survival through CTL activation, impair osteoblastic differentiation through IFN-\(\gamma\) secretion, and promote macrophage attraction and fusion through CCL1, IL4, and IL13 secretion, resulting in the formation of MNGCs. Our data also suggest that the over-expression of the MT1-MMP protein in T-deficient Nude mice might play a central role in implant vascularity and lamellar bone formation.

In conclusion, our data bring further insights into the mechanisms involved in the detrimental effect of lymphocytes Th1 and Th2, CTL, MNGCs, and inflammation factors in BMSC-induced ectopic bone formation and emphasize the putative interest of controlling the host adaptive immune reaction against autologous cell/biomaterial constructs in clinical practice for bone tissue engineering.

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Disclosure Statement

No competing financial interest exists. None of the authors have any conflicts of interest.

References


T-CELL IMMUNE ACTIVATION IS DETRIMENTAL TO BONE FORMATION


Address correspondence to:
Nathalie Rochet, MD, PhD
UFR Médecine F-06107
Université Nice Sophia Antipolis
28 avenue de Valombrose
Nice 06100
France
E-mail: Nathalie.rochet@unice.fr

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