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RESEARCH ARTICLE

Molecular Adjuvants Based on Plasmids Encoding Protein Aggregation Domains Affect Bone Marrow Niche Homeostasis

Maria Giovanna Sabbieti[1], Giovanna Lacava[1], Andrea Amaroli[2], Luigi Marchetti[1], Roberta Censi[3], Piera Di Martino[3] and Dimitrios Agas[1,*]

[1]*School of Biosciences and Veterinary Medicine, University of Camerino, 62032 Camerino (MC), Italy;* [2]*Department of Surgical and Diagnostic Sciences, University of Genova, Genova, Italy;* [3]*School of Pharmacy, University of Camerino, Camerino, (MC), Italy*

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Abstract: ***Background*:** During last years, DNA vaccine immunogenicity has been optimized by the employment of co-stimulatory molecules and molecular adjuvants. It has been reported that plasmid (pATRex), encompassing the DNA sequence for the von Willebrand A (vWA/A) domain of the Anthrax Toxin Receptor-1 (ANTXR-1, alias TEM8, Tumor Endothelial Marker 8), acts as strong immune adjuvant by inducing formation of insoluble intracellular aggregates. Markedly, we faced with upsetting findings regarding the safety of pATRex as adjuvant since the aggregosome formation prompted to osteopenia in mice.

***Objective*:** The present study provides additional evidences about the proteinaceous adjuvants action within bone marrow and questioned regarding the self-aggregation protein adjuvants immunotoxicity on marrow niches.

***Methods & Results*:** Using histological, biochemical and proteomic assays we shed light on pATRex effects within bone marrow niche and specifically we evidenced an aplastic-like bone marrow with disrupted cytokine/chemokine production.

***Conclusion*:** The above findings provide compelling support to the thesis that adjuvants based on plasmids encoding protein aggregation domains disrupt the physiological features of the bone marrow elements.

Keywords: Bone marrow, Bone marrow niche, Osteopenia, DNA vaccines, Adjuvants, Protein aggregates.

1. INTRODUCTION

The central dogma behind DNA vaccines is to induce immune responses against recombinant antigens encoded by genetically engineered DNA plasmids expressed *in vivo*. After immunization, host cellular apparatus enables the expression of plasmid-encoded genes, and consequently the activation of both major histocompatibility complex class I and class II molecules [1]. DNA vaccines provide high tolerability and safety and can be rapidly produced, but are typically hampered by reduced immunogenicity. An approach that has been effective in increasing DNA vaccine immunogenicity is the use of "vaccine cocktails" containing the DNA vaccine as well as plasmids encoding immunomodulatory proteins. Molecular adjuvant plasmids expressing cytokines, chemokines, or co-stimulatory molecules may be co-administered with the antigenic DNA vaccine plasmid [2]. Previously, we have demonstrated that plasmids encoding aggregation-promoting domains act as genetic adjuvants. These plasmids induce formation of insoluble intracellular aggregates (aggregosomes) that trigger caspase activation and apoptotic cell death leading to innate immune system activation. Of importance, we reported that a plasmid vector (pATRex) encompassing the DNA sequence for the von Willebrand I/A domain (VWA) of tumor endothelial marker-8 (TEM8, alias Anthrax Toxin Receptor-1) when given in combination with DNA encoding Tumor Associated Antigens (TAA) enhanced immune protection against various tumors (*e.g.* breast cancer, melanoma) and infectious disease (*e.g.* malaria) [3, 4]. Moreover, we addressed the question of whether there is any considerable immunotoxicity associated with the use of self-aggregating proteins as genetic adjuvants. Remarkably, we uncovered a "hidden" reactogenicity of pATRex-derived aggregates, which reflected in osteopenia. Indeed, we focused on long bones examination due to their peculiar feature as read-out system for immunotoxicity [5-8]. Namely, it has been observed a cortical and trabecular bone loss with concomitant disruption of bone mineral

*Address correspondence to this author at the School of Biosciences and Veterinary Medicine, University of Camerino, 62032 Camerino (MC), Italy; Tel: +39-0737-40 2715; E-mail: dimitrios.agas@unicam.it

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content and a decrease of bone forming markers in pATRex treated mice. The osteo-disruptive scenario observed after pATRex administration was also accompanied with bone remodeling failure and fatty infiltration within bone marrow reservoir [9]. The above observations clearly depict the uncomfortable hypothesis to employ these proteinaceous "sticky" adjuvants into practical vaccination protocols because of their deleterious skeletal effects.

Of importance, the study of the proteotoxic aggregates that chronically activate the innate immune system in amyloid and aggregosome associated disorders remains complex and under concurrent investigation.

In this view, we extended our analyses on pATRex effects on bone marrow landscape, a nest for function, homing, migration and selective retainment of innate and adaptive progenitor and mature immune cells [8, 10]. The bone marrow dynamic operational microareas (referred as niches) are depicted by interdependence and interconnectedness and establish numerous physical, autocrine or paracrine type of interactions involving cytokines/chemokines, growth factors and other signaling molecules, which guaranty the steady state functions of this composite machinery [8]. The bone marrow multi niche concept (some authors have coined the niches as endosteal and vascular in relation of their topography into bones) is thus characterized by an elegant poise between the hematopoietic and stromal components. Some reports have already described the ability of intramuscular injected plasmid to reach the bone marrow and to be assimilated by resident immune-components such as neutrophils and monocytes [11, 12]. Hence, in this study, we assessed whether plasmid encoding ATRex administration in mice could perturb bone marrow homeostasis and modify niche functional features. These results could exert a peculiar significance considering also the direct link between aggregosome (amyloid peptides) formation and neurodegenerative diseases.

2. MATERIALS & METHODS

2.1. DNA Plasmids

For DNA immunizations, large scale preparation of the plasmids was routinely performed by alkaline lysis using Qiagen Plasmid Maxi kits (Qiagen). To assure endotoxin free products, DNA plasmids were purified using either Endo Free Plasmid Kit (Qiagen) or Gen Elute HPSelect Plasmid Giga Prep columns (Sigma # NA0800).

2.2. Animals Trials

All the experimental protocols implicated in this study were achieved from our previously work [9] and thus, for animals, treatments and tissue collection the reader must refer to above cited article. This manuscript represents a sequential part of the initial one.

Briefly, female and male FVB and Balb/c mice (Harlan Italy SrL, Correzzana Milano, Italy) were used. Mice were kept in laminar-flow cage in a standardized environmental condition. Mice (6-8 weeks old) were randomized in four groups (6 mice for each group). The groups were intramuscularly (i.m.) injected into the hind limb on time per week for 3 weeks as follows: one group of mice (n = 6) received 100 μg of ATRex DNA (experimental) in saline; the second group of mice (n = 6) received 100 μg of ANTXR-1 DNA (control, coding for the parental ANTXR-1, alias TEM8) in saline; the third group of mice (n = 6) received 100 μg pcDNA3.1 (scaffold plasmid) in saline; the fourth group of mice (n = 6) received only saline.

Mice from all groups were sacrificed at 90^{th} days after the first injection by CO_2 narcosis according to the recommendation of the Italian Ethical Committee and under the supervision of authorized investigators. To evaluate the systemic effects of the injection sites, plasmids were injected either into the left or right quadriceps, and counter-lateral bone collected.

2.3. Histological Bone Analysis and Immunohistochemical Staining

Femurs, dissected from adhering tissues, were fixed in 4% PFA and decalcified as previously described [13]. Samples were embedded with Tissue-Tek OCT compound. Then, 12 μm thick sections of femurs were obtained by a rotatory -30°C air-dried microtome cryostat (Ames Cryostat Miles). Sections were stained with toluidine blue or with hematoxylin and eosin (H&E) stains.

Other sections, after rinsing with PBS and incubation with 0,3% H_2O_2 were incubated with blocking buffer (0.3% Triton X-100 and 1% BSA in PBS) containing 10% normal serum for 30-60 min at RT in a humidified chamber. Then, sections were incubated 1 h a RT with the following primary antibodies diluted 1:80 in blocking buffer: rabbit anti-Osterix (Santa Cruz Biotechnology, DBA, Milano, Italy); rabbit anti-CXCL12 (Abcam; Prodotti Gianni, Milano, Italy); mouse anti-nestin (Abcam; Prodotti Gianni, Milano, Italy). After washing in PBS, sections were incubated for 30 min at RT with a biotinylated goat anti-mouse IgG (Vector Laboratories, DBA Milano, Italy) or with a biotinylated goat anti-rabbit IgG (Bethyl Laboratories, Aurogene s.r.l., Roma, Italy) both diluted 1:200 in blocking buffer. Control experiments were performed by omitting the primary antibody. Slides were imaged using a Leica DM 2500 microscope.

2.4. Quantitative Analysis of Immunohistochemistry

High-resolution, 36-megapixel, digital micrographs were taken of defined bone marrow using a Leica DM 2500 optical microscope. Freely available software ImageJ software [version 1.34j, National Institutes of Health (NIH)] quantified pixel intensity.

2.5. Total Bone Marrow Cell (BMCs) Derivation and Cultures

Long bones (femurs, tibiae and humeri) from the mice groups were dissected and the marrow cavity was flushed as previously described [13]. Total Bone Marrow Cells (BMCs) were cultured for 3 days in DMEM containing 10% Heat-Inactivated-Fetal Calf Serum (HIFCS), penicillin and streptomycin (Invitrogen, Milano, Italy). Then, the culture medium was collected to study the BMCs release of cytokines and chemokines while cells were used to perform western blotting analysis.

2.6. Western Blotting

Proteins from BMCs were extracted in cell lysis buffer (Cell Signaling, EuroClone, Milano, Italy) after 3 days of culture, and the concentration was determined by the BCA protein assay reagent (Pierce, EuroClone). Western blotting was performed as previously described [14]. Membranes were immunoblotted in blocking buffer with rabbit anti-CXCL12 or with rabbit anti-CXCR4 (Abcam, Prodotti Gianni, Milano, Italy) both diluted 1:600. After washing with PBS-T, blots were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG IgG (Cell Signaling, EuroClone) diluted 1:100,000.

Immunoreactive bands were visualized using LiteAblot Turbo luminol reagents (EuroClone) and Hyperfilm- ECL film (EuroClone) according to the manufacturer's instructions. To normalize the bands, filters were stripped and re-probed with a monoclonal anti-α-tubulin (Sigma-Aldrich). Band density was quantified densitometrically.

2.7. Cytokines and Chemokines Assay

The cytokine/chemokine profiles in supernatants of 3-day cultured BMCs population were assessed by using Mouse Cytokine Array Panel A kit (R&D Systems, Milano, Italy) accordingly to the manufacturer's instructions. Briefly, after 3 days in culture BMCs supernatant (600 µl) from each experimental group was incubated overnight on nitrocellulose membranes spotted with specific antibodies. Chemiluminescence detection produced signals (blots) directly proportional to the amount of cytokine bound. Immunoreactive dots were visualized using LiteAblot Turbo luminol reagents (EuroClone, Milano, Italy) and Hyperfilm-ECL film (EuroClone, Milano, Italy) and quantitated densitometrically using Image J software.

2.8. Statistical Analysis

GraphPad Prism 5.0 for Macintosh was used for drawing graphs and for statistical analysis (GraphPad Software, San Diego, CA, USA). All experiments were repeated at least three times. t- student test was used to test for significant differences (*$p < 0.05$) between two groups.

3. RESULTS & DISCUSSION

Specialized cellular compartments within the bone cavities establish dynamic operational microareas designated as niches. The niche milieu encompasses a panorama of stem and progenitor elements as well as mature cells characterized by a complex symbiotic type of relationship [8, 15].

In this study, we showed the bone marrow niche modification of mice administered with plasmids encoding protein aggregation domains. Bearing in mind that these genetic adjuvants form intracellular protein aggregates and thus enhance the immunogenic host response, we faced with questionable results regarding the safety of the "aggregosome adjuvant".

Likewise, with biochemical, western blotting and histological methods, we shed light on pATRex effects on bone marrow reservoir due to the peculiarity of this multi-niche environment as the principal hematopoietic and osteogenic source [8].

As a first approach, histological sections of mice femurs were stained with toluidine blue and hematoxilin/eosin staining for the evaluation of the bone marrow structural details and bone marrow cellularity. Of note, pATRex-treated femurs evidenced an ectopic invasion of adipocytes within bone marrow, mainly located in the sub-metaphyseal region and gradually decreased towards the femur diaphyseal area (Fig. **1A**, **B**). Previous data demonstrated that adipocyte infiltration within bone marrow compartment could modify the clonal bias of certain HSC subpopulation, could disrupt the MSC and HSC commitment due to impaired cell-cell interaction and could alter production of key role niche elements such as cytokines and chemokines [16]. In this view, biochemical studies were performed to evaluate the cytokines/chemokines release in the culture medium of BMCs retrieved from pATRex-treated and ANTXR1-administrated group of mice. Specifically, an increase of the interleukin (IL)-1a, IL-1b, IL-6, IL-7, IL-17, IL-27, was observed in BMCs from pATRex-treated group (Fig. **2A**). Indeed, such cytokines are well known participants on osteoclast differentiation and function, myeloid differentiation, HSC proliferation and in full-blown inflammatory plateau [8, 17, 18]. On the contrary, cytokines devised as anti-inflammatory such as IL-1ra, IL-5 and IL-10 [8, 17, 19] were found slightly reduced in BMCs from pATRex-injected mice (Fig. **2B**). Also, given that cytokines exert a watchdog role in physiological conditions within bone marrow and control stromal components and HSCs homing and egression, we showed that pATRex-induced protein aggregates, provoke perturbations in the fine-tuned intramural cytokine trafficking. Thus, conventional wisdom would suggest that pATRex administration scattered pro-inflammatory signaling cascades, disrupting the HSC homeostatic niche framework.

Besides, niche ontogeny is characterized by interdependency of the various niche inhabitants, and explicitly (i) HSC functional attitudes are MSC/osteoblast-dependent [20], (ii) HSC and MSC pool play reciprocal regulation [8, 21, 22]. In this circumstantial, we questioned whereas aggregosomes could direct influence pre-osteoblast/MSCs population. Particularly, $Nestin^+$ cells have been identified as MSC subpopulation within bone marrow responsible for HSC regulation [23]. Moreover, $Osterix^+$ (Osx^+) osteolineage cells regulate the maturation of early B lymphoid precursors creating microniches for B cell development [24]. In addition, Osteopontin (OPN), matrix glycoprotein expressed by the osteoblasts, supports the migration and the adhesion of HSC toward to the osteoblastic niche and negatively regulates HSC proliferation, contributing to the maintenance of a quiescent state [25, 26]. In line, using immunohistochemical techniques we observed that MSC and osteoblast pool was reduced. Particularly, the decrease of Nestin+ and Osx+ pool, found in pATRex group compared with the control group, predicted variations of the endosteal niche cellular inhabitants (Fig. **3A**, **B**). In addition, the reduction of OPN in pATRex-treated mice (Fig. **3C**) could be ascribed to the direct consequence of the reduction of the bone progenitor/mature forming components.

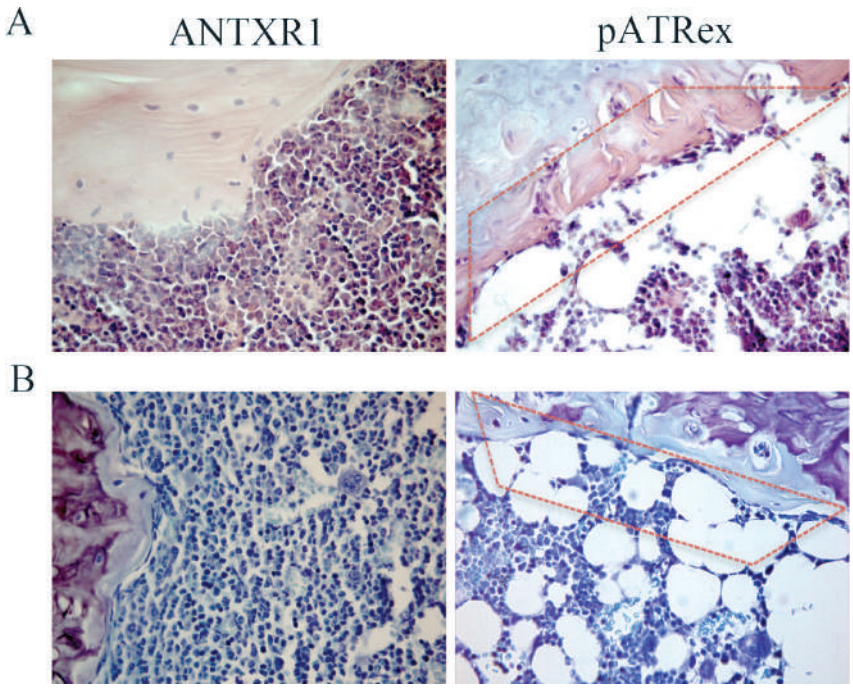

Fig. (1). Representative sections H&E (**A**) and toluidine bleu (**B**) stained of femour sub-metaphyseal area after ANTXR1 or pATRex administration. Note the fat cells infiltration near the endosteal niche (red dashed line). Magnification 40x. N= 6 per group. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

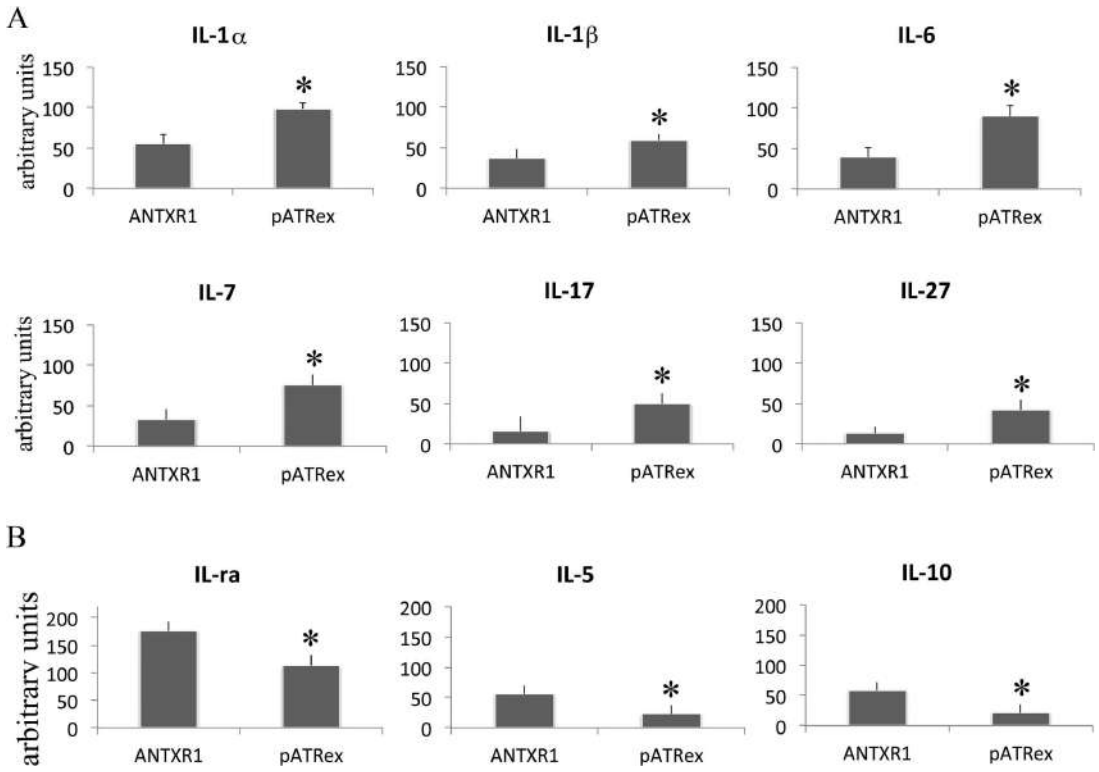

Fig. (2). Pro-inflammatory (**A**) and anti-inflammatory (**B**) cytokines and chemokines production from BMC cultures obtained by ANTXR1 and pATRex treated mice was analyzed using Mouse Cytokine Array Panel A kit (R&D Systems, Milano, Italy) accordingly to the manufacturer's instructions as specified in the M&M section. Immunoreactive spots were quantitated densitometrically using Image J software. ($p<0.05$) N= 6 per group.

The concurrent decrease in osteolineage cells with parallel increase of fat cells in pATRex-treated mice group might predict a deregulation of MSC fate toward adipogenesis than osteogenesis. Nonetheless, previous our results sustained the above posture. Namely, in BMSCs of pATRex treated mice, we found a decrease of Runx2 and Osx, both widely recognized as the main transcriptional factors of osteoblast differentiation [9].

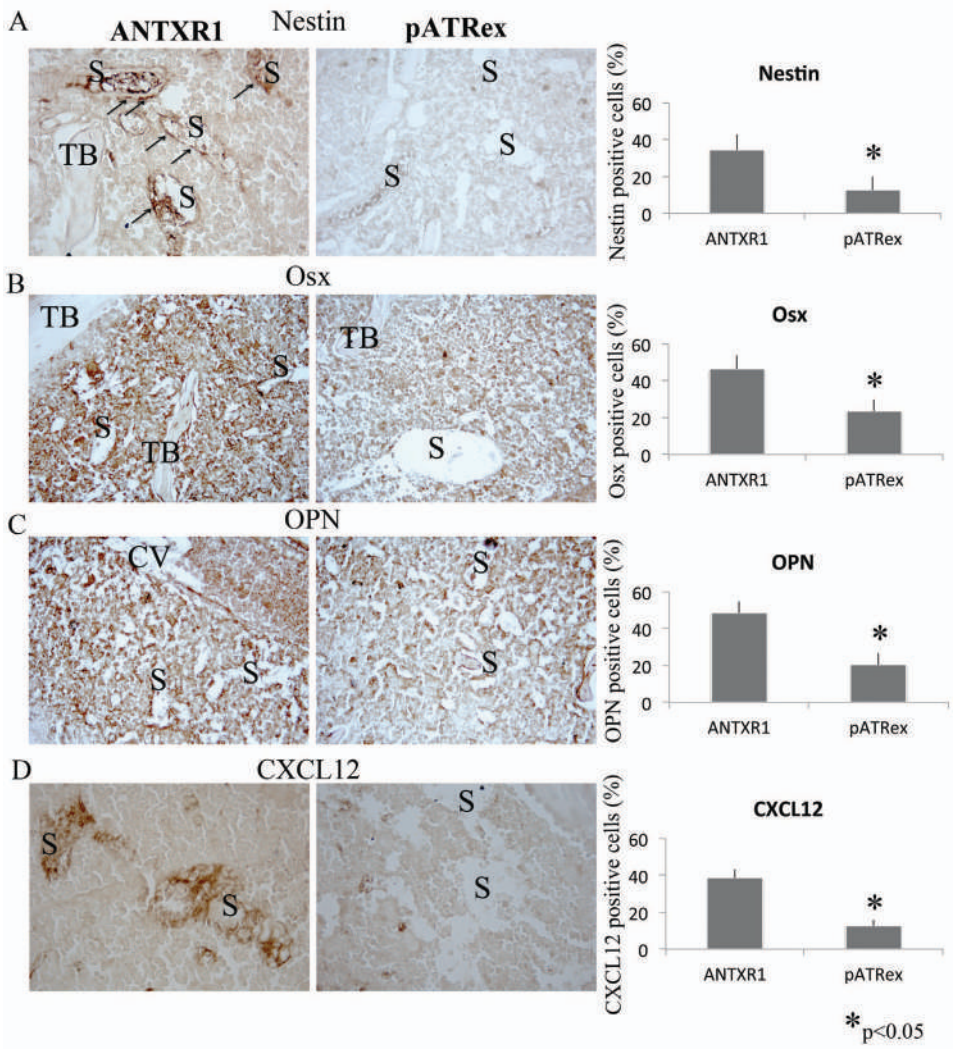

Fig. (3). Immunohistochemistry and quantification of Nestin (**A**), Osx (**B**), OPN (**C**) and Cxcl12 (**D**) expression in pATRex-treated and ANTXR1-treated mice. Note the disrupted distribution of Nestin (**A**) and Cxcl12 (**D**) immunostaining in pATRex-treated cells; on the contrary ANTXR1-treated samples exhibit a localization pattern, primarily around the perivascular mesenchymal and endothelial cells lining the sinusoid throughout the bone marrow (arrows). TB: trabecular bone; S: sinusoid. Magnification 40x. N= 6 per group. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

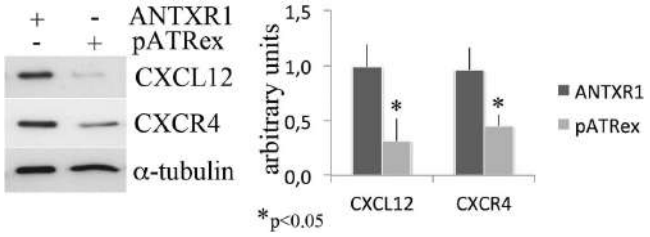

Fig. (4). Representative Western blotting of CXCL12 and its receptor CXCR4 of total bone marrow population from femours of ANTXR1 and pATRex-treated mice. ($p<0.05$). N= 6 per group.

Osteoblasts and MSCs express the soluble stromal-cell-derived factor 1 (also termed as CXCL12), that binds to its specific receptor C-X-C chemokine receptor type 4 (CXCR4) and prevents HSC mobilization supporting HSC maintenance [27, 28]. Interestingly, our findings revealed a reduced expression and a diminished perivascular distribution of CXCL12 in pATRex-treated mice in respect with control group (Fig. **3D**). As further evidence, western blotting analysis showed a reduce expression of CXCL12 and its receptor CXCR4 (Fig. **4**). Since CXCL12/CXCR4 signaling regulates osteoclast recruitment, bone resorption, hematopoietic chemotaxis, fusion and cell survival [29, 30], we speculated that peculiar vascular niche metabolic feature, attributed to CXCL12/CXCR4 signaling, has been modified in the bone marrow of pATRex-treated mice. This instance is toughly buttressed by the observation of bone marrow hypocellularity in pATRex treated mice. Surprisingly we observed morphological modifications, which reflects in architectural niche changes. The vascular sinuses (Fig. **5A**, **C**, **E**) were interspersed among adipocytes (Fig. **5A**, **C**) and the endosteal niche cellularity were decreased with disrupted bone lining cell micro-architecture (Fig. **5B**, **D**, **F**). The fact that there was no apparent change in appearance or distribution of the remaining hematopoietic tissue, predicting a decreased number of all hematopoietic cell lines in the bone

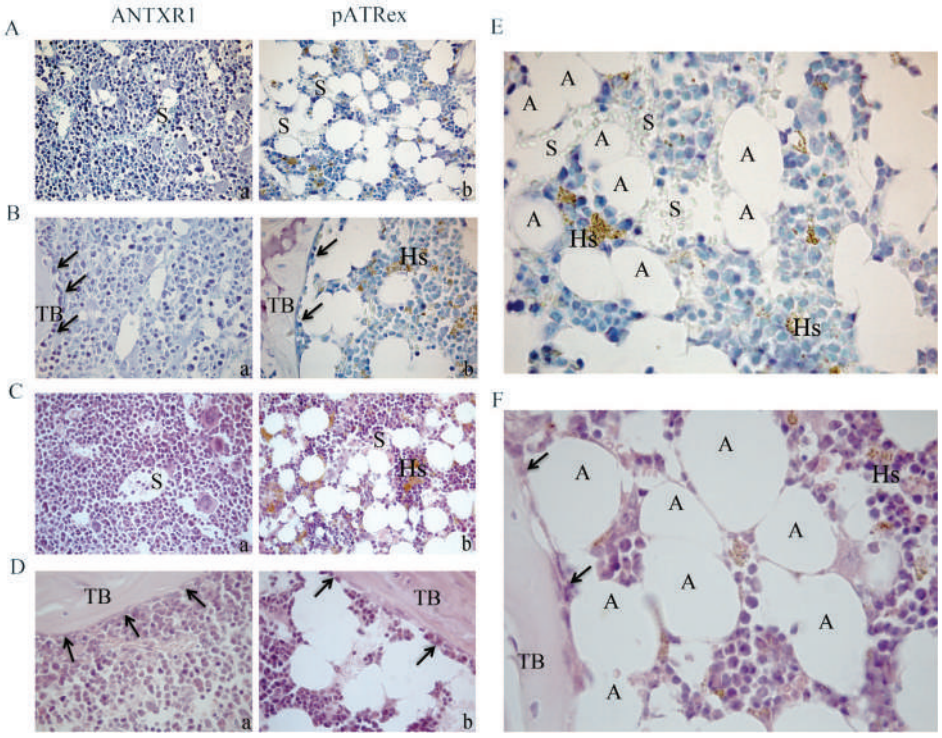

Fig. (5). Histological sections illustrate the bone marrow cellularity after ANTXR1 treatment with HSCs residing in close contact with vascular and perivascular cells but also megakaryocytes and other stromal cells (Aa, Ca); note also the distribution of the endosteal lining bone cells and the HSCs in close proximity (arrows) (Ba, Da). pATRex-treated group revealed a decrease in bone marrow hematopoietic cellular density at the perivascular (Ab, Cb) or endosteal (Bb, Db) compartments, probably due to adipocytes infiltration. Notably, after pATRex administration the hematopoietic and stromal cells were found scattered among the fat cells and the sinus micro-architecture toughly disturbed by the adipocyte pool (E, F). The distinct, brown-colored, closely packed granules observed referred as hemosiderin granules particularly evident in pATRex-treated group. Hs: hemosiderin; S: sinusoid; A: adipocyte. Magnification A, C 40x; B, D, 60x. Reconstruction E, F. N= 6 per group. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

marrow. In this scenario, H&E sections revealed an aplastic marrow, which appears devoid of hematopoietic cells, consisting primarily of vascular sinuses and adipose tissue. The aplastic-like marrow was primarily observed at the submetaphyseal level with a progressive reduction moving towards the diaphysis of the mice femour. Considering that approximately 70 to 80% of the marrow being hematopoietic tissue in mice [31] and that changes in bone marrow cellularity can be an indicator of systemic toxicity affecting multiple cell lineages [32], here we underscored the unseen reactogenicity of the aggregosome-induced adjuvants transduced in a pancytopenia-like condition.

CONCLUSION

In this report, we extended our knowledge concerning the safety of genetic adjuvants, which promote intracellular protein aggregates and sequentially induce frustrated autophagy and enhanced vaccine immunogenicity. Fully consistent with our previous observation in bone tissue, here we uncovered that protein aggregate formation, also seen in aggregosome disorders within a neurodegenerative proteinopathy scenario, disrupt bone marrow homeostatic features and drives to enfeebled physical, autocrine and/or paracrine progenitor/mature cell communication.

Considering that preceding studies evidenced that pATRex DNA administration did not trigger histopathological changes in a wide array of soft tissues [3], we undoubtedly sustain that bones [9] and bone marrow remain the preferential target tissues of these injectable adjuvants in mice. This hypothesis is also consistent with other findings underlining that amyloid β peptides from Alzheimer patients have been found stored in osteoporotic bone tissue with concomitant increase of osteoclastic activity [33].

Collectively, our data point on the fact that, albeit the options for gene-based immunomodulatory proteins are consistently developed, the real challenge resides in finding the optimal choice for each adjuvant that will lead to protective outcomes in clinical trials.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval is according to the recommendation of the Italian Ethical Committee and under the supervision of authorized investigators.

HUMAN AND ANIMAL RIGHTS

No human were used in this study. All the bone tissues implicated in this study were achieved from our previous work (CGT, Agas *et al*. 2016). The Institutional and national guidelines for the care and use of Laboratory animals were followed.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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