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REVIEW

Effect of aging on stem cells

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Abstract

Pluripotent stem cells have the remarkable self-renewal ability and are capable of differentiating into multiple diverse cells. There is increasing evidence that the aging process can have adverse effects on stem cells. As stem cells age, their renewal ability deteriorates and their ability to differentiate into the various cell types is altered. Accordingly, it is suggested aging-induced deterioration of stem cell functions may play a key role in the pathophysiology of the various aging-associated disorders. Understanding the role of the aging process in deterioration of stem cell function is crucial, not only in understanding the pathophysiology of aging-associated disorders, but also in future development of novel effective stem cell-based therapies to treat aging-associated diseases. This review article first focuses on the basis of the various aging disease-related stem cell dysfunction. It then addresses the several concepts on the potential mechanism that causes aging-related stem cell dysfunction. It also briefly discusses the current potential therapies under development for aging-associated stem cell defects.

Key words: Aging; Biological aging; Cellular aging; Adult stem cells; Premature aging; Mesenchymal stem cell; Stem cell renewal; Tissue regeneration

Core tip: Stem cells have the remarkable self-renewal capability and the amazing ability to differentiate into all cell types. It is generally believe that stem cells are the main source that provides cells to repair and re-generate damaged tissues and organs. However, there is now compelling evidence that the aging process has a deleterious effect on stem cells, and that the aging effects on stem cells may have play essential roles in the pathophysiology of the various aging-associated diseases. This review discusses briefly the relationship of aging-

associated stem cell dysfunction and the various aging-associated ailments, and several proposed concepts on the molecular mechanism of aging-related stem cell dysfunction.

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INTRODUCTION

Aging is an unavoidable physiological consequence of the living animals. Mammalian aging is mediated by the complex cellular and organismal processes, driven by diverse acquired and genetic factors[1]. Aging is among the greatest known risk factors for most human diseases[2-5], and of roughly 150000 people who die each day across the globe, about two thirds die from age-related causes[6].

In modern era, one of the emerging fields in treating human diseases is the "stem cells" research, as stem cells have the remarkable potential for use to treat a wide range of diseases. Accordingly, stem cells research has become a focal point of biomedical research since 1998, when Dr. James Alexander Thomson made the scientific breakthrough of successful generation of several embryonic stem cell lines from human blastocysts[7,8]. Stem cells are undifferentiated pluripotent cells that can give rise to all tissue types and serve as a sort of internal repair system[9]. Until the recent advance in development of induced pluripotent stem cells (iPSCs), scientists primarily worked with two kinds of pluripotent stem cells from animals and humans: Embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and non-embryonic "somatic" or "adult" stem cells, which are found in various tissues[10]. Because of potential ethical issues, "adult" stem cells have become the primary target.

Although stem cell science promises to offer revolutionary new ways of treating diseases, it is identified that aging affect the ability of stem (and progenitor) cells to function properly, which ultimately can lead to cell death (apoptosis), senescence (loss of a cell's power of division and growth), or loss of regenerative potential[11,12]. Aging may also shift gene functions, as reported for some genes such as, p53 and mammalian target of rapamycin (mTOR), which are beneficial in early life, but becomes detrimental later in life[13-15]. In this regard, a novel theory, namely "stem cell theory of aging", has been formulated, and it assumes that inability of various types of pluripotent stem cells to continue to replenish the tissues of an organism with sufficient numbers of appropriate functional differentiated cell types capable of maintaining that tissue's (or organ's) original function is in large part responsible for the aging process[1]. In addition, aging also compromises the therapeutic potentials of stem cells, including cells isolated from aged individuals or cells that had been cultured for many passages *in vitro*. Nevertheless, in either case, understanding the molecular mechanism involved in aging and deterioration of stem cell function is crucial in developing effective new therapies for aging- as well as stem cell malfunction-related diseases. In fact, given the importance of the aging-associated diseases, scientists have developed a keen interest in understanding the aging process as well as attempting to define the role of dysfunctional stem cells in the aging process.

In this review, we will first focus on the various aging disease-related stem cell dysfunction and then address the several concepts on potential mechanisms that cause aging-related stem cell dysfunction. We will also discuss current strategies for reversing age-related stem cell dysfunction. Finally, we will discuss up-to-date therapies for aging-associated stem cell defects, available-drugs, growth factors, *etc*.

DISEASES OF AGING FROM OLD STEM CELLS

Adult stem cells, also known as somatic stem cells, are found throughout the body in every tissues and organs after development and function as self-renewing cell pools to replenish dying cells and regenerate damaged tissues throughout life[16]. However, adult stem cells appear to age with the person. As stem cells age, their functional ability also deteriorates[12,17]. Specifically, this regenerative power appears to decline with age, as injuries in older individuals heal more slowly than in childhood. For example, healing of a fractured bone takes much longer time in elderly than in young individuals[18-21]. There is a substantial amount of evidence showing that deterioration of adult stem cells in the adult phase can become an important player in the initiation of several diseases in aging[22,23]. The following is some of the examples of aging-associated effects on stem cells.

Neural stem cells (NSCs) are multipotent and self-renewing cells and located primarily in the neural tissues. In response to a complex combination of signaling pathways, NSCs differentiate into various specific cell types locally in the central nervous system (CNS), like neurons, astrocytes, and oligodendrocytes[24]. NSCs in humans maintain brain homeostasis and it continuously replenishes new neurons, which are important for cognitive functions[25,26]. However, there is now strong evidence for the aging-associated cognitive deficits, such as olfactory dysfunction, spatial memory deficits, and neurodegenerative disorders, which are caused by deterioration of NSC proliferation and differentiation and enhanced NSC senescence as a consequence of aging[27,28].

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into cells of mesenchyme tissues, including osteoblasts (bone cells)[29], chondrocytes (cartilage

cells)[30], myocytes (muscle cells)[31] and adipocytes (fat cells)[32]. MSCs were first isolated from the bone marrow of guinea pigs in 1970's and after that it was isolated from almost every organ in mice including fat, liver, spleen, pancreas, kidney, lung, muscle, and brain[32]. Human MSCs have also been isolated from umbilical cord tissue and cord blood, placenta, bone and joints[33]. However, the major sources of MSCs are the bone marrow-derived MSCs (BM-MSCs) and the adipose tissue-derived MSCs (A-MSCs); and they are currently the most studied MSCs[32,34]. Aging also affects MSCs in humans and in animal models as indicated by the decrease in the bone marrow MSC pool and also shifts their lineage differentiation from one that usually favors osteoblastic differentiation to one that prefers adipogenic differentiation[35], which is largely responsible for the gradual and aging-associated shift of hematopoietic (red) marrows to fatty (yellow) marrows, and which also contributes significantly to the etiology of senile osteoporosis. It is also evident that with increasing donor age, MSCs from both bone marrow and adipose tissues have been shown to have reduced capacity to handle oxidative stress[36-38]. During the aging process, oxidative stress leads to hyperactivity of pro-growth pathways, such as insulin/IGF-1 and mTOR pathways, and the subsequent accumulation of toxic aggregates and cellular debris ultimately lead to apoptosis, necrosis, or autophagy[39]. In addition, in some non-skeletal tissues, particularly the hematopoietic system, MSCs is a key niche component for hematopoietic cells. Aging of MSCs has been shown to be detrimental with respect to this important function[35].

Adult skeletal muscle stem cells (satellite cells) have a remarkable capacity to regenerate[40,41]. Similarly, their regeneration capacity declines with aging, although it is not clear whether this is due to extrinsic changes in the environment and/or to cell-intrinsic mechanisms associated to aging. This impaired regenerative capacity of skeletal muscle during aging is due to accumulation of the altered progeny, which leads to progressive deterioration of tissue structure and function, manifesting after injury or in response to the depletion of memory B cells and naive T cells in the hematopoietic system in the elderly[41-44].

Hematopoietic stem cells (HSCs) are the blood-forming stem cells through the process of hematopoiesis[45]. They are located in the red bone marrow within marrow cavity of most bones. HSCs also produce immune cells of the body. Since blood cells are responsible for constant maintenance and immune protection of every cell type of the body, the constant production of billions of new blood cells each day by HSCs is very important for mammal life. HSC-derived monocytes can give rise to osteoclasts, macrophage and granulocyte. Osteoclasts are giant cells with numerous nuclei that work in synergy with osteoblasts through complicated bone coupling mechanisms to maintain bone homeostasis[35,46]. All these activities of HSCs are carefully modulated by a complex interplay between cell-intrinsic mechanisms and cell-extrinsic factors produced by the microenvironment; and aging altered this fine-tuned regulatory network, leading to aberrant HSC cell cycle regulation, degraded HSC function, and hematological malignancy[47].

MECHANISM FOR FUNCTIONAL DETERIORATION OF STEM CELL IN AGING

There are several potential mechanisms that are believed to contribute to the aging-associated stem cell dysfunction; and they probably are in part responsible for many aging-associated diseases. Figure 1 proposes some of the contributing factors/mechanisms that could be responsible for the aging-induced deterioration of stem cell functions and aging-associated diseases. This section summarizes some of these contributing factors/mechanisms and their potential roles in the aging effect on stem cells.

Microenvironment

Aging is characterized by common environmental conditions, such as hormonal, immunologic, and metabolic disorders[48-50] and these are considered as the critical microenvironmental factors affecting stem cell functions. Changes in these microenvironmental factors in response to aging are believed to be responsible for the changes in stem cell function with aging[51]. It has been shown that potentially underlying aging-related tissue degeneration, such as osteoporosis, could be due to impaired MSCs by surrounding micro-environmental pathologic factors[52,53]. It has also been shown that in mammals, metabolic alterations of hyperglycemia and hyperinsulinemia are important pathologic factors in aging and in MSC dysfunction[54]. However, the molecular mechanism in mediating stem cells dysfunction by microenvironmental signals is not yet fully understood.

Cells produce soluble (endocrine or paracrine) factors necessary for information exchange among cells of distant tissues and/or within the same organ[51]. Aging cells can influence an organ or tissue by secreting soluble endocrine or paracrine factors. Accordingly, aging of the endocrine glands has known to result in hormonal disturbances[50,55], which ultimately affects normal function and or differentiation of the stem cells. In humans, sex hormones, especially estrogen, are the most prominent endocrine factors that change with aging, and sex hormones discordance often leads to several significant diseases. Estrogen insufficiency also induces the biased differentiation of MSCs to adipocytes over osteoblasts[50,56,57]. Aging-related elevation in circulating levels of proinflammatory cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), can also cause differentiation disorders of MSCs[58,59].

DNA damage and telomere shortening

In mammals, spontaneous and extrinsic mutational

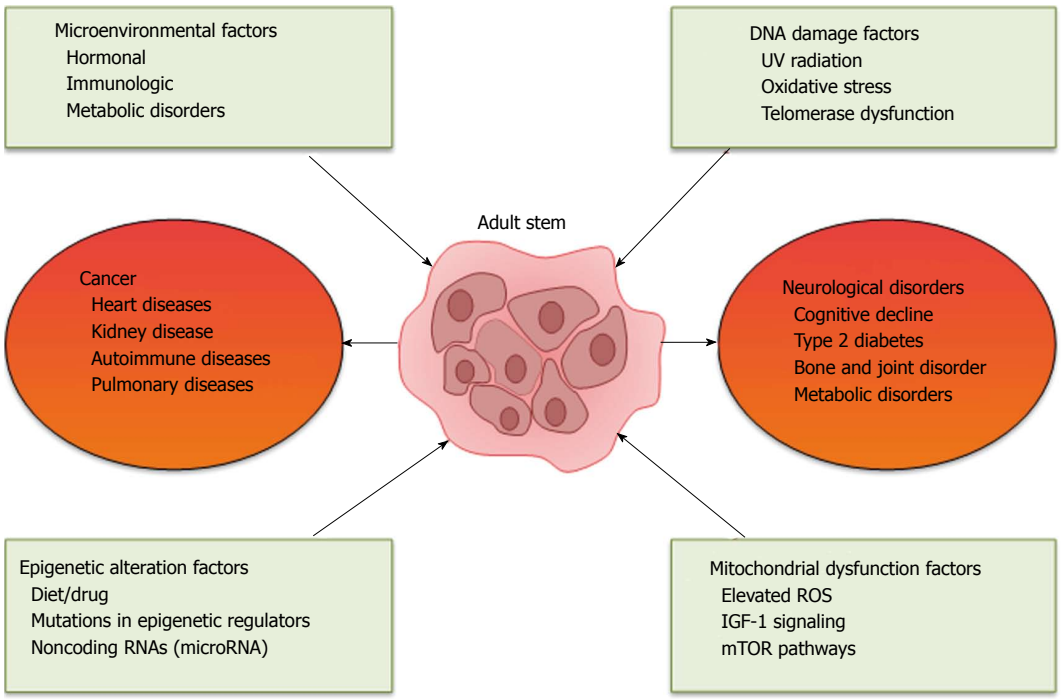

Figure 1 A proposed mechanism for the aging-induced deterioration of stem cell functions and aging-associated diseases. ROS: Reactive oxygen species.

events occur on DNA on daily basis. While most of the damaged DNAs are repaired by normal DNA repair mechanism, some of the mutated DNAs appear to escape from the repair mechanism and accumulate over time. Accordingly, there would be a significant accumulation of mutated or damaged DNAs in aging cells compared to young cells. The accumulation of damaged DNA may in part be responsible for the various cellular events of the aging process. In fact, this "mutational theory" is one of the earliest theories of the aging process[15]. DNA damage can be caused by environmental factors, like UV irradiation, and also can be the consequence of the cell's own metabolic processes [*e.g.*, generating reactive oxygen species (ROS)] that tend to accumulate with time[60]. DNA damage impaired stem-cell function in aging, which has been documented by the study that HSCs derived from aged mice harbored significant alterations in their DNA repair response[1,17]. DNA-repair proteins, such as FANCD1[61], MSH2[62] or ERCC1[63], are found to be deficient in adult mice with significant functional defects of HSCs and the dysfunction of MSCs in aging led to leukemia and aging-associated remodeling[64]. In addition, measures of DNA damage in HSCs, such as histone H2AX phosphorylation and comet tails, were also found to be increased with advancing age[65,66]. In satellite cells, H2AX phosphorylation was also accumulated with increasing age[67].

Premature aging can be resulted from defects in the DNA repair and telomerase pathway components in humans and mice[68]. In aging diseases, there has been significant interest in the telomere shortening that is now being used as a hallmark of aging, to which even stem cells are not immune[1,17]. A telomere is a region of repetitive nucleotide sequences at each end of a chromosome. It protects genome from nucleolytic degradation, unnecessary recombination, repair, or fusion with neighboring chromosomes[69]. Although stem cells express telomerase, the telomeres of HSCs, MSCs, NSCs, HFSCs and GSCs do shorten with age[70-72]. When telomeres become critically short, the cell becomes senescent, it ceases to divide and may undergo apoptosis. In fact, many aging-associated diseases, like the increased cancer risk[73,74], coronary heart disease[75-77], heart failure[78], diabetes[79], and osteoporosis[80], are caused by accelerated telomere shortening. Despite considerable evidence that telomere shortening causes reduction in life span, the telomere shortening concept of aging is still somewhat controversial, since laboratory mice lacking telomerase RNA component (TERC) showed no obvious abnormal phenotypes even after five generations[81,82].

Mitochondrial dysfunction

Mitochondria are ubiquitous intracellular organelles in mammals and are the main source of cellular adenosine triphosphate (ATP) that plays a central role in a variety of cellular processes. As mitochondria produce about 90% of cellular energy, the aging-related ROS generation, disruption in Ca^{2+} homeostasis, and increased cell apoptosis are three causes of mitochondria dysfunction that directly affects aging-related diseases[83]. In fact, there have been many studies suggesting a direct relationship between mitochondrial dysfunction and human stem cell aging[84-87]. Accordingly, in several cell systems, mitochondrial dysfunction has been shown to lead to respiratory chain dysfunction, which may be the result of the accumulation of mutations in mitochondrial DNA

(mtDNA)[88]. The elevated ROS in aging is mainly due to mtDNA mutation, as mitochondria is the primary cellular sources of ROS[89]. In addition, it has been confirmed that mitochondrial aging interact with other cellular pathways of aging, such as the IGF-1 signaling and the mTOR pathways, which presumed to play a major role in aging[90,91].

Epigenetic alteration

Epigenetics refer to changes in gene expression, which are heritable through modifications without affecting the DNA sequence. It has also been defined more broadly as the dynamic regulation of gene expression by sequence-independent mechanisms, including but not limited to changes in DNA methylation and histone modifications[92-94]. Epigenetic marks in stem cells are transmitted heritably to their daughter cells, priming lineage-specific loci for modification in downstream progenies[95]. Stem cell fates are regulated by epigenetic modifications of DNA that establish the memory of active and silent gene states[96,97]. Aberrant epigenetic regulation affects the organismal aging[98], age-associated dysfunction of stem cells, and predisposition to hematological cancers development[99]. For instance, DNA methylation specific to regions of the genome that are important for lineage-specific gene expression increased in aging HSCs[100] and the perturbations of their histone modifications (H3K4me3) may impair its self-renewal genes[101]. It has also been reported that mutations in epigenetic regulators, such as DNMT3a, TET2, and ASXL1, are frequently found in myeloid neoplasia[102]. Since most of the chromatin changes are intrinsically reversible, epigenetic alterations are therefore considered good therapeutic targets for molecular effectors and thereby are potential therapies for certain distinct pathologies[103,104]. Therefore, there has been immense interest in understanding these genome-scale regulatory mechanisms that lead to impaired gene expression, and that contribute to the decline of stem cell and tissue function with age.

MicroRNAs (miRNAs) are another key class of epigenetic mediators of stem cell dysfunction. They are a class of small noncoding RNAs composed of 18- to 25-bp nucleotides[105] that functions in RNA silencing and post-transcriptional regulation of gene expression[106-109]. It plays an important role in regulating stem cell self-renewal and differentiation by repressing the translation of selected mRNAs in stem cells and differentiating daughter cells[110]. In fact, non-coding RNA-mediated regulatory events as a part of the epigenetic mechanism to modulate mRNA degradation and/or protein translation that play important role in development and disease state[111]. MiRNAs, such as miR-17, regulates osteoblast differentiation of MSCs[112-114]. MiR-290–295 cluster seems to promote embryonic stem cell differentiation, self-renewal, and maintenance of pluripotency[110,115]. Moreover, recent findings show the involvement of miRNAs in senescence manipulation. These findings have led to the suggested use of these miRNAs as clinical biomarkers of stem cell senescence and their potentiality[116].

THERAPEUTIC APPROACHES FOR THE TREATMENT OF AGING-INDUCED STEM CELL DYSFUNCTION

In recent years with increasing understanding of stem cell behavior in different niche of the body offers promise for the development of potential therapeutic approaches to treat aging-associated dysregulation of adult stem cells and aging-related diseases. Some of the potential therapeutic approaches for the treatment of age-related stem cell dysfunction are discussed below.

Parabiosis

The concept of parabiosis is not new; however, in the past decade its role in reversing the effects of aging and enhancing rejuvenation has gathered substantial momentum. Recent findings suggest that aging-related cellular dysfunctions can be repaired successfully by modulating the molecular architecture of the tissue environment rather than inducing cell intrinsic changes alone[117]. Therefore, the effects of aging in an old individual can be modulated or reversed by the circulatory or systemic factors derived from the young blood through anatomical joining, parabiosis[40]. The fascinating results of parabiosis have been reported to rejenuvate brain[118], muscles[67], and liver tissues in the aged animals[119]. In skeletal muscle regeneration, serum derived from young mice activated the Notch signaling pathway and regulated the satellite cells proliferation of old mice *in vitro*[119]. In aged mice, through the parabiosis approach, systemic factors from young mice successfully reversed inefficient CNS remyelination, a regenerative process of CNS that produces new myelin sheaths from adult stem cells[118]. Despite the promising outcomes in animal models, there is persistence of contradiction in functions of factors identified in prominent parabiosis studies, rendering the concept highly controversial for use in humans. For instance, growth differentiation factor 11 (GDF-11) has been reported to show both positive[67] and negative corelations[120] with stem cell aging.

Retrotansposons

Retrotansposons are mobile DNA elements that can induce genetic instability and have been reported to be a cause of cellular dysfunction during aging[121]. The long interspaced nuclear elements (L1) are 6-kb long retrotransposons that code for RNA binding protein and endonuclease protein. There are 500000 copies of L1 elements in the human genome, and approximately 100 of such active elements replicated to induce genomic instabilities and to increase the risk of DNA damage. Elevated activity of L1 has been reported in aging-related pathological conditions[122]. The link between SIRT-6 (an important marker of longevity) and L1 offered more direct evidence for the role of L1 in aging-related genomic complications. SIRT6 are

known to repress the activity of L1 retrotransposons[123]. DNA damage-induced mobilization of SIRT6 to the site of repair and subsequent repression of L1 have been contemplated in the development of therapeutics for age-related neurological pathologies, such as dementia and cancer[124]. Suppression of L1 activity by overexpression of SIRT6 in senescence cells delayed the onset of L1-induced pathological conditions. High caloric diet activated the SIRT1 activity and has been reported to protect the animal from premature aging in Cockayne syndrome[125], whereas in the case of the mouse Alzheimer's disease model the caloric restriction slowed down the disease progression[126]. Other than modulation of SIRT6 expression, inhibition of reverse transcriptase (a critical enzyme for the L1 replication) is another way to attenuate L1 activity[127]. Several small non-coding RNAs, such as pi-RNAs, si-RNAs and L1 specific small RNAs, have also been reported to regulate the silencing of retrotransposons element activity in mouse germ cells and in aging human somatic cells[127].

Cellular reprogramming towards iPSCs

iPSCs are a type of pluripotent stem cell that can be generated directly from adult cells and the recent advances in this area have opened up many gateways for the research in cell-based therapeutics[128]. Cellular reprogramming of aged somatic cells towards iPSC enables the editing and resetting of the cellular clock by removing the characteristic feature of aging. The ability to derive iPSCs from aging-related pathological cells have enabled investigators to develop recombination-based therapeutic approaches to edit genetic defects responsible of premature and accelerated aging. The reprogramming of aged somatic cells to target stem can be used as an alternative source to get cells for transplantation and for genetic editing. Recent studies show encouraging effects of reprogramming in rejuvenation of senescent cells, as evident by elongated telomeres and reduced oxidative stress[129]. Human iPSC-based models for aging-related degenerative diseases have been tested to understand the disease dynamics in Parkinson's disease, Alzheimer's disease and in progeroid laminopathies[130]. Valuable information from these studies has resulted in the first clinical trial for progeroid patients[131]. In a mouse model of skeletal defect, human iPSC designed to express PAX7 were able to be differentiated into muscle progenitor cells that engrafted and repaired the defective dystrophin-positive myofibers formation. In case of Hutchinson-Gilford progeria syndrome (HGPS), reprogramming of HGPS fibroblasts by transduction with vectors expressing Oct4, Sox2, Klf4 and c-Myc has been reported to revert aging-associated markers, such as Lamin, to a "young" state[132].

Telomere lengthening

As discussed above, the telomere length is inversely linked to the chronical age, and thus it is believed that increasing the length of telomere may increase life span. Many advanced approaches are being developed to efficiently increase the telomere length and to protect cells from chromosome shortening. In *in vitro* cultured human cells, the delivery of RNA coding for telomere-extending protein has been reported to increase the cell proliferation rate[133]. In telomere-deficient mice, genetic editing to reactivate telomerase activity has been reported to reverse the aging symptoms[134]. Telomerase activation drugs and telomerase gene therapy are also alternative approaches that aim to increase the telomere length to protect the cells from premature aging[135,136].

CONCLUSION

From the various advances in stem cell research, it is clear that we grow old partly because our stem cells grow old with us. The functions of aged stem cells become impaired as the result of cell-intrinsic pathways and surrounding environmental changes. With the sharp rise in the aging-associated diseases, the need for effective regenerative medicine strategies for the aged is more important than ever. Fortunately, rapid advances in stem cell and regenerative medicine technologies continue to provide us with a better understanding of the diseases that allows us to develop more effective therapies and diagnostic technologies to better treat aged patients. However, there is a big ethical concern regarding the use of human embryos to procure embryonic stem cells and many countries already currently restrict experiments on embryos to the first 14 d. Additionally, the International Society for Stem Cell Research has issued guidelines advising researchers across the globe to stick with this 14-d window. Nevertheless, it seems that the human stem cell research in the next decade will likely bring enormous progress in the aging-associated disease therapies but may also reach a step closer to the edge of ethical concern of creation of "Frankenstein".

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REVIEW

Odd couple: The unexpected partnership of glucocorticoid hormones and cysteinyl-leukotrienes in the extrinsic regulation of murine bone-marrow eosinopoiesis

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Abstract

Granulopoiesis in murine bone-marrow is regulated by both intrinsic and extrinsic factors (including hormones, drugs, inflammatory mediators and cytokines). Eosinophils, a minor subpopulation of circulating leukocytes, which remains better understood in its contributions to tissue injury in allergic disease than in its presumably beneficial actions in host defense, provide a striking example of joint regulation of granulopoiesis within murine bone-marrow by all of these classes of extrinsic factors. We first described the upregulation of eosinopoiesis in bone-marrow of allergen-sensitized mice following airway allergen challenge. Over the last decade, we were able to show a critical role for endogenous glucocorticoid hormones and cytokines in mediating this phenomenon through modification of cytokine effects, thereby supporting a positive association between stress hormones and allergic reactions. We have further shown that cysteinyl-leukotrienes (CysLT), a major proinflammatory class of lipid mediators, generated through the 5-lipoxygenase pathway, upregulate bone-marrow eosinopoiesis *in vivo* and *in vitro*. CysLT mediate the positive effects of drugs (indomethacin and aspirin) and of proallergic cytokines (eotaxin/CCL11 and interleukin-13) on *in vitro* eosinopoiesis. While these actions of endogenous GC and CysLT might seem unrelated and even antagonistic, we demonstrated a critical partnership of these mediators *in vivo*, shedding light on mechanisms linking stress to allergy: GC are required for CysLT-mediated upregulation of bone-marrow eosinopoiesis *in vivo*, but also attenuate subsequent *ex vivo* responses to CysLT. GC and CysLT therefore work together to induce eosinophilia, but through subtle regulatory mechanisms also limit the magnitude of subsequent bone-marrow responses to allergen.

Key words: Bone marrow; Leukotriene; Eosinophil; Stress; Glucocorticoid

Core tip: The bone-marrow is exquisitely sensitive to regulation by systemic events, which selectively increase

production of different blood cell types to meet transient increases in demand following injury. An association between stress and allergy has long been known, but its mechanisms remain incompletely understood. The exploration of underlying mechanisms in a variety of murine models yielded evidence of separate but interrelated roles for adrenal glucocorticoid hormones and cysteinyl-leukotrienes in coupling systemic events to bone-marrow responses *in vivo*. We here discuss how these unlikely partners work together to promote eosinophilia but through subtle mechanisms also limit its magnitude.

Xavier-Elsas P, Masid-de-Brito D, Vieira BM, Gaspar-Elsas MIC. Odd couple: The unexpected partnership of glucocorticoid hormones and cysteinyl-leukotrienes in the extrinsic regulation of murine bone-marrow eosinopoiesis. *World J Exp Med* 2017; 7(1): 11-24 Available from: URL: http://www.wjgnet.com/2220-315X/full/v7/i1/11.htm DOI: http://dx.doi.org/10.5493/wjem.v7.i1.11

BONE-MARROW REGULATION BY INTRINSIC AND EXTRINSIC FACTORS

In both humans and mice, the lifelong production of blood cells (definitive hemopoiesis[1,2]; takes place in the bone-marrow of long bones, and encompasses the production, from a pool of hemopoietic stem cells (HSC), of both lymphoid (B cells, Natural Killer cells) and myeloid (erythroid, megakaryocytic, mononuclear phagocyte and granulocyte) lineages, through a series of increasingly committed (specialized, or developmentally restricted) stages, recognizable as morphologically, cytochemically and/or immunophenotypically distinct cell types[1,3-5]. Mature cells may then be exported to the circulation and remain there, until they are removed during their passage through the spleen (a typical fate for erythrocytes and platelets[6]) or emigrate to the tissues, ultimately undergoing apoptosis and clearance by resident phagocytes[7]. Emigration occurs either when inflammation follows injury (thereby allowing neutrophil granulocytes to exert short-term protective functions, in the absence or presence of infection[8]; or when chemoattractants selectively expressed in some sites attract leukocytes from a particular lineage (for instance, in the case of eosinophil granulocytes, enabling them to enter the mucosa of the digestive, respiratory and reproductive tracts to become long-term resident effector and regulatory cells[9].

Usually, peripheral clearance of senescent or apoptotic cells of bone-marrow origin is coupled to replenishment by a variety of mechanisms[10]. This is no small achievement, because specialized hemopoietic cell lineages, though ultimately derived from the same pool of pluripotent, self-renewing stem cells, differ largely in their numbers, requirements and properties[1-5]; accordingly, no single mechanism can account for the maintenance of their proportions across different compartments, nor for their lineage-selective increases or decreases often observed in immune reactions and disease[4,5,11,12].

Multiple factors intrinsic to the adult bone-marrow contribute to the maintenance of a steady output of these different cell types in very disparate proportions and rates. A major intrinsic factor is the differential expansion of hemopoietic lineages, driven by intense proliferation of lineage-committed progenitors (quantifiable *in vitro* as colony-forming units, or CFU), specified by unique profiles of gene expression under the control of master genes and transcription factors, in response to different hemopoietic growth factors or combinations thereof[1]. Progenitor expansion is adjusted to the turnover rate of the respective circulating forms of each lineage, so that relatively stable numbers of red cells, platelets and leukocyte subpopulations are replaced every day, enabling us to determine a range of "normal" blood cell counts, which may widely differ from one lineage to the other[1,3].

The original *in vitro* studies, which led to the purification and ultimately to cloning of a variety of colony-stimulating factors (CSF) of various nonlymphoid sources, endowed with selectivity for macrophage (M-CSF, or CSF-1), granulocyte (G-CSF), or granulocyte-macrophage (GM-CSF) progenitors, had suggested that hemopoiesis in steady-state conditions was driven by CSF-like molecules[3]. From this assumption one would predict that disruption of signaling by CSF-like molecules would entail profound deficiency in circulating leukocytes. This view must now be qualified, however, in view of the persistence of normal granulopoiesis in mice lacking the functions of GM-CSF and IL-3, two major CSF species[13]. Not all CSF, however, are irrelevant to steady-state granulopoiesis, as IL-5 seems necessary for normal production of eosinophils[9,13-15], G-CSF for that of neutrophils and M-CSF for that of macrophages[1]. Thrombopoietin and G-CSF, originally identified as CSF with lineage-selectivity for megakaryocytes/platelets and neutrophils, respectively, have been further characterized as multilineage regulators with complex actions, thereby overstepping the original boundaries of their function[1,3]. Therefore, while much remains to be learned about the intrinsic processes that drive definitive hemopoiesis in steady-state, it is likely that at least some CSF cytokines contribute to hemopoiesis in exceptionally demanding conditions, by mediating the actions of extrinsic factors linked to homeostatic disturbances or environmental changes on bone-marrow.

Increased demand on the bone-marrow imposed by systemic challenges, unlike regeneration of the entire hemopoietic environment[16], elicits lineage-selective responses, which may be short- or long-lived: For instance, hemorrhage and chronic hypoxemia are met with compensatory production of erythrocytes[17]; in other examples, bacterial infection elicits adaptive increases in neutrophil leukocytes[4,5,11], and helminth infection or allergic disease induce eosinophilia[9,14,18-20].

Importantly, the critical elements in these adaptations of bone-marrow to a transient stress are lineage-committed progenitors, rather than the HSC endowed with

self-renewing and long-term repopulating potential. This makes biological sense, since progenitors are closer than stem cells to terminally differentiated, functional blood cells, and the physiologically relevant increase in circulating blood cells will be faster, because it will require less rounds of cell division. By contrast, HSC, as a rule, are protected from such transient challenges for a good reason, since infection at least may severely impair their function[11].

GM-CSF and interleukin (IL)-3 may be more relevant to the stress (or emergency) myelopoiesis in systemic microbial infection[4,5,15], and, in the more restricted context of helminth infection and allergic disease, IL-5 plays an important role for its selectivity to the eosinophil lineage[9,14,19].

Importantly, however, in the case of neutrophil or eosinophil granulocytes, proliferative and maturation-promoting effects of these CSF on production are only part of their contribution to the adaptive hematological responses, since they also have important mobilizing effects on the reserve pool associated with bone-marrow and other sites, and they further extend the lifespan of selected hemopoietic lineages outside bone-marrow, thereby increasing the total number of cells belonging to these lineages in the periphery, and decreasing their turnover by a lineage-selective reduction in cell death rates[7]. The consistently positive action of the same CSF at multiple steps in the life cycle of granulocytes highlights the integration of these proliferative and non-proliferative cytokine effects, which translates in physiologically meaningful outcomes.

It is important that these granulopoietic/mobilizing/antiapoptotic cytokines are not restricted to the bone-marrow compartment, but are often produced by multiple cell types in the context of specific adaptive (specific) as well as innate (nonspecific) immune responses at distant sites. Nevertheless, cytokines acting on bone-marrow targets act early in this sequence, and due to the amplification of their effects through multiple rounds of cell division, they have long-lasting effects.

In the context of allergic disease or helminth infection, IL-5, the lineage-specific cytokine required for both constitutive and stress eosinopoiesis, is secreted in different contexts by different cells, especially by activated, allergen-specific, Th2 lymphocytes[1,9,14,15], and could contribute in various ways to the effects of allergen challenge. Recognition of allergen at the challenge site by TH2 lymphocytes, which subsequently secrete IL-5, is one way to couple allergen recognition in the peripheral sites to generation of a stimulus within the bone-marrow. Other possibilities include production of IL-5 by mast cells following recognition of allergen by specific IgE bound to FcεRI in the mast cell surfaces[15,20]. Secretion of IL-5 inside bone-marrow by lymphocyte populations[21] might also contribute, although it is unclear at present whether these would necessarily be conventional T lymphocytes, requiring MHC restricted allergen presentation by dendritic or B cells.

THE MODES OF OPERATION OF CYTOKINES AND OTHER EXTRINSIC REGULATORS OF BONE-MARROW FUNCTION

Cytokines can transduce the effects of immune reactions on the bone-marrow, by one of two ways: (1) systemic diffusion of the cytokine itself, from the inflammatory site to bone-marrow through the circulation; and (2) selective homing of cytokine-producing cells to the bone-marrow, followed by local cytokine production.

In the first case, the cytokine stimulus is widespread, but the response remains restricted because the relevant target cells are concentrated in bone-marrow or, if present elsewhere, are presumably absent or dormant. In the second case, such a systemically diffusible stimulus is not necessary or even relevant, since the effect of immunity on granulopoiesis is elicited through a local accumulation of cytokine-producing leukocytes inside the bone-marrow, which only activate the relevant target cells in their neighborhood. Both mechanisms depend on the stimulus not being constitutively present, or effective, but becoming so in the wake of allergen challenge.

These alternatives have clearly distinct counterparts in an experimental setting: In the first case, bone-marrow effects can be elicited by intravenous transfer of plasma from the appropriate donors to naive recipients[22], and effects of this transfer will be restricted to the bone-marrow as long as there are no responsive targets elsewhere; in the second, transfer of leukocyte populations capable of homing to the bone-marrow compartment will be sufficient, and responses will both be limited to the bone-marrow compartment, and associated with the physical presence of the transferred leukocytes in this compartment[23].

It should be noted that these various possibilities are not mutually exclusive, but may better describe events at different time points. This is probably relevant to the sequence of events elicited by allergen exposure of sensitized mice ("challenge"), thought to represent chronic processes that underlie allergic diseases, especially asthma[12,24], as discussed below in the context of eosinophil production, which is the prime target of IL-5 actions.

It is also important that CSF-like molecules are just a small fraction of the cytokines that might be influencing bone-marrow responses, which is defined by its ability to directly stimulate hemopoiesis. A much larger number of cytokines may be unable to act as primary hemopoietic stimulus, but remain quite effective in modifying the actions of primary stimuli to achieve particular effects. In the case of the eosinophil lineage, IL-5 is the best characterized (and highly selective) primary stimulus[9,15,20]; however, a number of cytokines discussed below, including eotaxin/CCL11, IL-13 and IL-17, do not stimulate directly eosinopoiesis, but interact with IL-5 to

achieve quite different outcomes[25,26].

Another important feature of cytokine-coordinated processes is the potential for interactions involving multiple partners. These are, in some cases, other cytokines as mentioned above; however, they may also include noncytokine mediators of inflammation such as proinflammatory or antiinflammatory lipid mediators, hormones, or vitamins. In the context of therapy, drugs or immunoregulatory leukocyte subpopulations may become partners for novel interactions. Generally speaking, the actions of a given cytokine may be only understood in context, which encompasses immunoendocrine and immunopharmacological interactions, in addition to cytokine network interactions.

THE EXPERIMENTAL ANALYSIS OF ALLERGEN-INDUCED EOSINOPHILIA

Eosinophils, a minor subset of circulating leukocytes, remain better understood in their contributions to tissue injury in allergic disease such as asthma[27,28], than in their presumably beneficial actions in host defense and tissue repair[9,14,15,29,30]. Nevertheless, eosinophils are very interesting cells, which produce a large number of specialized (mostly cationic, or "basic") proteins, a complex mixture of lipid mediators, and an impressive number of cytokines, which overstep the boundaries of the conventional TH1 and TH2 "profiles". In addition, eosinophils aided by antibody are capable of killing some tumor cell targets, and, at high effector/target ratios, some larval stages of worms[19]. It is both biologically puzzling and therapeutically serendipitous that eosinophil-depleting interventions in experimental models as well as in chronic treatments have no consistent adverse effects. Hence, the damage they could cause in people with a variety of diseases is prevented by such treatments, but no obvious function for eosinophils in an otherwise healthy subject is unveiled through eosinophil depletion[29]. This does not necessarily prove that eosinophils have become obsolete in the course of evolution; it nevertheless suggests that beneficial functions for eosinophils might be relevant in very specific conditions which have not yet been addressed in these previous studies. Two such examples in the recent literature are a beneficial role for eosinophils in liver regeneration[31] and a role for eosinophils in the recruitment of other leukocyte classes in response to CCL11[32].

Eosinophils are very suitable for the experimental analysis of stress granulopoiesis in mice, for a number of reasons.

Reliable identification and efficient detection of eosinophils

In mice and humans, eosinophils are easy to recognize and to detect in tissues, by a combination of surface marker expression and morphological criteria, including cytochemical reactions. Although nuclear morphology is not identical between human and mouse eosinophils, they are polymorphonuclear leukocytes presenting segmented, thick bands of chromatin when mature[9,14,15,33]. In both species, they contain numerous cytoplasmic granules of various sizes, and in the mouse a coarse type of granule, displaying the characteristic affinity for the acidic dye eosin, also stains positive in the cytochemical reaction for eosinophil peroxidase (EPO), which yields a brown color because of precipitated diaminobenzidine product formed in the presence of exogenous H_2O_2[34]. Murine EPO, unlike its homologues in other species, including humans[35], is resistant to cyanide, which makes this reaction a very useful marker for mouse eosinophils, as distinct from mouse neutrophils. Experimentally, the expression of a characteristic array of surface markers, including the receptor for the lineage-selective chemokine, CCL11 (or eotaxin-1), CCR3[36], as well as the cell surface lectin Siglec-F or Siglec-8[9], makes it easier to monitor the presence of eosinophils in cell suspensions by flow cytometry.

Quantitative changes can be accurately induced and monitored

The numbers and even the presence of eosinophils in individual animals can be manipulated conveniently in various murine models. Allergic sensitization and challenge, helminth infection, IL-5 overexpression and/or administration, IL-9[37] and more recently IL-33 infusion[38], have all been reported to induce eosinophilia in mice[9,14,22,39]. Eosinopenia, the opposite state, can be induced in variable degrees by a variety of neutralizing antibodies to IL-5[22,40] or by induced mutations, including selective deletion of an internal autoamplification site in the promoter of the GATA-1 transcription factor, through which the coding regions of the GATA-1 gene remain functionally intact and sufficient for differentiation of erythrocytes and platelets, while eosinophil production, which requires an early autoamplification step, fails on a permanent basis[41]. Even more conveniently for the experimenter, this model is suitable for reconstitution, since mature eosinophils can be transferred from wild type (BALB/c) control donors in high purity[32]. Alternative models, based on selective and conditional elimination of eosinophils by genetic engineering, have provided important insights in other experimental models[27,28,42]. It is reassuring that no obvious damage to the organism is associated with eosinophilia or eosinopenia per se, as documented even in IL-5 transgenic models. By contrast, damage to heart and nervous tissue, and extremely high eosinophilia, not induced by an external agent, coexist in humans with the so-called hypereosinophilic syndromes; there is, however, extensive evidence that, in these conditions, eosinophils are functionally activated and possibly abnormal[9,20]. In murine models[39], by contrast, no damage secondary to the induction of eosinophilia is likely to confound the interpretation of results.

Stimuli and procedures have a high degree of selectivity

Usually, marked changes in eosinophil counts occur in bone-marrow, spleen or blood without significant changes in total cell counts. This apparent selectivity is

due to eosinophils being a minority[9,14], amounting to 3% or less of circulating leukocytes in noninfected, nonallergic humans, for instance, while most other leukocyte populations are much larger (compare with up to 70% neutrophils in human blood). To reach significance, a much larger change in other leukocyte populations would be needed, because random fluctuation is larger in this case. Eosinophils have a specialized growth factor (IL-5), and differ from other leukocyte types in responses to other cytokines and mediators of inflammation, including rather selective chemoattractants, such as CCL11[33] and the cysteinyl-leukotrienes (CysLT)[43]. All of these differences contribute to the relative selectivity of the effects observed. Nevertheless, some stimuli, such as GM-CSF, elicit major responses in several hemopoietic lineages at once, including eosinophils and neutrophils[44]. It is relevant, in this context, that GM-CSF and IL-5 receptors, although distinct in their composition, share an essential signaling component, the common β chain (βc[13,44]). This subunit is also found in IL-3 receptors, although in mice there is evidence for a separate IL-3 receptor lacking βc[13]. Eosinophils and neutrophils present GM-CSF and IL-3 receptors bearing βc, while eosinophils (and basophils[42]) have IL-5 receptors, unlike neutrophils[8,13]. This implies that even though GM-CSF, IL-3 and IL-5 can stimulate eosinophil production through similar signaling pathways (mediated by βc), IL-5, unlike GM-CSF and IL-3, should not directly induce neutrophil production.

THE SPECTRUM OF CHALLENGE EFFECTS ON THE EOSINOPHIL LINEAGE INSIDE BONE-MARROW

We have first described the upregulation of eosinophil production in murine bone-marrow in mice sensitized and challenged with allergen in the airways. In this murine model of allergic eosinophilia, the critical stimulus is specific allergen challenge in the airways[22] or in alternative sites[45], and the major outcome is an increase in bone-marrow eosinophil-lineage cells (eosinophil peroxidase positive, or EPO^+, cells) in bone-marrow harvested 24 h after challenge, which is taken as direct evidence of allergen-induced eosinophilia of the bone-marrow *in vivo*. To keep a focus on bone-marrow events, we will not discuss here extramedullary effects of allergen challenge, such as the accumulation of eosinophil progenitors in the lungs[46] and the large increase in eosinophils in the spleen[47]. Nevertheless, these are interesting in themselves and share important mechanisms with events in bone-marrow[47].

Challenge-induced bone-marrow eosinophilia can be fully prevented in mice made specifically tolerant to the allergen before sensitization and challenge, as well in recipients of splenic T cells from these tolerized donors[48]. It is important, however, that the tolerance induction oversteps the boundaries of the original phenomenon, as tolerance-induced changes also affect neutropoiesis and therefore extends to another lineage[48].

This sensitization/challenge experimental setting provides a wealth of experimental opportunities, which have been explored in recent years. Because of the rapidity with which events in the airways translate into distant consequences in the bone-marrow, we hypothesized that a mediator released in the circulation would account for communication between the sites of challenge and of eosinopoiesis. Plasma transfer experiments from sensitized/challenged donors to naive recipients did support this view[22].

While *in vivo* observations suggest the relevance of the phenomenon, important information was provided by *ex vivo* protocols, defined as the *in vitro* analysis of changes induced by previous interventions *in vivo*. The outcome of *in vivo* interventions and the associated *ex vivo* observations is summarized in Table 1. In addition to rapidly inducing eosinophilia of bone-marrow *in vivo* (24 h), challenge also increases the magnitude of the responses of bone-marrow cells to IL-5. This effect was described as "priming" because it takes place *in vivo*, during the 24 h that follow challenge, but it remains undetected until cells are exposed to IL-5 in culture for several days - hence it corresponds to a silent change in developmental potential that is unveiled by subsequent IL-5 exposure[22,45]. It is analogous, but not identical, to priming for other cellular responses by exposure to IL-5 itself[49], since it distinguishes between *in vivo* allergen-challenged and -unchallenged sensitized mice, which do not necessarily differ in their circulating IL-5 levels[22]. The endpoint that defines priming is *in vitro* differentiation of precursors that had been exposed *in vivo* to allergen and presumably to newly released IL-5 as well. Nevertheless, these precursors are not IL-5-autonomous, since they do not complete differentiation in culture if exogenous IL-5 is not added[22]. This apparent paradox suggests that even though endogenous IL-5 has been shown be present *in vivo* in the bone-marrow after challenge[21], it is not sufficient to sustain eosinophil production over a week's culture.

Importantly, priming is a positive phenomenon: It rapidly increases the magnitude of the responses to IL-5 as well as to other eosinopoietic stimuli, such as IL-3[22]. Therefore, it is assumed to contribute to the eosinophilia of allergic disease, rather than to oppose it. It is detectable as early as 24 h and as long as one week after challenge of sensitized mice.

Priming, however, is paralleled by a distinct *ex vivo* effect[50], which we call immunoregulation of pharmacological responses, because it reduces the magnitude of the subsequent responses of cultured bone-marrow to some drugs, as well as to exogenously provided mediators and cytokines. In contrast to priming, which changes the response to the primary stimulus (IL-5), immunoregulation of pharmacological responses attenuates the response to secondary stimuli, such as nonsteroidal antiinflammatory drugs (NSAID) and proallergic cytokines, all of which require IL-5 to be effective. Hence, priming upregulates a core response to IL-5; by contrast, immunoregulation restricts

Table 1 The spectrum of GC-dependent effects on bone-marrow eosinopoiesis

In vivo treatment	Bone-marrow effects						Systemic factors		Ref.
	In vivo bone-marrow Eosinophilia	*Ex vivo* effects on response to GM-CSF (CFU counts) in bone-marrow culture		*Ex vivo* effects of challenge on responses to IL-5 or CysLT in bone-marrow culture			GC measurements and interventions targeting GC		
		Total	Eosinophil	Priming	Maturation of eosinophils in culture	Regulation of CysLT responses	Blockade by GC targeting	Plasma corticosterone	
None	Baseline	NA	NA	NA	Complete	NA	No effect	Baseline	[36,52]
Dexamethasone (5-20 mg/kg)	Baseline (BALB/c)	Increase	Increase	Primed by 24 h of injection	Incomplete, rescued by PGE2, anti-α4 integrin antibody	NE	RU486	NA	[51,36]
	Increased (C57BL/6)	Increase	NE	NE	NE	NE	RU486	N. A.	[53]
Surgical trauma	Increased (BALB/c) by 24 h of trauma	NE	NE	Primed by 24 h of trauma	Complete	NE	RU486 Metirapone	Stress level by 24 h of trauma	[52]
Sensitization and challenge	Increased (BALB/c, B6, BP-2) by 24 h of challenge	No significant increase	Increased by 24 h of challenge	Primed by 24 h of challenge	Complete	Attenuated by 24 h of challenge	RU486 Metirapone (eosinophilia, priming)	Stress level by 24 h of challenge (BALB/c), TNF-α induced	[22,25,45, 47,50,54,70]
Oral tolerance induction, sensitization and challenge	Increase prevented (BALB/c, BP-2) by 24 h of challenge	Increased by 24 h of challenge	Increased by 24 of challenge	Priming prevented (BP-2) by 24 h of challenge	Complete	NE	NA	NE	[48]

CFU: Colony-forming unit; NE: Not examined in the study; NA: Not applicable to the study.

a peripheral response to modifiers of IL-5 activity. While priming is assumed to promote an eosinophilic response, immunoregulation should, in principle, restrict further expansion of eosinophil numbers by exposure to other stimuli. These two effects do not cancel each other, but may interact, depending on the precise stimulation context and on their relative timing.

Indomethacin and aspirin, two NSAID with biochemically distinct mechanisms of action, proved stimulatory not only for IL-5-driven eosinopoiesis in bone-marrow culture, but for colony formation by myeloid progenitors of several lineages as well[50]. Unexpectedly, however, when bone-marrow from sensitized and challenged mice was studied, the ability of both NSAID to enhance eosinopoiesis was lost[50], and, depending on the experimental conditions, NSAID can even become inhibitory in this assay (manuscript in preparation). Therefore, immunoregulation of pharmacological responses comprises two aspects: (1) attenuation of the effectiveness of NSAID as enhancers of eosinopoiesis; and (2) inversion of its effects, leading to active suppression of eosinopoiesis when NSAID are present. Because pharmacological responses are usually not assumed to depend on the immune status of the organism, this apparently paradoxical observation has theoretical interest in itself. In its original description[50], no physiological relevance was ascribed to it. More recent results, as detailed below, substantially increased the scope of this effect and highlighted its potential to modulate eosinophilia in a biologically more relevant context.

The twin phenomena of priming and pharmacological immunoregulation highlight two important features of extrinsic control of bone-marrow: (1) changes can be both silent and durable, as in priming, thereby accounting for effects that may become visible only in the long-term; and (2) the apparent paradox of a drug response that depends on the immune status of the organism - challenged *vs* unchallenged mice - reflects the mechanism of action of the drug as well as the inflammatory events elicited by challenge. Both priming and immunoregulation remain silent effects, until they are unveiled by the appropriate stimuli (exposure to IL-5, in the first case; or to NSAID in the presence of IL-5, in the second). In both cases, challenge changes the properties of the target cell.

THE CENTRAL ROLE OF GLUCOCORTICOIDS IN EXTRINSIC REGULATION OF BONE-MARROW EOSINOPOIESIS

A central contribution of glucocorticoids (GC) to extrinsic bone-marrow regulation was first reported in a pharmacological setting, following exposure to dexamethasone, both *in vivo* and *in vitro*[51]. Subsequent experiments in a sterile trauma model indicated that stress-induced GC (corticosterone, in mice) selectively induced bone-marrow eosinophilia in the absence of

specific immune responses[52]. Finally, an essential role for corticosterone in allergen-induced bone-marrow eosinophilia was also demonstrated in a sensitization/challenge model[45], which necessarily involves specific immunity. Hence, evidence of a link between GC and bone-marrow eosinophilia was consistently provided by experiments ranging from pharmacological through physiological to immunological settings, which are also summarized in Table 1. This coherence of effects is to be expected from the well-established fact that GCs, synthetic or natural, act through the same receptor, which is blocked by RU486 (mifepristone[45,51-53]).

Dexamethasone did not induce bone-marrow eosinophilia *in vivo* in our original study, which used mice of the BALB/c background; however, it did prime bone-marrow for strongly enhanced responses to IL-5 *ex vivo* over a period of from 24 h[51] up to 4 wk after injection (manuscript in preparation); more recently, however, an important difference between strains of distinct backgrounds was observed for this drug effect, since bone-marrow eosinophilia was observed in C57BL/6 mice injected with dexamethasone, 24 h after injection, unlike BALB/c controls[53]. In both BALB/c and C57BL/6 mice, dexamethasone primed bone-marrow for increased eosinophil production in IL-5-stimulated cultures; dexamethasone did not replace IL-5 as a primary eosinopoietic stimulus, but greatly enhanced its effectiveness. However, dexamethasone significantly modified IL-5 effects, since a large fraction of the eosinophils produced in dexamethasone-exposed BALB/c cultures were cytologically immature and formed extensive homotypic aggregates[36,51], none of which had been observed in preceding studies of BALB/c[22,45] or C57BL/6[54] sensitized/challenged mice. Further studies[36] demonstrated the ability of PGE2 to synergize with dexamethasone in promoting terminal cytological maturation of these eosinophils in BALB/c bone-marrow cultures. Because neutralizing antibodies to VLA-4 (CD49; $\alpha4\beta1$ integrin) were able to dissociate the homotypic aggregates formed in dexamethasone-exposed cultures, leading to an increased recovery of fully mature eosinophils, we hypothesized that homotypic aggregation interfered with terminal maturation, and that release from aggregates allowed terminal maturation to proceed. Accordingly, PGE2 was shown to dissociate the same aggregates, through an effect on $\alpha4\beta1$ integrin expression[36].

By contrast, in the trauma model[52], the physiologically relevant GC, corticosterone, was elevated to stress levels 24 h after surgery and selectively induced eosinophilia in the bone-marrow, as well as primed for increased IL-5-dependent eosinopoiesis. These effects of trauma-induced GC were long-lasting, and significant at least for two weeks after surgery[52]. The direct contribution of glucocorticoids in this model was documented by blocking with RU486, as had been previously done with dexamethasone[51], and confirmed by two other independent approaches (metirapone treatment and adrenalectomy followed by trauma after a recovery period).

Unlike the response to dexamethasone injection, it is likely that the response to trauma adds to the GC surge other variables related to cell injury and innate immunity. One important such variable, is tumor necrosis factor-α (TNF-α), which may interact with corticosterone so as to modify its actions, in a way consistent with the differences observed between the pharmacological (dexamethasone) and the physiological (trauma) models, especially in the induction of bone-marrow eosinophilia and on cytological maturity of the eosinophils.

Finally, we recently demonstrated that the eosinophilia induced by challenge in sensitized mice involves endogenous GC[45], which are induced by a product of immune cell activation, TNF-α, because: (1) eosinophilia is abolished with equal effectiveness by RU486 or by anti-TNF-α neutralizing antibody; and (2) a corticosterone surge, reaching stress levels, is observed in wild-type controls, with or without RU486-pretreatment, but not in TNF-α type Ⅰ receptor-deficient (TNFRI-KO) mice.

Challenge-induced eosinophilia is sustained by IL-5 acting *in vivo*[21,22]; by contrast, priming requires endogenous GC to act *in vivo* to prime for an increased *ex vivo* response to IL-5 upon subsequent culture[45]. Although IL-5 has usually been considered the target of changes initiated by challenge, there is evidence that responses to IL-5 are self-limiting themselves, since exposure to IL-5, IL-3 or GM-CSF, presumably acting through βc, was shown to down-regulate IL-5Rα chain expression[55], thereby reducing IL-5 binding and strength of stimulation. Similar observations were reported with other extrinsic regulators, such as all-trans retinoic acid, which suppresses expression of IL-5Rα in culture of human hemopoietic cells[56]; in murine eosinopoiesis, the effects of all trans retinoic acid are effectively blocked by GC (Xavier-Elsas *et al*, submitted).

Together, these observations, summarized in Table 1, are consistent with the reported ability of TNF-α as well as IL-1β, both major inflammatory cytokines, to mediate, in animal models, an immunoendocrine response to tissue damage with stress-levels of adrenal GC[57]. They are equally consistent with the reported link between elevated levels of cortisol in humans subjected to stress and an increase in the frequency and severity of allergic reactions[58,59]. Against this background information, the extrinsic upregulation of bone-marrow eosinophilia by dexamethasone, trauma and allergy in our own studies is better explained as a paradoxical stimulatory effect of GC on progenitors and precursors of the eosinophil lineage. While this may go against the predominant view of GC effects in allergy[57,60-62], it is a reproducible effect with pathophysiological implications[58,59,63-65], which may appear less paradoxical as a result of its interaction with the 5-lipoxygenase (5-LO) pathway of arachidonate metabolism, as detailed below.

THE MULTIPLE ROLES OF 5-LO IN BONE-MARROW: SOLVED, UNSOLVED AND NOVEL QUESTIONS

The 5-LO pathway, which produces leukotrienes (LT), has been intensively studied over three decades in the

context of allergic disease[66,67]. While its involvement in the functional abnormalities of the airways in asthma is well-established, its roles in extrinsic regulation of bone-marrow remain incompletely explored, even though bone-marrow is believed to be central to chronic inflammation in asthma[12,24]. LT, especially CysLT (a class comprising LTC4, LTD4 and LTE4), besides making important contributions to asthma pathophysiology, have significant pharmacological effects on hemopoietic cells[68,69]. Such effects are of special interest in the case of eosinophils, which both produce and respond to LT[43], and play important roles in allergic disease. Type 1 CysLT receptors (CysLT1R) play important roles in the pathophysiology of human and experimental asthma, and CysLT1R antagonists, such as pranlukast, zafirlukast and montelukast, are currently approved for the treatment of asthma[47,66,67]. CysLT1R are expressed in several cell populations in the bone-marrow[68], and CysLT were shown to enhance colony formation by progenitors of different myeloid lineages in addition to eosinophils[69].

The stimulatory effect of NSAID on eosinopoiesis is of special interest not only because it is subject to immunoregulation of pharmacological responses, but because of what it tells us about the roles of COX and 5-LO in bone-marrow regulation.

NSAID, which block the cyclooxygenase (COX) pathway, were originally tested in the context of the effects of prostaglandin E2 (PGE2), a COX product, in murine bone-marrow culture. Eosinopoiesis is significantly suppressed by exogenously added PGE2 in bone-marrow cultures established from allergen-challenged mice, as well as from unchallenged controls. This suppressive effect of PGE2, which is unaffected by allergen challenge, is not surprising in itself, because nonselective inhibitory effects of PGE2 on hemopoiesis *in vitro* have long been known[36], and suppression of eosinopoiesis would seem to be just one particular example of this general phenomenon. On the other hand, if NSAID were working solely as COX inhibitors, they should be ineffective against exogenously added PGE2, which bypasses the COX pathway to act directly on PGE2 receptors. However, we showed that NSAID prevent the suppressive effects of PGE2 on eosinopoiesis, and further stimulate eosinophil production strongly (in this case, the eosinophils are cytologically mature)[70]. This rules out a mechanism involving only COX inhibition, which cannot protect against preformed COX products. Furthermore, blockade of the 5-LO pathway by genetical or pharmacological approaches abolishes the effectiveness of NSAID in promoting eosinopoiesis, which implies a 5-LO-mediated mechanism, quite distinct from simple COX inhibition. This alternative mechanism was shown to depend on CysLT, endogenously produced in bone-marrow culture, as evidenced by its absence in LTC4 synthase-deficient mice, and by its blockade by CysLT1R antagonists and CysLT1R deletion. In support of this view, exogenously added CysLT strongly stimulate eosinopoiesis, even in the absence of functional 5-LO or LTC4 synthase[70].

Importantly, this same CysLT-mediated mechanism was subsequently shown to underlie the stimulatory effects of eotaxin/CCL11 and IL-13, two cytokines central to allergic inflammation, on eosinopoiesis in naive bone-marrow culture[25]. Again, both depend strictly on functional 5-LO and CysLT1R to enhance eosinophil production, and both lose effectiveness when bone-marrow from sensitized-challenged mice is used.

It is clear, therefore, that extrinsic regulators of bone-marrow eosinopoiesis may be subject to immuno-regulation (NSAID, proallergic cytokines) or not (PGE2), depending on their mechanism of action.

Since IL-13 and eotaxin are produced during allergic episodes and present systemic effects[15,33,40], this suggests that CysLT in bone-marrow are proximal elements in a chain of events started by a distant allergic reaction, and therefore might play a role in the strong upregulation of eosinophil production following challenge. Consequently, one might predict that targeting CysLT production or signaling with drugs currently in use (respectively zileuton for production and montelukast and its analogues for signaling), would not only be beneficial in attenuating local allergic symptoms, but also in preventing increased eosinophil production. Such an effect of pranlukast has been previously reported in humans[71].

In ovalbumin-sensitized mice, we have observed the complete blockade of challenge-induced eosinophilia of the bone-marrow using both the leukotriene synthesis inhibitor diethylcarbamazine (DEC)[54] and 5-LO-activating protein inhibitor MK-886[47], which prevent production of CysLT; the same effect was observed with montelukast, which blocks CysLT1R[47]. Consistently with this hypothesis, DEC had no effect in mice lacking functional 5-LO. Together, this evidence supports an essential role for CysLT in challenge-induced eosinophilia, similar but distinct from that previously attributed to endogenous GC.

Further insight on the underlying cellular mechanisms is provided by the effects of DEC. Interestingly, DEC requires not only 5-LO to be effective[47] but inducible NO synthase (iNOS) as well[54]. This enzyme, which produces large amounts of NO in the course of cellular immune responses to a number of intracellular pathogens, had already been shown to be required for the suppressive effects of PGE2 on bone-marrow eosinopoiesis[72]; more recently, it was shown to account for similar effects of α-galactosylceramide (α-GalCer; an anticancer agent and immunomodulator)[23] and IL-17 (a powerful proinflammatory cytokine)[26] on bone-marrow. These observations, therefore, establish DEC as a pharmacological link between 5-LO and iNOS in bone-marrow, as discussed below. It should be noted that GC powerfully suppress iNOS expression[72], and this underlies their ability to block the suppressive effect of PGE2: *In vitro*, when iNOS expression is blocked by dexamethasone, this GC interacts with PGE2 to increase the production of mature eosinophils in culture[36], a somewhat unexpected interaction between an anti-inflammatory drug and a proinflammatory mediator.

It is therefore important that CysLT can counteract the effects of both IL-17[26] and α-GalCer[23] on eosinopoiesis, just as CysLT counteract those of PGE2[70]. Like GC,

therefore, CysLT target the iNOS-CD95-dependent proapoptotic mechanism that suppresses eosinopoiesis, as part of their eosinophilia-promoting actions.

Together, the available evidence suggests that challenge-induced eosinophilia in the bone-marrow is associated with both iNOS suppression (by GC) and 5-LO-mediated mechanisms; by contrast, its prevention is associated with iNOS-mediated mechanisms and blockade of 5-LO. It is therefore important to understand how these regulatory and effector elements (GC, 5-LO, iNOS) relate to each other.

If immunoregulation of responses to NSAID involved modulation of the COX pathway as opposed to the 5-LO pathway, responses to exogenously added CysLT in the bone-marrow would not depend on the immune status of the mouse. However, just like for NSAID, responses to LTD4 are strongly immunoregulated in murine bone-marrow (manuscript in preparation). This suggests that challenge not only requires CysLT to increase eosinophil production, it also profoundly attenuates the effectiveness of CysLT, thereby limiting the magnitude of responses to drugs and cytokines which are mediated by endogenous CysLT.

Because the effects of challenge on bone-marrow are counteracted with similar effectiveness by blockade of endogenous GC signaling, and by blockade of CysLT1R signaling, this raises the issue of the relationship of endogenous GC to CysLT in the context of sensitization and challenge.

While the similar actions of endogenous GC and CysLT might seem unrelated or even incompatible, recent studies point to a critical partnership of these mediators *in vivo*. This prompted us to address in the following section how these quite dissimilar classes of extrinsic regulators might work together to induce eosinophilia in murine bone-marrow, and to further limit the magnitude of this response to challenge through subtle regulatory mechanisms.

MAKING SENSE OF AN UNLIKELY PARTNERSHIP: CHALLENGE-INDUCED EOSINOPHILIA AS A SELF-LIMITING PROCESS STARTED BY GC AND AMPLIFIED BY CYSLT

The observations summarized above may appear paradoxical in several respects: (1) GC are usually thought of as antiallergic agents, not as promoting allergy; (2) GC are believed to suppress the generation of lipid mediators from arachidonate metabolism (eicosanoids) and should accordingly prevent the generation of CysLT; (3) even though GC are essential to the effects of challenge on the bone-marrow, dexamethasone alone cannot reproduce all of these effects; (4) GC (anti-allergic agents) and CysLT (proallergic agents) seem to elicit the same outcome - increased eosinophil production - and therefore constitute a highly unlikely couple; and (5) the effects of CysLT, furthermore, are attenuated after challenge, and may even become suppressive, in a clear inversion of the original signal provided by these mediators.

GC and CysLT form indeed an odd couple: GC are widely used as anti-inflammatory agents and for the long-term maintenance in asthma control; by contrast, CysLT account for some of the most visible manifestations of asthma and allergy, and CysLT antagonists are useful for asthma control. So GC and CysLT would be expected to be natural antagonists, not partners. However, the eosinopoiesis-enhancing effects of dexamethasone are observed at lower concentrations that its anti-inflammatory and anti-allergic effects[51], and are compatible, in terms of glucocorticoid activity, with the GC surges associated with acute stress[45,52]. So, GC promotion of eosinopoiesis by dexamethasone might be dose-dependent and self-limiting over time.

This priming effect of GC is reproduced by surgical trauma in the absence of allergen sensitization[52], and is therefore independent of underlying allergic processes. When the relevant GC, corticosterone, is released by challenge of sensitized mice, this release depends on TNF-α type 1 receptors, and is therefore part of the nonspecific host response to aggression, mediated by proinflammatory cytokines[45]. The effect of challenge seems to last about one week[54], although surgical trauma has a longer-lasting impact on bone-marrow[52], and dexamethasone may have a priming effect demonstrable in bone-marrow culture as long as one month after a single injection (manuscript in preparation). Our interpretation is that the duration of GC effects is significantly curtailed by factors operating *in vivo* during trauma or allergic challenge, and, in a sense, this makes the extrinsic regulation of bone-marrow eosinopoiesis by allergen challenge a self-limiting process.

On the other hand, the results of complete prevention of challenge-induced eosinophilia by a variety of interventions that target CysLT production or signaling (DEC, MK886, 5-LO deficiency; montelukast) can only be understood if one assumes that despite the elevation in endogenous GC levels induced by challenge[45], the 5-LO pathway is operative and CysLT are produced. Resistance of CysLT to GC even at therapeutic levels has been reported in human studies[67] and underlies the rationale for using antileukotrienes as complementary to GCin asthma control[62,66,67].

If both endogenous GC and CysLT are present *in vivo* after challenge, what is their relationship? They might be present simultaneously, but at different, unconnected sites and therefore act independently of each other; alternatively, they might be coupled. Prevention of challenge-induced eosinophilia by GC or by CysLT blockade to the same extent might seem to argue against either having an independent effect on bone-marrow, since one would logically expect an additive effect of blocking both targets, which is not observed. However, bone-marrow of naive mice[70], or from sensitized mice pretreated with RU486 before challenge (manuscript in preparation), does respond to CysLT in culture in the absence of exogenously

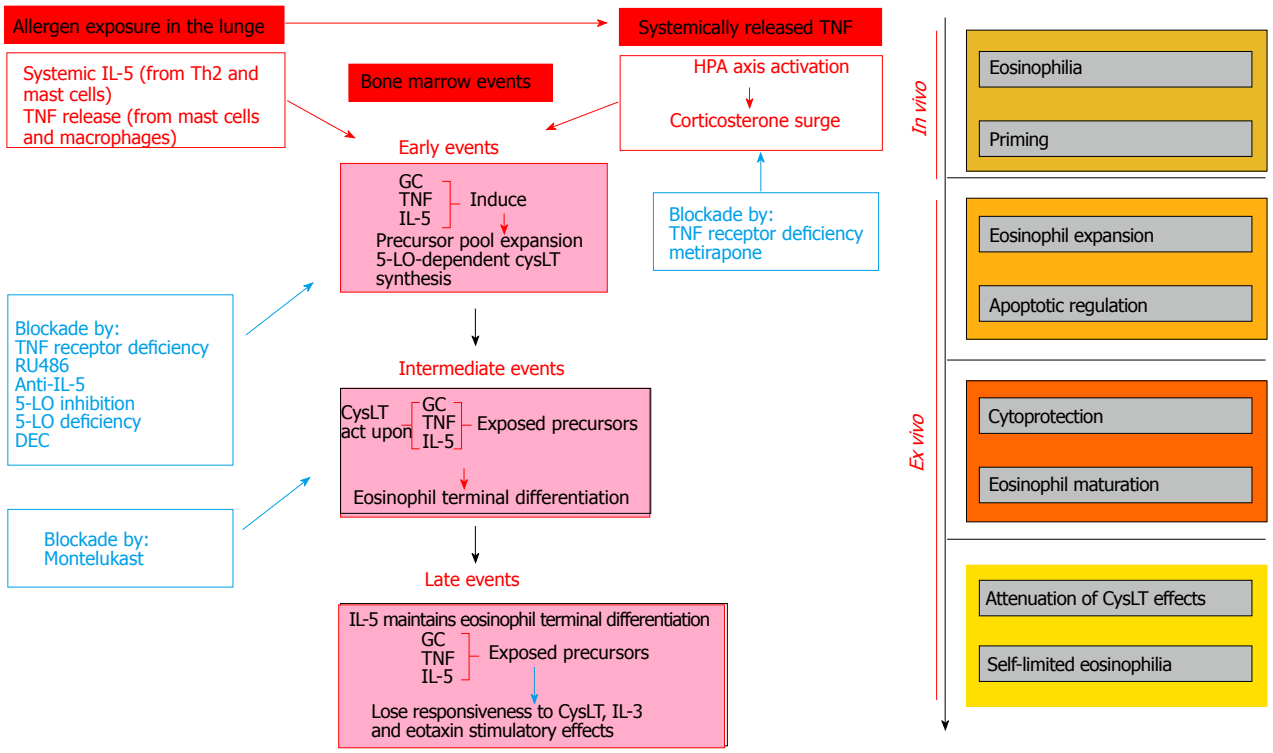

Figure 1 Events outside and inside bone-marrow following allergen challenge. The sequence of critical events in the lungs, endocrine system and bone-marrow is outlined on the left as a flow chart, and their impact on the establishment of bone-marrow eosinophilia is depicted on the right as a timeline. On the left, we outline the contributions of cytokines (IL-5, TNF-α), adrenal GC hormones, and CysLT at early, intermediate and late phases after challenge, as have been characterized by genetical, immunological and pharmacological tools in bone-marrow culture (*i.e.*, *ex vivo*; refs. provided in Table 1). Events promoting allergic inflammation are shown in pink boxes; interventions opposing allergic inflammation are shown in light blue boxes. Systemic events preceding the local bone-marrow response (left side, lungs; right side, endocrine system) are shown in red boxes. RU486 (mifepristone) is a blocker of GC receptor; metirapone is an inhibitor of adrenal GC biosynthesis. The combination of IL-5, TNF-α and adrenal GC is considered to be critical for the entire sequence of events in the bone-marrow, due to long-lasting effects of exposure during the initial 48 h of culture[51]. CysLT act downstream from GC[45,47] in the same sequence of events. Challenge promotes expansion of eosinophil precursors and their maturation in the presence of CysLT *in vivo*, but also attenuates responses to CysLT during subsequent exposure *ex vivo*, thereby limiting the magnitude of the resulting eosinophilia (represented in shades of orange at the right). TNF-α: Tumor necrosis factor-α; IL: Interleukin; CysLT: Cysteinyl-leukotrienes.

added GC. Also, bone-marrow from unsensitized C57BL/6 mice shows eosinophilia *in vivo* after dexamethasone administration[53], in the absence of any known CysLT inducer, just as dexamethasone enhances eosinopoiesis in culture without addition of CysLT. The effectiveness of both partners in the absence of the other is thus established, showing that they have independent pharmacological effects outside the framework of sensitization/challenge. Nevertheless, blocking either inside this framework achieves full prevention of the effects of challenge. This suggests that during challenge they become functionally coupled *in vivo*, which does not necessarily occur following dexamethasone administration. Coupling is here characterized by continuity in time (one event follows the other) and by dependence of the latter event on the former (blockade of the former event prevents the latter). However, it is not synonimous with causality in the usual sense: The first event might be just permissive for the second, not necessarily its immediate cause. Coupling is a term applicable to events that take place at separate moments in separate sites, just as it is to events that take place at separate moments in very close sites or even at exactly the same site, provided the second event is reproducibly prevented by blockade of the first. These distinct possibilities are illustrated by the two modes of cytokine action discussed in section 2.

Our hypothesis (coupling of GC and 5-LO in response to challenge) is consistent with the observed difference between the effects of challenge and of *in vivo* exposure to dexamethasone in the BALB/c strain. Challenge induces eosinophilia and primes for better responses to IL-5. Dexamethasone does not induce eosinophilia, but does good priming. Challenge effects are GC-dependent, with both eosinophilia and priming being abolished by RU486. Hence, even though GC are central to challenge, there is a hitherto unidentified factor present *in vivo* during challenge in addition to elevated GC, which modifies the ultimate effects of GC on bone-marrow, by coupling GC to CysLT. Below we develop the hypothesis that this unidentified factor is TNF-α, also produced in the course of challenge, and capable of inducing both GC[45] and CysLT[73,74].

Further insight can be provided by a comparison of dexamethasone and challenge: Dexamethasone-exposed eosinophils are cytologically immature and show no resemblance to circulating eosinophils[36,51]; challenge-induced eosinophils[22], as well as those induced by CysLT *in vitro*[70], are fully mature. In a sense, dexamethasone is an incomplete enhancer of eosinopoiesis, for it increases

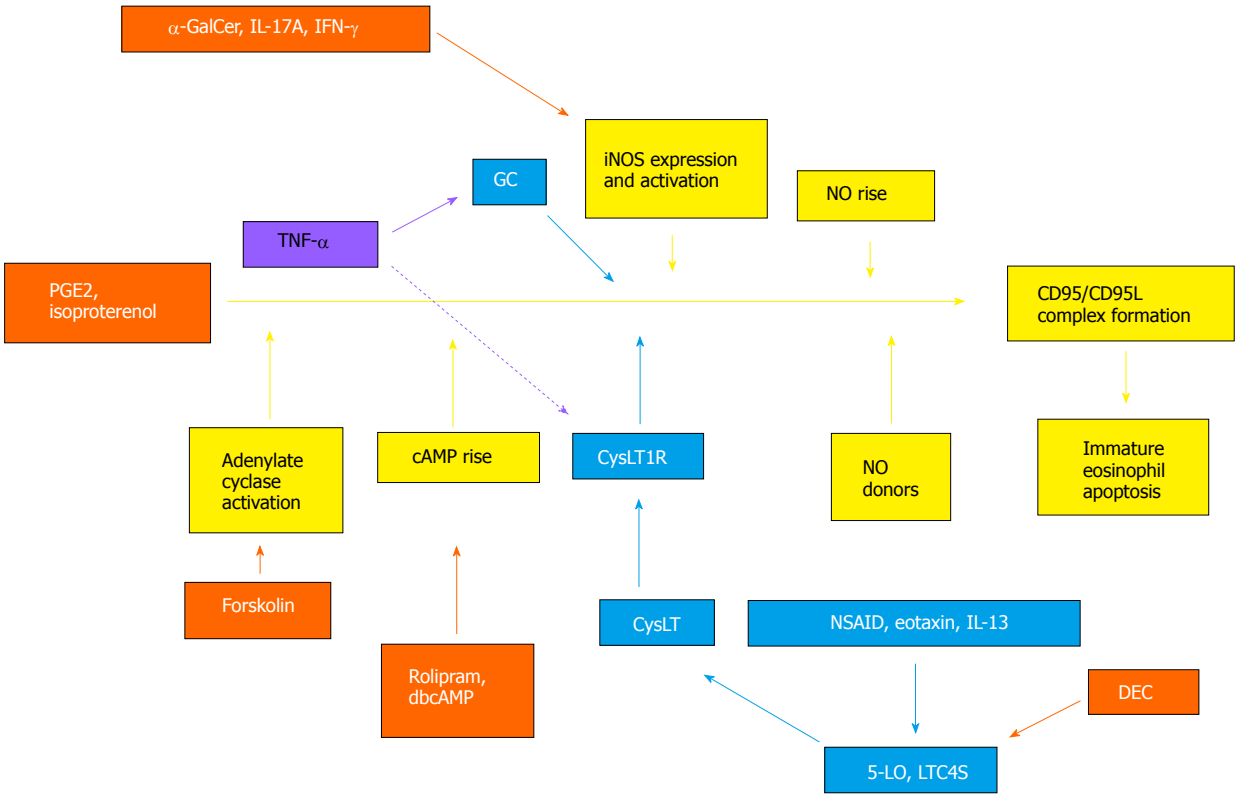

Figure 2 A graphical abstract of the main events identified in extrinsic regulation of bone-marrow eosinopoiesis, and of the hypothetical interactions of tumor necrosis factor-α with the underlying mechanisms. Colored boxes and arrows identify different classes of agents and their actions as follows: Orange, extrinsic suppressors of murine bone-marrow eosinopoiesis both *in vitro*[23,26,72,73] and *in vivo*[47,54]; light blue, extrinsic enhancers of eosinopoiesis *in vitro*[25,36,51,70] and *in vivo*[45,47,52,53]; yellow, essential components of a proapototic sequence (iNOS-CD95L-dependent pathway[54,72,73]) which is susceptible to activation by the first (orange-labeled) and blockade by the second (light blue-labeled) sets of extrinsic regulators; lavender, TNF-α, presenting both constitutive (continuous arrow) and challenge-induced (discontinous arrow) effects, inside the bone-marrow, besides its extramedullary actions[45] as activator of the HPA axis (not shown). TNF-α: Tumor necrosis factor-α.

the number of eosinophil-lineage cells but prevents their full maturation. The maturation sequence, which involves downregulation of α4 integrin in dexamethasone-exposed immature eosinophils, can be completed in the presence of PGE2[36] or IL-17[26] *in vitro*, just as it is completed in the presence of CysLT *in vivo*[47].

To reconcile all of these apparent paradoxes, we propose a model in flow chart format (Figure 1) which has the following essential tenets: (1) GC and CysLT become functionally coupled *in vivo* as a consequence of allergen challenge, so that TNF-α-dependent GC signaling makes it possible for CysLT generated locally in the context of allergic challenge to induce bone-marrow eosinophilia; (2) because the upstream permissive element (GC) and the downstream effector element (CysLT) are coupled, blocking the former will prevent the actions of the latter; (3) as a result of this coupling, full maturation of the eosinophils produced can be achieved *in vivo* after challenge; and (4) nevertheless, this mechanism is not operating unchecked *in vivo*, for its operation makes it less effective in subsequent rounds of allergen challenge, as shown by the attenuation of the proallergic effects of CysLT *ex vivo*, and by the observation that the positive effects of GC on the eosinophil lineage tend to less marked following chronic (repeated challenge) than acute (single challenge) exposures. Attenuation of CysLT effects *ex vivo* in murine bone-marrow would be consistent with observations that allergen challenge leads to a large increase in CysLT production in humans, and that a mechanism of desensitization to CysLT effects may limit the untoward effects of these potent bronchoconstrictors[75], although at present it is unclear whether and how this applies to hemopoietic effects.

In our model, we propose a role for TNF-α, not only in inducing GC production through HPA axis activation, but in coupling GC surge to CysLT production. The hypothesis of a complex relationship of TNF-α produced after challenge to actions of GC and CysLT is illustrated in Figure 2, which provides a graphical abstract of the essential biochemical events identified so far in the extrinsic regulation of bone-marrow eosinopoiesis by allergen challenge, drugs and cytokines. Coupling of GC to CysLT is portrayed, based on the available evidence, as a transient relationship between events taking place within a single cell target (eosinophil precursor inside the bone-marrow), since these events all impinge upon a signaling sequence that begins at surface receptors and ends at apoptotic cell death[72,73] which is limited to immature eosinophils and their immediate precursos[36]. In this continuous sequence of signaling events, GC and CysLT act as suppressors

of apoptosis (indicated in light blue colored boxes and arrows) by blocking distinct signaling steps upstream from iNOS; conversely, inhibitors of CysLT production or action, including DEC, promote apoptosis (indicated in orange-colored boxes and arrows, for DEC as well as a wide panel of pharmacological agents which act at CysLT-unrelated steps) by acting on targets upstream from iNOS[23,26,45,47,54,70,72,73]. In addition to its systemic effects on adrenal release of GC during allergen challenge, which are not shown in the picture, TNF-α is hypothesized to have separate effects on GC and CysLT-mediated responses: A constitutive effect permissive for GC action on eosinophil precursors (solid lavender line), and an adaptive (coupling) effect permissive for GC control of CysLT responses in the same cell target (discontinuous lavender line). TNF-α has been reported by others to induce CysLT production and the expression of critical enzymes in CysLT biosynthesis; in addition, LTD4 duplicates these effects, providing a mechanism for amplification of TNF-α actions[74]. It at present is unclear whether these observations from other groups apply to bone-marrow, and, if so, how TNF-α induction of CysLT might be dependent on GC signaling.

To test the validity of these models, some points are critical, foremost the definition of the site, timing and mechanism of coupling of GC to CysLT, and of the role played by TNF-α therein. To define the mechanisms of attenuation of CysLT-dependent response is equally essential, including the roles played by GC hormones themselves and by changes in CysLT receptor types, expression or intracellular signaling. These steps should help us put in proper perspective the paradoxical enhancement of eosinophil production by GC, which, despite being at odds with the prevailing views of the contributions of GC and eosinophils to immune responses, is likely to shed some light on the puzzle of stress-related mechanisms in allergic disease.

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ORIGINAL ARTICLE

Retrospective Study

Statin escape phenomenon: Fact or fiction?

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Abstract

AIM

To evaluate the presence of the so called "statin escape" phenomenon among hyperlipidemic subjects attending a lipid clinic.

METHODS

This was a retrospective analysis of 1240 hyperlipidemic individuals followed-up for ⩾ 3 years. We excluded those individuals meeting one of the following criteria: Use of statin therapy at baseline visit, discontinuation of statin treatment at most recent visit, change in statin treatment during follow-up and poor compliance to treatment. Statin escape phenomenon was defined as an increase in low-density lipoprotein cholesterol (LDL-C) levels at the most recent visit by > 10% compared with the value at 6 mo following initiation of statin treatment.

RESULTS

Of 181 eligible subjects, 31% exhibited the statin escape phenomenon. No major differences regarding baseline characteristics were found between statin escapers and non-statin escapers. Both escapes and non-escapes had similar baseline LDL-C levels [174 (152-189) and 177 (152-205) mg/dL, respectively]. In comparison with non-escapers, statin escapers demonstrated lower LDL-C levels at 6 mo after treatment initiation [88 (78-97) mg/dL *vs* 109 (91-129) mg/dL, $P < 0.05$], but higher levels at the most recent visit [103 (96-118) mg/dL *vs* 94 (79-114) mg/dL, $P < 0.05$].

CONCLUSION

These data confirm the existence of an escape phenomenon among statin-treated individuals. The clinical significance of this phenomenon remains uncertain.

Key words: Statin; Escape; Cholesterol

Core tip: This was a retrospective study aiming to evaluate the presence of the so called "statin escape" phenomenon among hyperlipidemic subjects attending a lipid clinic and elucidate any potential confounding factors. This study confirms the limited bibliography reporting on statin escape phenomenon and its quite high prevalence. However, due to the small number of eligible participants, we were not able to identify potential predictors for the statin-escape phenomenon or establish an association between statin escape and incidence of cardiovascular disease. In this context, further investigation on the underlying pathophysiology of this phenomenon and its potential clinical ramifications is required.

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INTRODUCTION

Statins remain the cornerstone therapy for primary and secondary cardiovascular prevention, mainly due to their ability to reduce low-density lipoprotein cholesterol (LDL-C)[1]. Nevertheless, a notable cardiovascular risk remains in statin-treated individuals, which has been attributed to other residual factors, such as hypertension, diet and adherence to therapy[2]. Recently, the so called "statin escape" phenomenon has been reported as an independent cardiovascular risk factor in patients with acute myocardial infarction on prolonged statin treatment[3]. This phenomenon was first described in small studies including patients with familial hypercholesterolemia[4,5] and afterwards in the Expanded Clinical Evaluation of Lovastatin (EXCEL) study[6]. The latter reported an increase in LDL-C levels after the first year of statin treatment, despite a marked decrease in those levels 1 mo after treatment initiation[6]. So far there have been few reports on this phenomenon and its underlying mechanisms remain obscure[5,7-9].

The aim of this study was to provide additional data on the possible statin escape phenomenon based on the experience of a lipid clinic and try to elucidate potential risk factors.

MATERIALS AND METHODS

This was a retrospective (from 1999 to 2013) observational study as previously described[10-12]. Briefly, dyslipidemic adults followed-up for ⩾ 3 years in the Outpatient Lipid Clinic of the University Hospital of Ioannina in Greece were included. A complete assessment of serum lipid profile along with cardiovascular risk factors and concomitant treatment was available. The study protocol was approved by the Institutional Ethics Committee.

Demographic characteristics as well as various clinical and laboratory data were recorded at the baseline visit, at 6 mo and the most recent visit. These included: (1) age, gender, and smoking status; (2) body mass index (BMI) and waist circumference; (3) fasting glucose levels and glycated hemoglobin (HbA1c); (4) blood pressure (BP); (5) estimated glomerular filtration rated (MDRD - eGFR); and (6) a complete fasting lipid profile, including total cholesterol (TCHOL), triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C), LDL-C and non-high density lipoprotein cholesterol (non-HDL-C). The methods of blood sample collection and biochemical assessments have been previously described[10].

The evaluation of adherence to medication was based on the Hellenic national e-prescription web database. Subjects were classified according to their compliance with treatment as good or poor compliers if they refill ⩾ or < 80% of their expected prescriptions over time, respectively. We excluded those individuals meeting one of the following criteria: Use of statin therapy at baseline visit, discontinuation of statin treatment at most recent visit, change in statin treatment during follow-up and poor compliance to treatment. Statin escape phenomenon was defined as an increase in subject LDL-C levels at the most recent visit by > 10% compared with the value at 6 mo following initiation of statin therapy[8].

Statistical analysis

Continuous variables were tested for normality by the Kolmogorov-Smimov test and logarithmic transformations were performed if necessary. Data are presented as mean ± standard deviation (SD) and median [interquartile range (IQR)] for normal and non-normal distributed data, respectively. χ^2 tests were performed for categorical values. The difference of variables between ⩾ 2 groups was assessed by analysis of variance (ANOVA) and *post hoc* least significant difference tests were used for the comparison of variables or ratios of interest between the groups. Paired sample *t* tests were performed to assess the change of variables within each study group. Analysis of covariance (ANCOVA) was performed to assess the difference of variables between 2 subject groups, after adjusting for their baseline values. Binary logistic regression was performed to elucidate potential predictors for statin escape phenomenon. Two tailed significance was defined as $P < 0.05$. Analyses were performed with the Statistical Package for Social Sciences (SPSS), v21.0 software (SPSS IBM Corporation, Armonk, New York, United States).

RESULTS

Of 1240 hyperlipidemic individuals, 181 were considered eligible for the present analysis (Figure 1). Study participant baseline characteristics are shown in Table 1. Of 181 eligible subjects, 56 (31%) exhibited the statin escape

Table 1 Baseline characteristics of study participants

Variable	Escape group	Non-escape group
n	56	125
Gender (male), %	43	52
Current smoking, %	9	14
Age, yr	56 (51-63)	57 (49-65)
Waist, cm	97 (90-101)	98 (90-105)
SBP, mmHg	134 (127-146)	140 (129-150)
DBP, mmHg	83 (79-95)	87 (80-92)
Follow-up, yr	4 (3-6)	4 (4-7)
Metabolic syndrome, %	39	40
Hypertension, %	59	57
Diabetes, %	11	9
Stroke, %	5	4
Coronary heart disease, %	7	1[a]
Abdominal aortic aneurysm, %	2	0
Carotid stenosis ⩾ 50%, %	0	2
Peripheral arterial disease, %	0	1
Statin therapy and median dose, % (median dose)		
Atorvastatin	38 (20 mg)	34 (20 mg)
Rosuvastatin	29 (10 mg)	24 (10 mg)
Simvastatin	21 (40 mg)	26 (40 mg)
Fluvastatin	7 (80 mg)	6 (80 mg)
Pravastatin	0	1 (40 mg)
β-blocker, %	9	7
Thiazides, %	11	19
Pioglitazone, %	4	1
Antipsychotics, %	0	1
Levothyroxine, %	4	5
Clopidogrel, %	2	2
Proton-pump inhibitors, %	4	4

Median follow-up duration = 4 years (IQR: 3-6 years). Values are expressed as median (IQR), unless percentages as shown. [a]$P < 0.05$ for the comparison with the escape group. DBP: Diastolic blood pressure; IQR: Interquartile range; SBP: Systolic blood pressure.

phenomenon and 125 (69%) did not. There were no differences between these 2 groups apart from the higher baseline prevalence of coronary heart disease noticed in the escape group (7% *vs* 1%, $P < 0.05$). As shown in Table 1, there was no difference between the 2 groups regarding statin treatment. No participant received any non-statin lipid-lowering therapy (*i.e.*, fibrate, ezetimibe). In addition, no difference was found regarding drugs interfering with cholesterol or statin metabolism (*i.e.*, β-blockers, thiazides, pioglitazone, atypical antipsychotics, levothyroxine, clopidogrel or proton-pump inhibitors; Table 1).

Baseline lipid and metabolic profile did not differ between the 2 study groups (Table 2). Six months after the initiation of statin treatment, LDL-C levels were lower in the escape compared with the non-escape group [88 (78-97) mg/dL *vs* 109 (91-129) mg/dL, $P < 0.01$; Figure 2]. On the contrary, LDL-C levels at the most recent visit were lower in the non-escape compared with the escape group [103 (96-118) mg/dL *vs* 94 (79-114) mg/dL, $P < 0.01$; Figure 2]. Similarly, non-HDL-C levels were lower six months after the initiation of statin therapy in the escape compared with the non-escape group among non-diabetic individuals [107 (97-121) mg/dL *vs* 132 (115-153) mg/dL, $P < 0.01$; Table 2]. On the other hand, higher non-HDL-C levels were noticed in the former group at the most recent visit (Table 2). TRG significantly declined by 11% and 18% in the escape and non-escape group during follow-up, respectively ($P < 0.01$ respectively for the change within each group; Table 2). Despite the fact, that the non-escape group exhibited higher TRG levels than the escape group 6 mo after the initiation of statin therapy [104 (83-140) mg/dL *vs* 97 (69-117) mg/dL, $P < 0.05$], there was no difference between 2 groups regarding TRG levels at the most recent visit and the change of TRG levels during follow-up (P = NS for the comparison between 2 groups). On the other hand, HDL-C levels did not change during follow-up and were not different between 2 groups (Table 2).

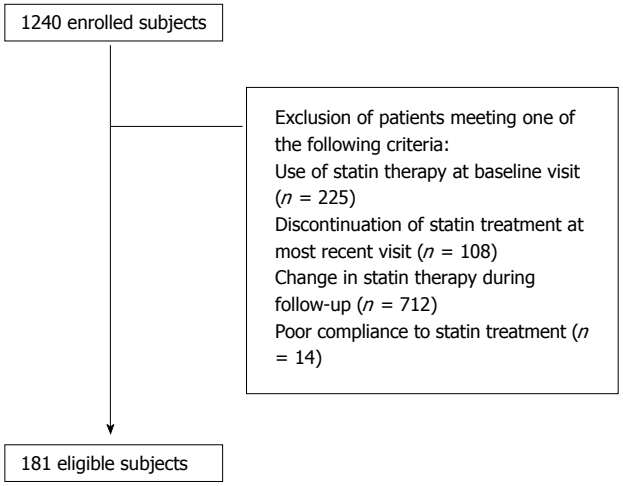

Figure 1 Flow chart of subject eligibility.

There was no significant difference between the 2 groups regarding BMI change. As also shown in Table 2, glucose levels did not change during follow-up and were not different between the 2 groups. eGFR declined by 0.5 and 4.1 mL/min per 1.73 m^2 in the escape and non-escape group, respectively ($P < 0.05$ respectively for the change within each group), but the difference between the 2 groups was not significant. The same was true for the change in diabetics' HbA1c levels (Table 2, P = NS for the comparison between the 2 groups).

Binary logistic regression assessing baseline characteristics along with the changes in BMI, eGFR or HbA1c levels during follow-up did not reveal any significant predictor for the statin escape phenomenon.

During a median follow-up of 4 years, 1 of 56 escape individuals and 6 of 125 non-escape subjects were diagnosed with incident cardiovascular disease (P = NS for the comparison between the 2 groups).

DISCUSSION

The present report confirms the existence of statin escape phenomenon in clinical practice.

Two small studies including patients with familial hypercholesterolemia were the first to notice a paradox rebound cholesterol increase following statin dose

Table 2 Lipid and metabolic profile of study participants

	Baseline visit	Visit at 6 mo	Most recent visit
TCHOL, mg/dL			
Escape group	258 (233-283)	162 (147-174)	182 (170-201)
Non-escape group	259 (235-295)	184 (162-206)[a]	172 (154-193)[a]
TG, mg/dL			
Escape group	117 (89-175)	97 (69-117)	104 (87-129)
Non-escape group	132 (99-181)	104 (83-140)[a]	108 (79-130)
HDL-C, mg/dL			
Escape group	53 (47-68)	55 (43-64)	54 (48-68)
Non-escape group	53 (46-65)	52 (44-60)	56 (46-62)
LDL-C, mg/dL			
Escape group	174 (152-189)	88 (78-97)	103 (96-118)
Non-escape group	177 (152-205)	109 (91-129)[a]	94 (79-114)[a]
Non-HDL, mg/dL[1]			
Escape group	204 (181-223)	107 (97-121)	127 (116-143)
Non-escape group	209 (182-241)	132 (115-153)[a]	118 (102-137)[a]
BMI, kg/m^2			
Escape group	27.3 (23.5-29.9)	27.2 (23.5-30.1)	27.6 (24-30.2)
Non-escape group	27.9 (25.5-30.6)	28.3 (25.1-30.9)	28.4 (25.5-31.5)
Fasting glucose, mg/dL			
Escape group	95 (88-105)	95 (87-129)	95 (88-106)
Non-escape group	93 (87-103)	94 (88-104)	96 (89-106)
HbA1c, %[2]			
Escape group	8.5 (6.7-8.6)	6.6 (5.6-5.9)	6.7 (6.6-7.1)
Non-escape group	8.4 (7.7-10.9)	6.7 (6.3-7.9)	6.9 (6.3-7.6)
MDRD-eGFR, mL/min per 1.73 m^2			
Escape group	77 (69.6-86.7)	76.6 (67.9-84.8)	76.5 (65.4-81)
Non-escape group	81 (70.7-91.4)	79.7 (69-89.7)	76.9 (65.5-85.7)

Values are expressed as median (IQR). To convert from mg/dL to mmol/L multiply by 0.0555 for glucose, 0.02586 for TC, HDL-C, LDL-C, and 0.01129 for TG. [1]Non-HDL-C levels refer to non-diabetic individuals (n = 164); [2]HbA1c values refer to diabetic individuals (n = 17). [a]P < 0.05 for the comparison with the escape group. BMI: Body mass index; MDRD-eGFR: Estimated glomerular filtration rate according to The Modification of Diet in Renal Disease (MDRD) Study equation; HbA1c: Glycated hemoglobin; HDL-C: High-density lipoprotein cholesterol; IQR: Interquartile range; LDL-C: Low-density lipoprotein cholesterol; non-HDL-C: Non-high-density lipoprotein cholesterol; TCHOL: Total cholesterol; TG: Triglycerides.

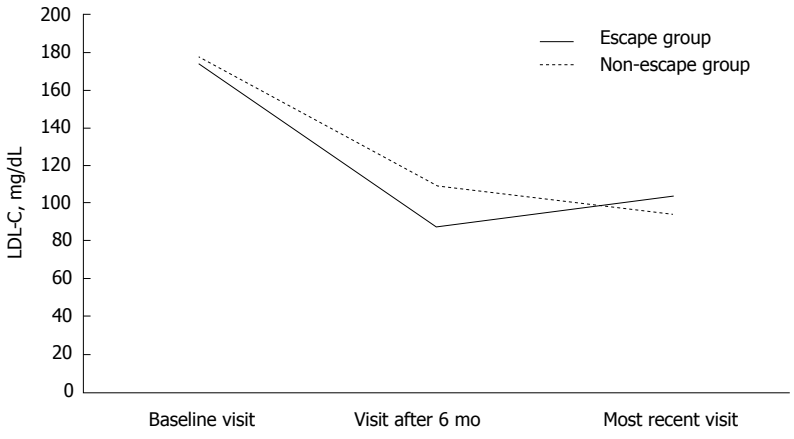

Figure 2 Change in low-density lipoprotein cholesterol levels during follow-up. [a]P < 0.05 for the comparison between the 2 groups. LDL-C: Low-density lipoprotein cholesterol.

increase[4,5]. Since then, the EXCEL study along with others, has described this so called statin escape phenomenon[3,5-7]. Our results showing an initial marked LDL-C reduction but followed by a > 10% LDL-C increase after prolonged statin treatment in subjects exhibiting the statin escape phenomenon are in line with the results of these studies[3,5,7]. Similar to previous studies, we did not find any predictors for this phenomenon[3,5,7]. A recent study showed that statin escape phenomenon not only exists, but also might be an independent predictor of cardiovascular disease[3]. The mechanisms attributing to the statin escape phenomenon have not yet been elucidated. The failure of statin therapy to decrease LDL-C levels on a long-term basis may be attributed to poor compliance with lipid-lowering treatment and diet. Particularly, an increased intake of cholesterol in the diet may contribute to intermittent variations in cholesterol levels. In addition, weight changes or a poor glycemic

control in diabetic individuals could also cause a LDL-C increase, which could be wrongfully considered as statin escape phenomenon. After excluding subjects with these characteristics, one study concluded that only 1.2% of 161 study participants exhibited the statin escape phenomenon, although 28% of those were initially considered to meet the criteria of statin escape[7]. Despite the fact that no data regarding diet and exercise was available in our study, there was no significant difference between groups in terms of BMI change, glycemic control and kidney function.

We also assessed non-HDL-C levels in non-diabetic individuals considering that atherogenic dyslipidemia may alter LDL-C changes[10]. Statin escapers had higher non-HDL-C levels after prolonged statin therapy in comparison with non-escapers, although they had a higher non-HDL-C reduction 6 mo after treatment onset.

Although we checked for adherence to therapy, our study might have included non-compliant individuals. It may be possible that the escapers adhered less to statin therapy and diet after seeing a large drop in their LDL-C levels. Another possible explanation for the statin escape phenomenon could be the concomitant therapy, since a variety of drugs could increase LDL-C lowering action of statins by inducting cytochromes CYP450-3A4 and 2C9[13,14]. According to a few experimental studies, statin escape phenomenon could be attributed to a slow increase in the 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity or to an increase in proprotein convertase subtilisin kexin-like 9 (PCSK9) levels caused by prolonged statin therapy[9,15-19].

Our data suggest that statin escape phenomenon is indeed noticed in clinical practice, although its clinical significance remains uncertain. Patients with larger than anticipated initial LDL-C lowering should be carefully monitored.

COMMENTS

Background

A few studies have reported on the so called "statin escape phenomenon", which describes an increase in low-density lipoprotein cholesterol (LDL-C) levels after prolonged statin therapy despite an initial marked decrease. Statin escape phenomenon has been recently reported as an independent cardiovascular risk factor.

Research frontiers

Very few studies have reported on statin escape phenomenon and its underlying mechanisms remain obscure. The present study contributes to clarifying whether this phenomenon exists in clinical practice.

Innovations and breakthroughs

This was a retrospective observational study with a small sample size. However, only the EXCEL study, which is the only randomized trial reporting on statin escape phenomenon and a retrospective cohort had larger samples. The small number of eligible participants did not allow any analysis to identify potential predictors for the statin-escape phenomenon. Additionally, due to small sample and low incidence of cardiovascular disease this study did not have the power to stablish an association between statin escape and incidence of cardiovascular disease. Nevertheless, this study confirms the limited bibliography reporting on statin escape phenomenon and its quite high prevalence (28%-31%).

Applications

This study suggests that further investigation on the underlying pathophysiology of the statin escape phenomenon and its potential clinical ramifications is required.

Peer-review

This study is well written and the patients have been well selected although several variables could have influenced the results.

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ORIGINAL ARTICLE

Observational Study

Discernment scheme for paraquat poisoning: A five-year experience in Shiraz, Iran

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Abstract

AIM

To evaluate various schemes for paraquat poisoning and different variables that influence the outcome of acute paraquat poisoning.

METHODS

In a cross-sectional study, the information about all cases of acute paraquat poisoning who were admitted to teaching hospitals affiliated to Shiraz University of Medical Sciences, in a five year period (September 2010 to September 2015) were evaluated. The variables included: Demographic data, medical assessment, therapeutic options, laboratory findings, and the outcomes. Data were

analyzed using SPSS, version 22. Significant difference between groups was tested using t-test for continues outcomes and χ^2 test for categorical. The significance level was considered to be $P < 0.05$.

RESULTS

A total of 104 patients (66.3% male) were evaluated. The mean age of the female patients was 22.81 ± 9.87 years and the male patients' was 27.21 ± 11.06 years. Ninety seven (93.3%) of all the cases were suicide attempts with mortality rate of 43.2%. Despite the necessity for emergency hemodialysis during the first 6 h of intoxication, none of the patients had dialysis during this time. Immunosuppressive and corticosteroid medications were not administrated in adequate dosage in 31.1% and 60% of the patients, respectively. Ingestion of more than 22.5 cc of paraquat and increase in creatinine level were the most important predictors of mortality.

CONCLUSION

Treatment should start immediately for these patients. Moreover, creating a clinical guideline according to the findings can have an impact on the treatment procedure which seems to be necessary.

Key words: Mortality; Paraquat; Poisoning; Prognosis; Suicide

Core tip: In developing countries with an agriculture economy poisoning by means of herbicides is very common. Paraquat is a highly toxic compound and consumption of 30 mg/kg is lethal in humans. In this study, we have analyzed multi-center data of patients with paraquat poisoning between September 2010 and September 2015, establishing the largest series of paraquat poisoning in the Middle East. Based on the data, medical knowhow that affects its current management as well as different variables which influence the outcome were evaluated.

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INTRODUCTION

Due to widespread usage of herbicides in agricultural industry reports of human poisoning has been on the rise around the world[1-3]. Paraquat (1,1′-dimethyl-4,4′ bipyridinium dichloride) is a well-known compound used in agriculture and it is a suitable due to its wide range of effects on weeds and instability in the environment[4,5].

Consumption of 30 mg/kg (equal to around 3-6 g of paraquat ion) is lethal in adults[6-8]. In the case of oral intake, it is quickly absorbed through the luminal tract, and 95% of its tissue distribution occurs within the first 6 h. Kidneys play a vital role in disposing paraquat from the body, and its maximum disposal is carried out during the first 12 to 24 h[6]. Symptoms include: Burning sensation in the mouth, throat, chest, epigaster, nausea, vomiting, abdominal pain, and diarrhea, which can be stopped after 2-3 d, if the patient is still alive[9]. Additionally if a patient has consumed more than 20 mg/kg of paraquat ion, his/her survival rate is very low[10]. I think (equal to 10 cc of 20% solution) is not a proper statement, because 20 mg/kg is more explanatory itself. The main mechanism in being poisoned with paraquat is the formation of superoxide ions, active oxygen radicals, NADPH oxidation, lipid peroxidation of cell membrane, and destruction of the cell membrane structure[4,11]. Despite progressions in critical care domain, to this point there has not been any effective treatment for paraquat poisoning. Some studies have indicated some improvement in prognosis of patients, ensuing the prescription of absorbents, treatment by immunosuppressive medications, radiotherapy and hemodialysis[12-14]. The main objective in this research was to study the clinical symptoms, laboratory abnormalities and the outcome of paraquat poisoning in a 5-year period in Fars province, Iran.

MATERIALS AND METHODS

Study population and data collection

In this retrospective descriptive analytical study, a total of 104 records of paraquat poisoning patients in three main tertiary hospitals in Shiraz, Iran, from September 2010 to September 2015 were evaluated. This research was conducted following the approval of Shiraz University of Sciences Ethics Committee. The required data were manually obtained from the patients' records. The data included; age, gender, consumed paraquat amount, occurrence of vomiting after consumption, the gap between poison consumption and treatment initiation, the treatments modalities, hospitalization duration, laboratory abnormalities, and sequela. Patients who had not signed the written informed consent for using their records, those who had taken other medications or poisons simultaneously, and those who had a history of cardiac, pulmonary, renal or hepatic diseases were excluded from the study.

Statistical analysis

The retrieved data were analyzed using SPSS, version 22. The continuous variables were described by standard deviation ± mean and the categorical were reported in the form of frequencies. Significant difference between groups was tested using *t*-test for continues outcomes and χ^2 test for categorical. In all cases, the significance level was considered to be $P < 0.05$. In order to assess the performance of laboratory changes in predicting the death occurrence, the area under receiver operating characteristic (ROC) curve and sensitivity, specificity, positive and negative predictive value were studied. The

Table 1 Baseline demographics of the subjects, 2010-2015

Variable	Total = 104
Gender	
Male (%)	69 (66.3)
Female (%)	35 (33.7)
Duration of hospitalization	
1-6 d (%)	61 (58.7)
7-13 d (%)	29 (27.9)
More than 14 d (%)	14 (13.5)
Mean (d)	6.73 ± 5.73
Time interval (d)	1-27
Cause of poisoning	
Occupational exposure (%)	4 (3.8)
Suicidal (%)	97 (93.3)
Accidental (%)	3 (2.9)
Habitat	
Rural (%)	80 (76.9)
Urban (%)	24 (23.1)
Type of poisoning	
Ingestion (%)	101 (97.1)
Injection (%)	3 (2.9)
Outcome	
Recovery (%)	59 (56.7)
Death due to complications (%)	45 (43.3)

confidence interval was 95%.

RESULTS

Overall characteristics of patients

In this research, a total of 104 patients poisoned by consuming paraquat were studied in a period of five years. The demographic data of the patients are presented in Table 1.

The duration of hospital stay for the patients was 6.73 ± 5.73 d (between 1 and 27 d) on average. Poisoning with paraquat in males was 1.9 times higher than females. The highest rate of poisoning prevalence was among females under 20 and males between the ages of 20 and 30. The mean age of the female patients was 22.81 ± 9.87 (between 1 and 61 years) and the male patients' was 27.21 ± 11.06 (between 15 and 60 years) (P = 0.045).

Clinical manifestations at presentation

Majority of the patients (76 cases; 73.1%) had vomited before being admitted to the hospital and the most common symptom during admission was nausea (74 cases; 71.1%). However, prevalence of epigasteric pain and inflammation of the oral mucosa was (29 cases; 27.9%) and (28 cases; 26.9%). No dysrhythmia was observed on the electrocardiogram at the time of presentation or during hospitalization, excluding agonal arrhythmia in dying patients.

Emergency management of poisoned patients

The most common decontamination method carried out for the patients was gastric lavage in 94 cases (90.4%). Charcoal alone or along with Fuller's earth was prescribed for gastric decontamination in 60 (57.7%) and 17 cases (16.3%), respectively. Gastric lavage was carried out in all cases that Fuller's earth or charcoal was prescribed. Only in 17 cases, gastric lavage was the sole method carried out.

Medical knowhow and inappropriate treatments

In 91 cases (87.5%), treatment was carried out by corticosteroids and in 39 (37.5%) by cyclophosphamide. In 50 cases (48.1%) N-acetyl cysteine (NAC), in 34 (32.7%) vitamin E, and in 32 (30.8%) vitamin C were prescribed as antioxidant medications.

In none of the 45 deceased patients, treatments were carried out completely. The most common type of managements were prescribing corticosteroid medications in 43 cases (95.6%), gastric lavage in 42 (93.3%), charcoal in 35 (77.78%), and NAC administration in 22 (48.9%) patients. Lack of attention in prescribing cyclophosphamide, dexamethasone and vitamin E as the most commonly ignored treatments in deceased patients, had occurred in 28 cases (62.2%), and lack of attention in prescribing NAC had occurred in 23 cases (51.1%). Methylprednisolone was not prescribed in 9 cases (20%), and in 27 cases (60% of the deceased patients), it was prescribed insufficiently.

Since, hemoperfusion was not available in any of the tertiary hospitals in Shiraz, Iran therefore; hemodialysis was carried out for extracorporeal removal of paraquat. Only 3 cases had expired due to the severity of poisoning during the early hours of admission and before performing hemodialysis. Nonetheless, initiating hemodialysis was delayed in all cases, at least for 6 h, mainly due to the delay in receiving the results of viral marker status. About 54% of the patients were hemodialyzed again due to increase in renal biomarkers after the first day.

Chest radiographic findings

Lung radiography was done for 45 cases. Table 2 presents the frequency of the positive lung radiography findings in the studied patients.

Correlation between the amount of consumed poison and prognosis

The amount of consumed poison in patients was 34.61 ± 55.36 mL (between 1.5 and 300 mL) on average. While the deceased patients had consumed 66.63 ± 72.61 mL of poison on average, this amount was 10.18 ± 5.77 mL on average for the patients who survived (P = 0.001); that is, 85.5% of the patients who had consumed less than 10 cc and 12.7% of the patients who had consumed between 10 and 20 cc of poison were discharged after recovery. On the other hand, 91.1% of the patients who had consumed more than 20 cc of poison ultimately expired (P = 0.000).

Figure 1, illustrates the ROC curve related to poison consumption and mortality rate in patients. Table 3 shows the cutoff point for the amount of poison consumed and patients' mortality, by considering the minimum positive predictive value of 90%. Based on poison consumption

Table 2 Chest radiographic findings of the 45 survivors and non-survivors among paraquat poisoned patients

Radiographic findings	Time interval of radiographic study (d)	No. of patients (percent in the survivors/non-survivors groups)
Non-survivors (n = 18)		
Pneumothorax (%)	2-5	2 (11.1)
Pneumomediastinum/emphysema (%)	1-2	2 (11.1)
ARDS (%)	1-10	4 (22.2)
Lung fibrosis (%)	4-14	10 (55.6)
Survivors (n = 27)		
Pneumothorax (%)	1-5	2 (7.4)
Lung fibrosis (%)	4-32	4 (14.8)
Normal (%)	1-8	21 (77.8)

ARDS: Acute respiratory distress syndrome.

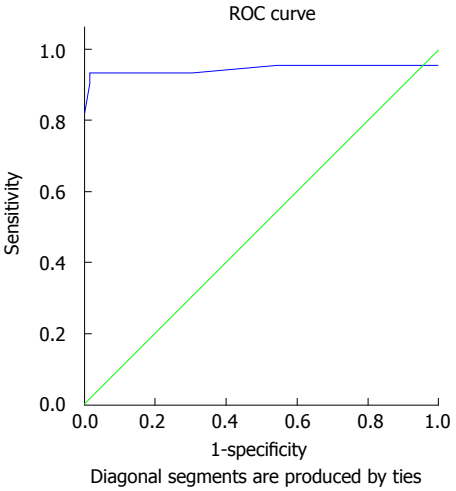

Figure 1 Receiver operating characteristic curve related to the position amount consumption in relation with patients' death. Based on the area under the curve, the confidence level was determined to be 95% for the poison consumption of 0.945 [between 0.87-1.00 (P = 0.000)]. ROC: Receiver operating characteristic.

rate and ROC curve, the best cutoff point was calculated at 22.5 cc or higher (considering the minimum positive predictive value of 90%).

On average patients' were deceased after 4.8 ± 4.62 d of hospitalization, (between the first and 21st days). Total of 9 cases expired during the first day of hospitalization who had consumed about 35 and 300 cc of poison.

Correlation between laboratory abnormalities and prognosis

This study indicates that maximum average levels of serum creatinine was 2.50 ± 1.80 (between 0.6 and 9.5), maximum average of blood urea nitrogen 16.42 ± 29.18 (between 6 and 66), maximum average of AST levels 114.52 ± 246.85 (between 8 and 1509), and maximum average of ALT 301.43 ± 145.31 (between 8 and 1803) were observed after the third day of admission.

Figure 2, illustrates the ROC curve related to Levels of serum creatinine, blood urea nitrogen (BUN), aspartate aminotransferase (AST), and alanine transaminase (ALT) after the third day of admission and mortality rate in patients. Table 4 shows the cutoff point of serum creatinine level, BUN, AST, and ALT on the third day in relation with patients' mortality by considering the minimum positive predictive value of 90%. The best cutoff point (considering the minimum positive predictive value of 90%) was calculated based on serum creatinine level, BUN, AST, and ALT on the third day and ROC curve. This cutoff point was calculated to be 1.95 or higher for creatinine level, 25 or higher for BUN level, 24.5 or higher for AST level, and 12 or higher for ALT level on the third day.

DISCUSSION

Being poisoned with herbicides in developing countries of South, East and Southeast Asia with an agriculture economy is very common[15]. In this study, a total of 104 paraquat poisoning cases in Fars province, which is one of the agriculture hubs in Iran, were studied in a 5-year interval. More than 65% of all the cases were male. The gender ratio of the paraquat poisoning in other studies was reported to be between 55% and 70% in males[5,6,10]. However, the mean age of female patients which was around 23 years, was 4 years lower than the male patients; this was in accordance with Kim's study[16]. The highest prevalence was observed in teenage girls and males between the ages of 20 and 30, which are considered as the active population. Around 77% of the patients were from rural areas. Other studies, also showed that poisoning was more common among the rural population, from 56% to 73%[6,13]. Around 93% of the cases were suicide attempts, which is in accordance with the results from other studies[5,6,13].

The most common clinical symptoms in patients included nausea, epigasteric pain and inflammation of the oral mucosa, which were in line with results from Cherukuri *et al*[9]. However, Sandhu *et al*[12] had reported that all patients diagnosed with paraquat poisoning experienced nausea and vomiting, but oral mucosal ulcers were reported in only 59% of the patients.

Gastric lavage was carried out as the most common type of emergency procedure in about 90% of all the cases, prescribing charcoal in about 58% and prescribing

Table 3 The cutoff point for the amount of poison consumption and patients' death

Variable	Area under the ROC (the minimum positive predictive value of 90%)	Cutoff point	Positive predictive value	Negative predictive value	Sensitivity	Specificity
Amount of consumed poison	0.945 (0.87-1.0)	22.5	93.3	98.3	93.3	98.3

ROC: Receiver operating characteristic.

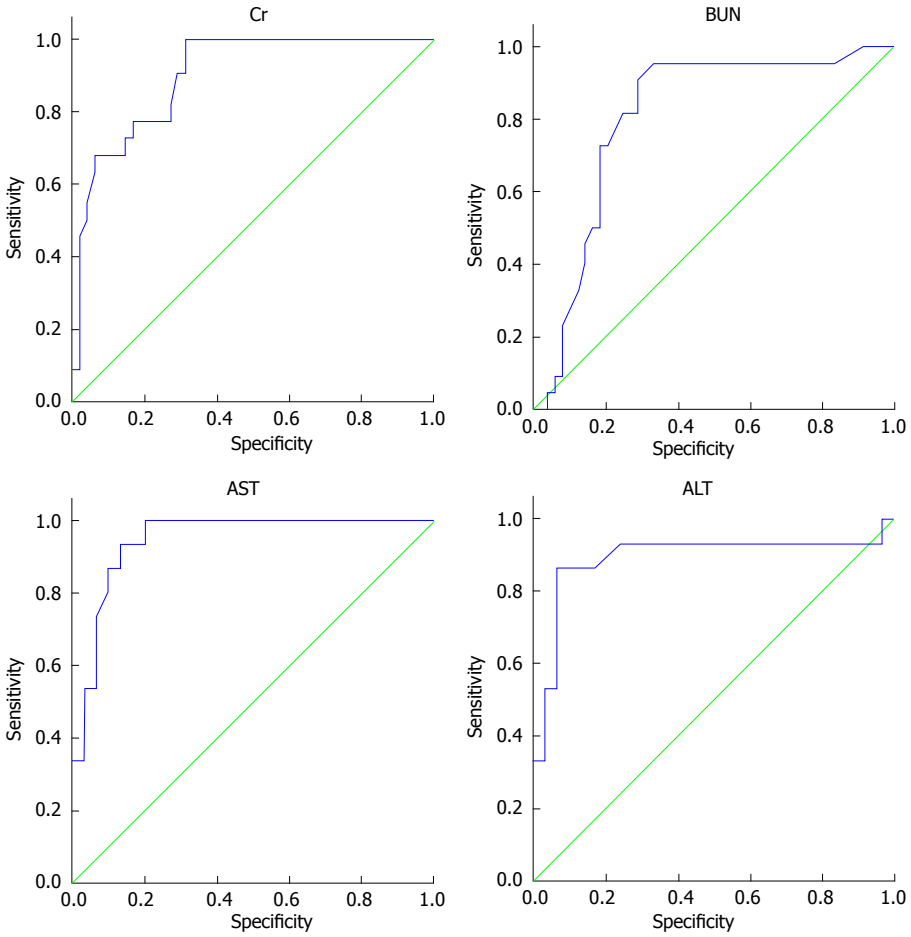

Figure 2 Receiver operating characteristic curve related to levels of serum creatinine, blood urea nitrogen, aspartate aminotransferase and alanine transaminase on the third day of hospitalization in relation with patients' death. Based on the levels under the curve, confidence interval of 95% was determined for creatinine on the third day 0.90 [between 0.83-0.97 (P = 0.000)], for BUN on the third day 0.80 [between 0.69-0.91 (P = 0.000)], for AST on the third day 0.85 [between 0.58-1.00 (P = 0.008)], and for ALT on the third day 0.79 [between 0.57-1.00 (P = 0.028)]. BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine transaminase.

charcoal along with Fuller's earth was carried out in 16% of the all the patients in the present study. In Senarathna's study[17], it was shown that Fuller's earth was prescribed in about 75%, and charcoal prescription was carried out in about 22% of the cases; however, in 16% of their patients, both were prescribed. Nevertheless, it is worth mentioning that Fuller's earth (magnesium citrate) and charcoal have similar effects[18], and there is no need for their simultaneous prescription. Although some of the old studies had proposed the necessity for gastric lavage in paraquat poisoning[19,20], Wilks *et al*[21] showed that gastric lavage can lead to increase in mortality rate in cases where the patient has consumed lesser than 30 cc of poison. It seems that gastric cleansing was inappropriate in the studied patients.

Research has suggested that paraquat can reach plasma concentration peak in one hour due to rapid absorption[22,23], and subsequently accumulate in targeted tissues. However, there is a chance of re-distribution from tissues to plasma, as well. Paraquat distribution half-life is around five hours in human, and around 6 h after its consumption, it reaches the maximum of tissue concentration in the lungs[24,25]. Considering the pathology of free radicals in paraquat poisoning, some older studies have proposed the use of antioxidant medications such as vitamin E and vitamin C in order to reduce tissue injuries. However, the impact of such treatments has not been proven[26,27]. Also, NAC, as a proper source of

Table 4 Cutoff point for the levels of serum creatinine, blood urea nitrogen, aspartate aminotransferase and alanine transaminase on the third day in relation with patients' death considering the minimum positive predictive value of 90%

Laboratory variables in the third day of admission	Area under the ROC (the minimum positive predictive value of 90%)	Cutoff point	Positive predictive value	Negative predictive value	Sensitivity	Specificity
Serum creatinine	0.90 (0.83-0.97)	1.95	90.9	70.8	90.9	70.8
BUN	0.80 (0.69-0.91)	25	95.4	66.7	95.5	66.7
AST	0.85 (0.58-1.000)	24.5	100	66.78	100	72.7
ALT	0.79 (0.57-1.000)	12	93.3	10.34	88.9	9.1

BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine transaminase; ROC: Receiver operating characteristic.

sulphydryl groups, could play a great role in scavenging free radicals[28]. In this research, 33% of the patients were treated with vitamin E, while vitamin C was prescribed for about 30% and NAC for about 48% of the patients. It seems that other researchers in various studies did not choose similar antioxidant medications; Cherukuri *et al*[9] in their study showed that treatment with vitamin E was done in about 18%, while vitamin C was prescribed in 25% and NAC in 50% of their patients. About 15% of the patients in Delirrad's[6] study were treated with NAC. However, in Sabzghabaee[10] and Sandhu *et al*[12]'s studies, all patients were treated with antioxidant medications. In their study on 9 cases, Yasaka *et al*[30] showed that mortality rate in patients who were treated with vitamin E reached 78%, however, in Hong *et al*[31], study on 5 cases they showed that all treated patients survived. Some previous studies have proposed the impact of treatment by pulse corticosteroids and cyclophosphamide in preventing pulmonary fibrosis[31,32]. In this study, 87% of the patients were treated with corticosteroids and 37% with cyclophosphamide. Other researches have shown to use similar antioxidant medications, but they did not use similar immunosuppressive and corticosteroid medications. As Cherukuri *et al*[9] showed in their study, treating with pulse methylprednisolone was carried out in 38% of patients, while treating with cyclophosphamide was carried out in 22% of the cases. In Delirrad's[6] study, 54% were treated with corticosteroids, and 22% of the cases were treated with cyclophosphamide. On the other hand, all patients were treated with corticosteroids in Sabzghabaee[10] and Banday's studies[4].

Our study suggests that consuming more than 22.5 cc of 20% paraquat can lead to poor prognosis in the patients, and this is in accordance with the results of Hosseinian Amiri *et al*[5] and Delirrad *et al*[6], studies. In addition, Buckley *et al*[33] showed that consuming around 10 to 20 cc of this poison could lead to fatal complications.

Thus, results from our study indicates that on average, patients' mortality occurred on the fifth day of hospitalization, which is in accordance with Afzali's[34] study. Also, in 9 cases who had consumed around 35 to 300 cc of the poison, death occurred on the first day of admission. In fact, consuming higher doses of poison can lead to death during the first few hours through acute multi organ failure[1].

Even though hemoperfusion has been introduced as an effective treatment in washing off the poison from plasma in the first 6 h[35], but for the patients in this study hemodialysis was performed, due to lack of hemoperfusion facilities. However, this measure was not performed for any of the patients during the first 6 h of admission. It seems that the main reason for this delay was that they had to wait for receiving the results for viral markers for hemodialysis. Nevertheless, based on Marashi *et al*[36], considering the high mortality rate due to poisoning and the low probability of viral infections, there is no need to study the viral markers, and in such cases hemodialysis should be carried out by the device allocated for patients diagnosed with hepatitis B.

Pulmonary fibrosis is among the known complications in paraquat poisoning which occurs approximately 7 to 14 d after poisoning along with acute respiratory failure[37,38]. This complication transpires due to body's inability to repel the free radicals that leads to the destruction of cell membrane and lipid peroxidation[39]. In this study, among the expired patients, pulmonary fibrosis was the most common radiography finding, which was observed in almost 22% of the patients (more than 55% of the expired patients), which was initially observed on the fourth day. Also, Hsu *et al*[40] showed that pulmonary fibrosis had led to death in about 25% of patients. Our radiography findings in deceased patients were acute respiratory distress syndrome (ARDS), pneumothorax, and pneumomediastinum, which is in accordance with the results of Weng *et al*[41].

Unfortunately, despite various studies, limited variables have been identified for predicting prognosis. Although one of the best prognostic criteria is to determine paraquat serum concentration and to use nomogram[18], but this factor could not be studied due to lack of serum paraquat concentration measurement as a common laboratory test in Iran.

This research showed that the maximum average of serum creatinine levels increased on the third day (up to around 2.5 mg per deciliter), and that the serum creatinine average decreased, subsequently. Serum creatinine level on the third day higher than 1.95 mg per deciliter was accompanied with a poor prognosis in our patients. Ragoucy-Sengler and Pilerire[42] showed that an increase in serum creatinine lower than 0.03 mg during five hours accompanies an acceptable prognosis. On the other hand, Roberts *et al*[43] showed that an increase more

than 0.05 mg during 12 h could lead to poor prognosis. However, according to Levey *et al*[44], creatinine level has insignificant value, even for assessing the kidney damages.

According to other studies, other biomarkers for renal function were not appropriate factors in predicting patients' sequela[23]. This study showed that BUN level higher than 25 on the third day was accompanied with a poor prognosis.

Studying the changes in liver enzymes showed that the maximum average of AST and ALT levels on the third day were 114 and 145, respectively. AST level higher than 24.5 or ALT level higher than 12 on the third day was accompanied with poor prognosis. Considering the fact that these levels are in the normal range for AST and ALT, it seems that they are not appropriate factors for predicting severe poisoning. Furthermore, in their study, Almasi *et al*[45] showed that exposure to paraquat could lead to liver cell damage and increase in AST and ALT levels in rats, which could be treated by prescribing ginger extract. It seems that routine treatment by NAC played a role in improving liver cells' performance and decreased AST and ALT in a number of patients in this study. Since prescribing NAC, as an antioxidant, is an approved treatment for paraquat poisoning, hence it seems that liver biomarkers are not appropriate factors in predicting the patients' sequela.

This research showed that cardiac dysrhythmia is not a common finding in paraquat poisoning, which was in line with the results from Noguchiet *et al*[46]. In contrast, some other herbicides such as glyphosate, glufosinate and chloracetanilide herbicides (*e.g.*, alachlor, metachlor, butachlor, and propanil) appeared to have significant cardiotoxicity[47-51]. Even though other types of herbicides such as; glyphosate, glufosinate and chloracetanilide herbicides are available in Iran, by reviewing the published literature, we could find only one case report of butachlor dermal exposure[52]. It seems that, paraquat as a highly toxic compound is recognized by those who are seeking to commit suicide.

This research showed that a standardized treatment protocol was not used in all paraquat poisoning cases and in some cases, unnecessary or improper measures were carried out for the patients and in contrast or in some cases, a patient was deprived of necessary treatments.

Since there is no charcoal hemoperfusion available in our hospitals, but hemodialysis, which is the alternative choice was not used for extracorporeal elimination in the right time. Therefore, it is necessary to immediately carry out hemodialysis in these patients, along with training physicians and assistants working in teaching hospitals.

It seems that due to lack of paraquat poisoning treatment guidelines, patients are deprived of proper treatment by antioxidant and immunosuppressive medications. Providing treatment guidelines for this type of poisoning could assist in choosing a suitable treatment method. Finally, to better identify this type of poisoning systematic and meta-analysis reviews must be performed.

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COMMENTS

Background

Poisoning with herbicides in developing countries of South, East and Southeast Asia with an agriculture economy is highly common. Paraquat poisoning, is a highly mortal toxicity and rapid management is required to increase patient survival. However, without a standard guideline, there are no agreement on therapeutic strategies conducted in different healthcare facilities. The use of hemoperfusion or hemodialysis during the first hours of admission, followed by administration of immunosuppressive and corticosteroid medications, as well as antioxidants, is currently accepted to be the conventional treatment protocol for these cases. However, in practice, negligence is responsible for low survival rate of patients with paraquat poisoning.

Research frontiers

Reviewing the published literature, it seems that paraquat poisoning has the most prevalence rate in Fars province Iran, amongst different parts of Middle-Eastern countries. The research purpose was to evaluate the accuracy of treatment strategies, conducted to treat this fatal poisoning, as well as to demonstrate prognostic factors regarding long-term survival outcome.

Innovations and breakthroughs

Medical treatment for paraquat poisoning is improving in developing countries. This study represents the largest series of paraquat poisoning cases in the Middle-East ever reported. The current data suggests that therapeutic inaccuracies are common amongst healthcare providers. On the other hand, it was determined that there hasn't been any particular protocol used to treat patients diagnosed with paraquat poisoning. This indicates the necessity to develop a guideline for treating paraquat poisoning in order to provide better healthcare services to these patients. In case there is no access to charcoal hemoperfusion, hemodialysis should be used as an alternative choice in extracorporeal elimination; however, due to delay in reaching the results from viral marker study, hemodialysis was not carried out in most of the patients in the six-hour golden time. The importance of immediate use of extracorporeal removal techniques and omitting the viral markers results, also accurate use of immunosuppressive, corticosteroid and antioxidant medications should be considered as the main treatment protocols.

Applications

Results from this research indicated that the increase in renal biomarkers in the third day could be used in prognosis of the patients; hence, by identifying the patients who are at risk, it could be used as a guideline in intensive treatment measures.

Terminology

Paraquat poisoning is a lethal toxicity in patients who have consumed this herbicide, which is characterized by a rapidly progressive multi-organ failure in severe cases and progressive lung fibrosis in moderate cases. The most important treatment for paraquat poisoning is extracorporeal elimination within the first 6-h of toxicity. By using extracorporeal elimination technics, paraquat ion removal takes place through a machine performing blood circulation outside the body. By using charcoal hemoperfusion, paraquat cleansing will exceed that of hemodialysis. During hemoperfusion, blood is pumped through a cartridge containing activated charcoal.

Peer-review

Kavousi-Gharbi *et al* from Shiraz University of Medical Sciences, Shiraz, Iran investigated cross-sectionally the information about all cases of acute poisoning by the herbicide paraquat (1,1'-dimethyl-4,4' bipyridinium dichloride) admitted

to 3 main teaching hospitals of Shiraz University in a 5-year period (September 2010 to September 2015). A total of 104 patients (66% male) with a mean age 26 ± 11 years were evaluated. The mortality rate was 43%. Despite the necessity of emergency hemodialysis in first 6 h of intoxication, none of the patients had dialysis during this time.

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LETTERS TO THE EDITOR

Comments on eurytrematosis in Brazil and the possibility of human infection

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Abstract

The manuscript "Eurytrematosis: An emerging and neglected disease in South Brazil" discusses some aspects of *Eurytrema sp*. fluke as an animal pathogen and based in some aspects of the parasitism in cattle and the life cycle of *Eurytrema sp*. Authors suggest the possibility of human infection, once there is no research on this subject in Brazil. In human cases reported, the mechanism of infection was not disclosed, so it keeps the discussion opened. Although we focused on animal eurytrematosis, we speculated the possibility of human infection by *Eurytrema sp*. in Brazil, but after all, the only way to determine it, would be a study searching for people infected through coprological or serological tests.

Key words: Veterinary parasitology; Cattle; Pancreas; Eurytrema coelomaticum; Pathology

Core tip: The possibility of human infection by flukes of the genus Eurytrema in Brazil is reviewed. Based on the life cycle of the parasite and the high prevalence of infection in cattle, the possibility is suggested, although only an investigation with coprological or parasitological tests could give some reliable information.

Schwertz CI, Henker LC, Mendes RE. Comments on eurytrematosis in Brazil and the possibility of human infection. *World J Exp Med* 2017; 7(1): 40-41 Available from: URL: http://www.wjgnet.com/2220-315X/full/v7/i1/40.htm DOI: http://dx.doi.org/10.5493/wjem.v7.i1.40

TO THE EDITOR

The manuscript "Eurytrematosis: An emerging and neglected disease in South Brazil"[1] discusses some aspects of *Eurytrema sp.* fluke as an animal pathogen, regarding it's prevalence, subclinical disease and possible productive losses related to parasitism. Additionally, based in some aspects of the parasitism in cattle and the life cycle of *Eurytrema sp.*, the authors suggest the possibility of human infection. Since this work is

an editorial, the aim was to make comments on an important topic, regarding its current research status and future directions that will promote development of this subject.

We have read with interest the letter to the editor by Pinto *et al*[2]. Although, authors seem to have made an error of interpretation, as they say on the manuscript that eurytremiasis was suggested by Schwertz *et al*[1] to be a neglected and emerging human disease in Brazil. We would like to make clear that our manuscript reviews aspects about bovine's eurytrematosis, and suggests that the disease is neglected and emerging as an important pathogen for cattle in south Brazil, since we basically work with animal diseases. Furthermore, the majority of veterinarians believe the parasite is non pathogenic, information contradicted by us[2,3]. Based on the previously cited arguments, we only suggest the possibility of human subclinical infections, there is no research on this topic in Brazil. Also, at the time of writing the manuscript[1], no molecular identification had been conducted on specimens of *Eurytrema sp.* in Brazil. Based on this information, we speculate the parasite present in Brazil could be *E. pancreaticum*, which could be also present in human beings. Nowadays, our research group has already established by molecular technics that the parasite present in south Brazil is *E. coelomaticum*[3], which is not described as a human pathogen in the literature.

Pinto *et al*[2] criticize the life cycle of *Eurytrema sp.* showed in our editorial, once there is no evidence of infection through the ingestion of metacercariae over the pasture. In fact, it was a mistake to suggest this mechanism of infection without scientific support; although we believe that it could be possible, based on the high prevalence of the parasitism and the questionable probability of accidental ingestion of insects such as *Conocephalus spp.* by 70% of cattle in some regions. The liberation of metacercariae over the pasture by live grasshoppers, in our opinion, could better justify the high prevalence of infection by *Eurytrema sp.* where it occurs, although there is no scientific evidence of this for now. It is inconceivable, to think that 70% of dairy cattle in the area, in 100% of farms, were infected only by the ingestion of these insects. Specially taking into account that when someone walks in the field their agility is noted. Furthermore is quite uncommon to find dead specimens available to be ingested by ruminants. We have found up to 2578 *E. ceolomaticum* flukes in a pancreas of one cattle, and the average was 532[3,4].

According to Headley[5], the fact that *E. pancreaticum* has already been identified in human beings should not be ignored and more epidemiological data must be obtained and analyzed to establish the form of transmission to human beings, thereby discovering the potential of this fluke as a threat to human health. In the case reported by Ishii *et al*[6], it was not possible to determine how the person got infected, but the author presume that she accidentally ingested metacercariae in or from an infected grasshopper.

Pinto *et al*[2] defend that there is no possibility of human infection by *Eurytrema sp.* in Brazil. Still, the species that occurs in Brazil is *Eurytrema coelomaticum*, as we later established[3] and the human cases reported in the literature are due infection by *Eurytrema pancreaticum*[6]. In the editorial[1], we mentioned the possibility of infection but we have not focused on this aspect and we have not discussed this in detail as thoroughly as Pinto *et al*[2]. We have detailed the current research status and future directions of eurytrematosis in cattle. The arguments proposed by them[2] make clear that the possibility of human infection by *Eurytrema sp.* in Brazil is low, but after all, the only way to determine it, would be a study searching for people infected through coprological or serological tests[7].

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MINIREVIEWS

Recent clinical trials of cancer immunogene therapy in companion animals

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Abstract

This mini-review presents the results of veterinary clinical trials on immunogene therapy published from 2014 to 2016. A variety of tumors, among them melanoma (canine and equine), mastocytoma (canine), mammary adenocarcinoma (canine) and fibrosarcoma (feline) were treated by using diverse strategies. Non-viral vectors were usually employed to transfer genes of cytokines, suicide enzymes and/or tumor associated antigens. In general terms, minor or no adverse collateral effects were related to these procedures, and treated patients frequently improved their conditions (better quality of life, delayed or suppressed recurrence or metastatic spread, increased survival). Some of these new methodologies have a promising future if applied as adjuvant treatments of standard approaches. The auspicious results, derived from immunogene therapy studies carried out in companion animals, warrant their imperative usage in veterinary clinical oncology. Besides, they provide a strong preclinical basis (safety assays and proofs of concept) for analogous human clinical trials.

Key words: Cancer; Gene therapy; Immunotherapy; Companion animals; Comparative oncology

Core tip: Cancer immunogene therapy is a major growing area among human clinical trials. Until August 2016 there were about 2409 registered gene therapy trials, where 1554 were aimed to cancer, and among them 864 corresponded to immunotherapy. Working with veterinary cancer bearing patients can significantly speed up translational research and benefit both veterinary and human patients. New data demonstrated the safety and efficacy of different immunotherapy approaches. Following our previously published review on the subject covering from 1996 to 2014, this new mini-review is focused on veterinary cancer immunogene therapy covering published work in the field from 2014 to 2016.

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INTRODUCTION

More than 20 years elapsed from the publication of the pioneering work of Quintin-Colonna *et al*[1] in 1996 on *ex vivo* interleukin-2 (IL-2) immunogene therapy for canine melanoma and feline fibrosarcoma. As we had previously discussed in a previous review covering this subject from the early beginnings up to 2014[2], nearly all veterinary cancer gene therapy clinical trials involved the stimulation of immune responses against tumor cells. In this new review we are covering the three years period from January 2014 to December 2016. As we did in the preceding review, we focused our interest only on reports of clinical data collected from client-owned patients with spontaneously arising tumors.

Why clinical trials of cancer immunogene therapy in companion animals do actually matter? Despite some progresses treating tumors in companion animals and human patients, there is still a need of new therapeutic approaches for diverse malignancies because of the lack of long lasting effective treatments and the unwanted side effects of most of them. In addition, spontaneous tumors in companion animals offer a very useful translational model because pets display relatively large sizes, diversity of genetic backgrounds and intact immune systems. They usually offer a strong support as proof of concept in a setting similar to the population of human patients, while directly providing data about toxicity and long-term efficacy. Nowadays there is a revival of the idea that researchers should turn to canine clinical trials to advance cancer therapies[3].

Up to date we found 57 reports about veterinary immunogene therapy clinical trials: 45 canine, 7 feline and 5 equine. As seen in Table 1, the fourteen newly reported trials mainly involved non-viral gene transfer (13/14), sometimes enhanced by physical methods (10/13: 7 electrotransfer and 3 jet injection), while a viral vector was used in 1 trial (1/1: poxvirus). Reports about using genetically modified bacteria[4,5] or suicide gene expressing encapsulated mammalian cells[6] as vectors are not discussed in this mini-review.

New trials (7/14) were mainly reported from only three countries: 3 from United States (2 canine, 1 feline), 2 from Belgium (canine) and 2 from Italy (canine). The remaining five studies were: 1 from Argentina (canine), 1 from France (feline), 1 from Germany (equine), 1 from Slovenia (canine), 1 from United Kingdom (canine), 1 from South Africa (canine) and 1 multi-centric from Ukraine, Russia and Italy (canine).

Most of the current veterinary trials (6/11 canine, 1/2 feline and 1/1 equine) employed cytokine genes, mainly IL-2-and IL-12 as single (6/8) or combined genetic agents (2/8), followed by antigens (5/11 canine, 1/2 feline, 1/1 equine).

Immunogene therapy was applied alone (10/14), or combined with chemotherapy (3/14) or suicide gene (1/14). Most trials were focused on feasibility and safety (8/14) with few treated animals and often carrying diverse kind of tumors. On the other hand, larger controlled trials (4/14) allowed the determination of clinical efficacy.

CYTOKINE-BASED IMMUNOGENE THERAPY

Only five cytokine genes were assayed in veterinary clinical settings in the latest three years. Granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates dendritic cells that then induce antitumor immune responses. The result is twofold: Direct destruction of the injected lesion and enhanced antigen presentation, leading to an immune response against metastatic melanoma. T-cells treated with GM-CSF have demonstrated increased antitumor responsiveness.

IL-2 is a naturally occurring glycoprotein secreted by T-cells to augment the immune response and was first used in clinical cancer studies in the early 1980s. This glycoprotein promotes T-lymphocyte proliferation and stimulates cytotoxic T-cells and natural killer cells. IL-2 has been used as immunotherapy for nearly 40 years. The treatment with the recombinant protein displays systemic cytotoxicity that deteriorates the patient's quality of life.

Interferon-β (IFN-β) simultaneously displays antiproliferative and anti-angiogenic effects as well as immunomodulatory activities. It was among the first cytokines approved for clinical use. Even though clinically effective, the completion of IFN-β therapy is often impaired by its inherent systemic toxicity that negatively affects the patient's quality of life.

IL-12 and IL-18 exhibit different immune regulatory roles including activation of cytotoxic T-lymphocytes and natural killer cells, production of interferon-γ (IFN-γ). In addition they have antiangiogenic properties and induce apoptosis in tumor cells. Although they produce from moderate to severe side effects, significant antitumor activity was demonstrated in clinical trials for recombinant IL-12 and IL-18 proteins.

Combination with suicide gene therapy and whole tumor cells extracts for canine melanoma

Spontaneous canine melanoma is a highly aggressive tumor. It appears mainly in the oral cavity, footpads, digits and mucocutaneous junctions, displaying a clinical behavior analogous to aggressive human melanoma. Both diseases are resistant to chemotherapy, radiotherapy and treatments with modulators of biological responses. They share comparable location selectivity and metastatic phenotypes.

As a continuation of encouraging data obtained with a surgery adjuvant treatment that consisted of complete or cytoreductive surgery followed by a combination of suicide gene therapy with a subcutaneous vaccine[7], a

Table 1 Veterinary cancer immunogene therapy trials 2014-2016

No.	Genes	Tumor	Vector	Mode	Results	Ref.
1	hIL-2 + hGM-CSF cIFN-β + HSV-tk	MEL	Plasmid lipofection: HSV-tk + IFN-β + GCV. Plasmid lipofection: hIL-2 and hGM-CSF	*In vivo* (SG) + (IFN-β) - i.t. *In vivo* (hIL-2 + hGM-CSF) + (TV) - s.c./+ SX	Combined treatment (n = 301)/surgery controls (n = 162). Complete surgery arms: Local disease-free patients: 83%/11%. Metastasis-free patients: 89%/44%. Median survival: > 2211 d/109 d. Metastasis-free median survival: > 2211 d/134 d	Finocchiaro *et al*[8]
2	tthIL12	SCC MEL	Plasmid/EGT	*In vivo* - i.t. EGT	(n = 4) Dose escalation and safety. Two confirmed local and distant responses. No severe side effects	Cutrera *et al*[9]
3	cIL-12	AMB, PCY, SCC, STS	Naked plasmid + bleomycin/ gemcitabine EGT	*In vivo* - i.t./+ CHT	(n = 13) 2 CR, 4 PR, 5 SD, 2 PD. No severe side effects	Cutrera *et al*[12]
4	hIL12	MCT	Naked plasmid + cisplatin or bleomycin electrotransfer	*In vivo* - i.t./+ CHT	(n = 18) 13 CR, 2 PR, 1 SD, 2 PD. No severe side effects	Cemazar *et al*[14]
5	hIL12	FSA, MAC, MCT, OSA, SCC, SCH	Plasmid/EGT	*In vivo* - i.t.	(n = 9) Safety studies and demonstration of immunogenic and anti-angiogenic effects. No significant clinical benefits	Cicchelero *et al*[10]
6	hIL12	ADC, FSA, MEL, OSA, SCH	Plasmid/EGT + metronomic cyclophosphamide	*In vivo* - i.t. EGT/+ metronomic CHT	(n = 6) No significant unwanted side effects. Combined therapy slowed down tumor progression and improved QOL	Cicchelero *et al*[13]
7	fIL-2	FSA	Canary pox virus	*In vivo* - p.t.	Combined treatment (n = 25)/ Surgery + brachytherapy controls (n = 23). Disease-free patients: 41%/72%. Median survival: > 730 d/287 d	Jas *et al*[11]
8	eIL-12 + eIL-18 hgp100 htyr	MEL	Plasmid like	*In vivo* - i.m./p.t.	(n = 27) No differences between eIL-12 + eIL-18 without (n = 9) or with either hgp100 (n = 9) or htyr (n = 9). Tumor size reduction on day 120 approximately 20%	Mählmann *et al*[15]
9	CSPG4	MEL	Plasmid/EGT	*In vivo* - i.m./ + SX	(n = 14) VAC/(n = 13) CTR Median survival: VAC 532 d/ CTR 205 d	Riccardo *et al*[23]
10	hp62	MAC	Plasmid	*In vivo* - i.m.	(n = 7) 5PR (50%-75% reduction) 2 SD. Safety verified	Gabai *et al*[22]
11	htyr	MEL	Naked plasmid - Jet injection	*In vivo* - i.m./ + SX ± RX	Retrospective study (n = 38). Responding patients median survival: 870 d. Non-responding patients median survival: 240 d	McLean *et al*[19]
12	htyr	MEL	Naked plasmid - Jet injection	*In vivo* - i.m./ + SX ± RX	Retrospective study (n = 32). Median survival: 335 d. Progression-free median survival: 160 d	Treggiari *et al*[20]
13	CSPG4	MEL	Plasmid/EGT	*In vivo* - i.m./ + SX	(n = 23) VAC/(n = 19) CTR Median survival: VAC 684 d/ CTR 220 d	Piras *et al*[24]
14	htyr	MEL	Naked plasmid - Jet injection	*In vivo* - i.m./ + SX ± RX	Retrospective study (n = 24). Eleven percent of manageable post-vaccination adverse effects. Safety verified. Forty-two percent died of unrelated causes or still alive	Sarbu *et al*[18]

The No. 1-8 genes are cytokines; the No. 9-14 genes are antigens; the tumors in No. 1-6 and 9-13 are canine; the tumors in No. 7 and 14 are feline; the tumors in No. 8 is equine. ADC: Adenocarcinoma; AMB: Acanthomatous ameloblastoma; FSA: Fibrosarcoma; MAC: Mammary adenocarcinoma; MCT: Mast cell tumor; MEL: Melanoma; PCY: Plasmocytoma; OSA: Osteosarcoma; SCC: Squamous cell carcinoma; SCH: Schwannoma; STS: Soft tissue sarcoma; CSPG4: Chondroitin sulfate proteoglycan-4; GM-CSF: Granulocyte macrophage colony-stimulating factor; gp100: Glycoprotein 100; HSV-tk: Herpes simplex thymidine kinase; IFN-β: Interferon-β; IL: Interleukin; p62: Protein 62; tyr: Tyrosinase; CR: Complete response; CTR: Control; GT: Gene therapy; PR: Partial response; SD: Stable disease; GCV: Ganciclovir; SG: Suicide gene; TV: Tumor vaccine; VAC: Vaccinated; i.m.: Intramuscular, i.t.: Intratumoral; p.t.: Peritumoral; s.c.: Subcutaneous; CHT: Chemotherapy; RX: Radiotherapy; SX: Surgical excision; c: Canine; e: Equine; f: Feline; h: Human; tt: Tumor targeted.

new local and vaccine formulation was assayed[8]. This new trial involved the injection (at the end of surgery) of DMRIE/DOPE lipoplexes carrying two therapeutic genes: Canine IFN-β and herpes simplex virus thymidine

kinase. In parallel, a subcutaneous genetic vaccine made of lipoplexes carrying human GM-CSF and IL-2 genes and tumor formalized extracts was periodically administrated. Taking surgery-only-treated (ST) as controls, it was found that the combined treatment (CT) that followed complete surgery (CS) significantly increased the portion of local distant metastases-free (M0) from 44% to 89% and disease-free canine patients from 11% to 83%. Even in the case of cytoreductive surgery (CR), CT had a better control of the systemic disease (M0: 82%) than ST (M0: 48%). Furthermore, taking the ST group as control, CT displayed a considerable > 17-fold (CS) and > 13-fold (CR) increase of metastasis-free survival (MFS), and 7-fold (CS) and 4-fold (CR) increase of overall survival. The remarkable rise of CS-recurrence-free survival (RFS) and CS-MFS (both > 2251 d) and CR-MFS (> 1321 d) revealed that CT was transforming a fast terminal disease into a chronic one. Finally as a surgery adjuvant, this CT was able to considerably postpone or preclude distant metastasis and postsurgical recurrence, while it extended disease-free and overall survival, and preserved the quality of life. The long-term safety and efficacy of this treatment was supported by the high number of canine patients involved and the wide follow-up (> 6 years) with negligible or null toxicity. Besides, this work confirmed that the most advantageous clinical situation is to implement this approach as a surgery adjuvant acting on the minimal residual disease.

Single treatments for canine and feline solid tumors

Electrotransfer was the preferred method for introducing IL-12 plasmids into cells. In a first report, the peptide-cytokine VNTANST-IL12 could successfully target expressed cell-surface vimentin while displaying the antitumor effects of cytokine IL-12. So, a tumor-targeted IL-12 plasmid DNA (ttIL12 pDNA) was safely delivered at clinical levels by electrotransfer to four tumor bearing canine patients, inducing antitumor immune responses in both treated and untreated tumors including metastatic lesions[9].

In a parallel study, nine dogs with spontaneous cancer were subjected to three consecutive intratumoral IL-12 electrogene therapy (EGT) sessions to assess their clinical, anti-angiogenic and immunological effects[10]. Serum and tumor tissue displayed transitory increases of IFN-γ and IL-12. Intratumoral IL-12 EGT did not produce clinically significant results, even though some anti-angiogenic and immunostimulatory activities appeared as well as transitory objective responses did. It was not possible to get a sustained tumor regression during the trial. Without severe side effects, safety was verified.

Fibrosarcoma is a lethal disease in cats that is frequently found at standard vaccine injection sites. Because of the high local recurrence rates after surgery alone, the addition of adjuvant treatments has been under investigation for the last few years. A randomized, controlled clinical study was performed to determine the safety and efficacy the of a recombinant canarypox virus carrying the feline IL-2 gene (ALVAC IL-2)[11]. Additionally to surgery and post-surgical brachytherapy and as adjuvant treatment, the vector was transferred to cats with a first occurrence of feline fibrosarcoma. The pet patients were distributed at random to a control group (23 cats), low dose injected group (25 cats) and high dose injected group (23 cats). The treatment consisted of six successive intratumoral injections of ALVAC IL-2. With limited mild local reactions, it was well tolerated. Injected animals displayed a significantly longer median time to relapse (> 730 d in the low dose injected group) than in the control group (287 d). The risk of relapse of treated cats displayed a considerable decrease: 56% at one year (injected groups compared to control group) and 65% at two years (low dose injected group compared to control group).

Combination with electro-chemotherapy for canine solid tumors

In a further report, a study demonstrated the efficacy and safety of recurring cycles of *IL-12* gene therapy plus chemotherapy for long-term treatment of aggressive tumors[12]. In this case 13 canine patients were subjected to various rounds of electro-chemo-gene therapy (ECGT) with canine IL-12 plasmid DNA (pDNA) and either gemcitabine or bleomycin. This approach was versatile, being effective for many histotypes, and allowed fast eradication or debulk of large tumors (sarcoma excluded). It affected both treated tumors and non-treated metastatic tumors. Without severe adverse events reported, even after multiple treatment cycles, ECGT with IL-12 pDNA proved to be a very valuable approach for many types of spontaneous cancers including refractory, large and multiple tumor loads. Being this phase Ⅰ trial designed for dose escalation/safety assessment, no data about long term quality of life and progression free survival could be obtained.

By combining with metronomic cyclophosphamide, the outcomes of three successive intratumoral IL-12 electrogene transfer treatments were explored in six dogs with spontaneous tumors[13]. This treatment induced erythema and swelling of the tumor, a transient increase in IL-12 levels, and a continuous increase in IFN-γ and thrombospondin-1 concomitant with a continuous decrease in vascular endothelial growth factor. While the treatment improved the quality of life and weight, it slowed tumor progression in most of the patients (4/6).

Mast cell tumors (MCTs) are common cutaneous neoplasms in dogs, accounting for up to 21% of all canine skin tumors. Peritumoral *IL-12* gene transfer was combined with electro-chemotherapy (cisplatin or bleomycin) targeting mast cell tumors in 18 canine patients[14]. Without emerging side effects, one month after the therapy a considerable local tumor control was evidenced. The complete responses rate increased up to 72% during the observation period. *IL-12* gene electrotransfer produced detectable serum IL-12 and/or IFN-γ levels in 78% of patients. This study showed a high antitumor efficacy of the combined treatment that also prevented recurrences or distant metastases.

In addition the safety and feasibility of this approach was demonstrated.

Combination with antigens for equine melanoma tumors

Equine melanoma has a high incidence in grey horses. In a clinical trial[15], 27 melanoma-bearing grey horses were allocated into 3 groups (n = 9) and intramuscularly vaccinated on days 1, 22, and 78 with lipoplexes carrying DNA plasmids bearing equine *IL-18* and *IL-12* genes alone or combined with either human glycoprotein 100 or tyrosinase. In all groups, by day 120 the relative tumor volume significantly decreased about 20%. The combination of the two cytokines was safe, but the addition of DNA vectors encoding the human melanoma antigens did not potentiate their effects.

ANTIGEN-BASED IMMUNOGENE THERAPY

As it happens in dogs, in cats malignant melanoma appears as locally aggressive and highly metastatic regardless of the primary site of origin. The standard modalities used for treatment are surgery and radiotherapy, with poor results in the long-term. Following the studies about the use of xenogeneic antigen vaccination for spontaneous canine malignant melanoma[16,17], new data about intramuscular needle free jet injection of a plasmid containing human tyrosinase gene and its expression as a melanoma xeno-antigen against feline and canine melanoma were reported.

In a retrospective study the safety of the canine melanoma DNA vaccine (Oncept®) following surgery in 24 feline patients was assessed[18]. The vaccine appeared to be well tolerated, with a low number of reported adverse events. Since most cats finally died from this disease, controlled prospective studies are necessary to determine the immunogenicity and efficacy of this vaccine originally designed for canine melanoma in cats with melanoma.

Furthermore, two additional independent retrospective analyses on the use of this DNA vaccine for canine melanoma[19,20] were in agreement on safety issues with previously published results[16,17]. In the first case 38 canine patients were evaluated: The median survival time of responding patients (42%) was 870 d, while it was 240 d in the case of non-responding ones (58%)[19]. Even though a relatively small number of patients was analyzed, melanotic oral melanoma appeared to give survival advantage as compared to its amelanotic counterpart. As expected, surgery with clean margins also improved the treatment outcome. In the second case, 32 oral melanoma patients received the DNA vaccine after surgery displaying a median survival time of 335 d[20]. No significant differences were found when compared to other adjuvant treatments. Because of the low statistical power due to small number and group heterogeneity, the authors proposed controlled studies to assess whether the addition of any adjuvant treatment to surgery, including immunogene therapy, is able to significantly prolong survival in cases of canine oral melanomas. A very recent paper[21] analyzing the outcome of 69 canine melanoma patients after human tyrosinase DNA vaccination, reported a median survival time of 455 d, a value that is similar to those published earlier[19,20]. Since the authors observed responses in patients with macroscopic disease, they suggest that the vaccine could be considered as palliative treatment in dogs with remaining tumors or recurrence.

Mammary tumors are the most common tumors of unspayed female dogs with a prevalence of 40% of all tumors, and about half of them are malignant. The p62 protein (SQSTM1) is a major player in selective macro-autophagy and acts as a signaling hub for many signal transduction pathways. It is noteworthy that p62 is expendable for normal tissues, but critical for development and survival of tumors. The effects of intramuscular p62 DNA vaccine on mammary tumors of seven dogs were tested[22]. Locally advanced lesions decreased (5/7) or stabilized (2/7) their growth while the overall toxic effects of p62 were absent. The observed antitumor activity was associated with lymphocyte infiltration and tumor encapsulation *via* fibrotic reaction.

Chondroitin sulfate proteoglycan-4 (CSPG4) is an early cell surface progression marker associated with tumor cell migration, invasion and proliferation. In a first trial, the immunogenicity, safety and therapeutic efficacy of a human CSPG4 DNA-based vaccine were evaluated[23]. Canine patients with stage Ⅱ-Ⅲ surgically excised CSPG4-positive oral malignant melanoma were treated by adjuvant intramuscular electrotransfer with human CSPG4-encoded plasmid from 6 to 20 mo. Overall (653 d *vs* 220 d) and disease-free (477 d *vs* 180 d) survival times were significantly longer in 14 vaccinated dogs as compared with 13 non vaccinated controls. No clinically relevant local or systemic side effects were found. This suggested that xenogeneic electrovaccination against CSPG4 can prevail over the host tolerance to the "self" antigen, being successful in treating canine malignant melanoma.

In a second trial, the same treatment was performed in 23 dogs with surgically excised CSPG4-positive oral canine melanoma. In parallel, 19 control dogs with CSPG4-positive tumors were subjected only to surgery[24]. Vaccinated dogs displayed a one year survival rate of 74%, with a median survival time of 684 d, and one year disease-free interval rate of 48%. Non-vaccinated dogs showed one year survival rate of 26%, with a median survival time of 200 d, and a one-year disease-free interval rate of 26%. The involvement of a fair amount of canine patients together with the relatively wide follow-up (2 years) with absent or minimal adverse side effects, added to the results of the previous report[23], assure the significant efficacy and the long-term safety of this treatment.

CONCLUSION

The suitability of comparative oncology using large animal naturally occurring cancer models to test new

cancer drugs, particularly gene medicines, was widely discussed[2,25].

This approach can primarily benefit pets that do not have efficient treatments for some malignancies and ultimately human patients. The proof of concept and safety results obtained in companion animals can be readily used for designing clinical trials against the corresponding human malignancies.

Some immunogene therapy approaches similar to those presented here were also tested in human patients before 2014: Xenogeneic tyrosinase[26] and gp100[27], IL-2[28], GM-CSF[29] and IL-12[30]. On the other hand, encouraging results were recently reported about a clinical trial of *AdCD40L* gene therapy combined with cyclophosphamide chemotherapy for human melanoma[31] and p62 protein (SQSTM1) gene therapy for some human solid tumors[32]. These trials were respectively based on previous veterinary trials involving canine melanoma[33] and canine mammary adenocarcinoma[22] patients. In addition, our Unit is currently involved in a phase I clinical trial for human advanced melanoma based on the safety and efficacy of our previous long lasting veterinary clinical trials[7,8].

Because of safety reasons and the cost of producing the suitable vectors, most of the veterinary gene therapy protocols were done with non viral vectors, being electrotransfer mainly used in the latest period (2014-2016).

Four controlled trials[8,11,23,24] showed statistically significant tumor control and survival time increase, while maintaining a good quality of life. These results strongly support further developments in immunogene therapy. Meanwhile, new immune-mediated gene therapy approaches are emerging for treating equine melanoma with a bacterial antigen[34] and canine lymphoma with a chimeric antigen receptor[35]. A different gene therapy approach is also under investigation for feline oral squamous cell carcinoma with RNAi oligonucleotides targeting feline CK2α and CK2α′ (TBG-RNAi-fCK2αα′)[36].

As we discussed earlier[2], proper cancer models involving companion animals spontaneously induce the investigators to work in the duality between the veterinary perspective and the potential application to human medicine. However, these are not contradictory objectives, and more trials in humans based on the equivalent trials in pets will possibly come soon.

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ORIGINAL ARTICLE

Basic Study

Role of LIGHT in the pathogenesis of joint destruction in rheumatoid arthritis

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Abstract

AIM

To characterise the role of substitutes for receptor-activator nuclear factor kappa-B ligand (RANKL) in rheumatoid arthritis (RA) joint destruction.

METHODS

Synovial fluid (SF) macrophages isolated from the knee joint of RA patients were incubated with 25 ng/mL macrophage-colony stimulating factor (M-CSF) and 50 ng/mL LIGHT (lymphotoxin-like, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes) in the presence and absence of 25 ng/mL RANKL and 100 ng/mL osteoprotegerin (OPG) on glass coverslips and dentine slices. Osteoclastogenesis was assessed by the formation of multinucleated cells (MNCs) expressing tartrate-resistant acid phosphatase (TRAP) on coverslips and the extent of lacunar resorption pit formation on dentine slices. The concentration of LIGHT in RA and osteoarthritis (OA) synovial fluid was measured

by an enzyme-linked immunosorbent assay (ELISA) and the expression of LIGHT in RA and OA synovium was determined by immunohistochemistry using an indirect immunoperoxidase technique.

RESULTS

In cultures of RA SF macrophages treated with LIGHT and M-CSF, there was significant formation of TRAP + MNCs on coverslips and extensive lacunar resorption pit formation on dentine slices. SF-macrophage-osteoclast differentiation was not inhibited by the addition of OPG, a decoy receptor for RANKL. Resorption pits were smaller and less confluent than in RANKL-treated cultures but the overall percentage area of the dentine slice resorbed was comparable in LIGHT- and RANKL-treated cultures. LIGHT significantly stimulated RANKL-induced lacunar resorption compared with RA SF macrophages treated with either RANKL or LIGHT alone. LIGHT was strongly expressed by synovial lining cells, subintimal macrophages and endothelial cells in RA synovium and the concentration of LIGHT was much higher in RA compared with OA SF.

CONCLUSION

LIGHT is highly expressed in RA synovium and SF, stimulates RANKL-independent/dependent osteoclastogenesis from SF macrophages and may contribute to marginal erosion formation.

Key words: Receptor-activator nuclear factor kappa-B ligand; Osteoclast; Rheumatoid arthritis; Resorption; LIGHT

Core tip: Rheumatoid arthritis (RA) is an inflammatory joint condition characterised by the formation of marginal erosions due to the activity of bone resorbing osteoclasts. Osteoclasts can be formed from macrophages by both receptor-activator nuclear factor kappa-B ligand (RANKL)-dependent and RANKL-independent mechanisms. LIGHT is a potent RANKL substitute that induces significant osteoclast formation from cultures of RA synovial fluid macrophages; this results in comparable levels of resorption to that seen in RANKL-treated cultures. LIGHT also stimulated RANKL-mediated lacunar resorption. LIGHT is highly expressed in RA joints and synovial fluid and is likely to play a key role in the pathogenesis of marginal erosion formation in RA.

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INTRODUCTION

Rheumatoid arthritis (RA) is a common inflammatory arthropathy which affects approximately 1% of the adult population[1]. RA is characterized by a heavy lymphocyte and plasma cell infiltrate in the synovium, synovial overgrowth; and extension of inflammatory tissue into bone; this leads to the formation of periarticular marginal erosions by osteoclasts, multinucleated cells which are specialised to carry out lacunar bone resorption. The osteoclast is a member of the mononuclear phagocyte system and is formed from $CD14^+$ macrophage precursors in the presence of macrophage-colony stimulating factor (M-CSF) and receptor activator for nuclear factor κB ligand (RANKL), a tumour necrosis family (TNF) superfamily member[2-5]. RANKL is expressed by activated fibroblasts, osteoblasts and lymphocytes which also produce osteoprotegerin (OPG), a decoy receptor for RANKL that inhibits osteoclastogenesis[6,7]. The RANKL-OPG axis is key to controlling osteoclast formation and survival. A monoclonal antibody directed against RANKL, denosumab, has been shown to increase bone mineral density and to reduce pathological bone resorption in osteoporosis and RA[8-11].

Several studies have shown that osteoclasts can be formed by a RANKL-independent pathway promoted by other TNF superfamily members[12-17]. LIGHT (lymphotoxin-like, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes) is the most potent RANKL substitute identified to date[14,18,19]. LIGHT (TNFSF14) is a type 2 transmembrane glycoprotein which is expressed on activated T lymphocytes, monocytes and dendritic cells[20-22]. LIGHT binds to two membrane-bound members of the TNFR superfamily, herpes virus entry mediator (HVEM), which is expressed by many inflammatory cells including T cells, B cells, monocytes and dendritic cells, and lymphotoxin β receptor (LTβR), which is expressed by many cell types such as fibroblasts, endothelial cells, stromal cells and monocytes but not lymphocytes[23-25]. LIGHT also binds to decoy receptor 3 (DcR3), a soluble non-signaling receptor which has been shown to modulate its function *in vitro* and *in vivo*[23,26]. LIGHT is expressed constitutively by dendritic cells and activated T cells and is mainly found in lymphoid tissues[20-22]. LIGHT has been shown to induce osteoclast formation by a RANKL-independent mechanism from monocytes[14,19].

The role LIGHT plays in inducing pathological bone resorption in RA joints is uncertain. LIGHT levels are increased in the serum of patients with RA, and blocking the action of LIGHT has been shown to reduce the severity of murine collagen-induced arthritis[14,27]. Synovial fluid (SF) macrophages are known to differentiate into osteoclasts in the presence of RANKL and M-CSF[28]. In this study we have examined the role LIGHT plays in the pathogenesis of inflammatory joint destruction by determining whether LIGHT alone can stimulate osteoclastogenesis from SF macrophages isolated from RA joints. We have also examined whether the concentration of LIGHT is higher in RA than non-inflammatory osteoarthritis (OA) SF and compared the

expression of LIGHT in RA and OA synovium.

MATERIALS AND METHODS

Patients samples and reagents

SF samples were derived from six patients (four females, one male) undergoing therapeutic arthrocentesis for inflammatory joint disease. Non-inflammatory OA control SF was derived from four patients (two females, two males) undergoing unicompartmental knee replacement. All patients diagnosed with RA were seropositive for rheumatoid factor and met the ACR/EULAR diagnostic criteria for RA[29]. RA and OA synovial tissue was derived from biopsy specimens. All patients gave informed consent and the Oxford Clinical Research Ethics Committee approved the study.

For all tissue culture incubations, α-minimum essential medium (α-MEM) (Invitrogen, United Kingdom) was supplemented with 2 mmol/L (w/v) glutamine, 10 μg/mL streptomycin, 100 IU/mL benzyl penicillin, 10% (v/v) heat-inactivated fetal bovine serum (FBS). Recombinant soluble human RANKL was purchased from Peprotech Europe (London, United Kingdom); all other cytokines were purchased from R and D Systems Europe (Abingdon, United Kingdom). Primary antibodies for immunohistochemistry were purchased from Abcam (Cambridge, United Kingdom).

Isolation and culture of SF macrophages

SF macrophages were isolated from aspirates by centrifugation at 2500 rpm for 10 min at 4 ℃; macrophages were subsequently used for osteoclastogenesis (see below) while the cell-free supernatant was frozen and stored at -80 ℃ for ELISA measurement of LIGHT levels. Following centrifugation of SF aspirates, the resultant cell pellet was re-suspended α-MEM/FBS and then added at 1 × 10^6 cells/well to 96 well tissue culture plates containing 5 mm dentine slices. After 2 h incubation, dentine slices were removed from the wells and washed vigorously in MEM/FBS to remove non-adherent cells; the cell suspension was subsequently transferred to a 24-well tissue culture plate containing 1 mL of MEM/FBS supplemented with various factors. As positive controls, SF macrophage cultures were maintained in the presence of 25 ng/mL M-CSF plus 50 ng/mL soluble RANKL. As negative controls, cultures were maintained with M-CSF alone. SF macrophages were also cultured in the presence of LIGHT (50 ng/mL) ± OPG (100 ng/mL). All SF macrophage cultures were incubated for up to 14 d, during which time the entire culture medium containing all factors was replenished every 2-3 d.

Characterisation of osteoclast formation and activation

Tartrate-resistant acid phosphatase: After 14 d in culture, cells were fixed in formalin and stained histochemically for tartrate-resistant acid phosphatase (TRAP), an osteoclast marker, using naphthol AS-BI as a substrate, in the presence of 1.0 mol/L tartrate.

F-actin ring formation: Multiple rows of podosomes containing an F-actin core are often localised in the sealing zone of osteoclasts. To detect F-actin ring structure, dentine slices were fixed with 4% formaldehyde for 5 min and then permeabilised for 6 min in 0.5% Triton X-100 in phosphate buffered saline (PBS) and rinsed with PBS. The cells on dentine slices were then incubated with tetramethyl-rhodamine isothiocyanante-conjugated phalloidin (Sigma-Aldrich) for 30 min and observed using a fluorescence microscope (Olympus).

Lacunar resorption: Functional evidence of osteoclast formation was determined using a resorption assay system. Circular dentine slices (4 mm in diameter) were prepared from elephant tusk blocks, kindly supplied by the Customs and Excise, United Kingdom, and sterilised in absolute alcohol overnight. After 14-d incubation, dentine slices on which cells had been cultured, were removed from wells, rinsed in PBS, incubated in 1.0 M ammonium hydroxide for 24 h and sonicated in distilled water for 5-10 min. All cellular debris was thus removed from the dentine slice permitting examination of its surface for evidence of lacunar resorption. The slices were washed in distilled water, stained with 0.5% (w/v) toludine blue in 1.0% (w/v) boric acid pH 5.0 and examined by light microscopy. To quantify the lacunar resorption, dentine slices were photographed and the resorbed areas highlighted and measured using Adobe Photoshop CS3 and Image J (NIH, Bethesda, MD, United States).

Quantification of LIGHT levels in synovial fluid of OA and RA patients

Human LIGHT enzyme-linked immunosorbent assay (ELISA) kit (R and D Systems Europe) was used to determine the concentration of soluble LIGHT in the synovial fluid derived from RA (n = 5) and OA (n = 4) patients, as per manufacturer's instructions. The upper and lower detection limits of the ELISA were 31 pg/mL and 2 ng/mL, respectively.

Immunohistochemistry of RA and non-inflammatory OA synovial tissue

Formalin-fixed, paraffin-embedded synovial biopsies of RA and OA synovium were cut (3 μm), deparaffinized with xylene and rehydrated through a series of graded alcohols. After blocking endogenous peroxidase with 0.2% (v/v) hydrogen peroxide in 80% alcohol for 30 min, antigen retrieval was performed in 500 mL 10 mmol/L Tris + 1 mol/L EDTA (BDH, United Kingdom) buffer (pH 8.5) using a microwave for 20 min. Immunohistochemistry was performed using an indirect immunoperoxidase technique with 3,3-diaminobenzidine chromogen (EnVision™ + Dual Link System-HRP, Liquid DAB + Substrate Chromogen System, Dako, United Kingdom). Sections were incubated with an anti-LIGHT antibody (anti-hLIGHT/HVEM-L, R and D systems, United States) 1:5 overnight at room temperature followed by 30 min incubation with labeled polymer and 10 min in 3,3-diaminobenzidene. Slides were counterstained using Mayer's haematoxylin for 3 min, blued in 2% hydrogen sodium carbonate, dehydrated,

cleared in xylene and mounted using DePeX (Surgipath, United Kingdom). All sections were examined by light microscopy.

Ethical consideration

All tissue specimens and blood samples from RA and OA patients were taken after informed consent and ethical permission was obtained for participation in the study.

Statistical analysis

The statistical review of the study was performed prior and after the study was conducted and with consultation with a biomedical statistician. Data is represented as mean ± SEM. For assessment of osteoclast resorption, the area of lacunar resorption was normalized and expressed as a percentage of RANKL-induced lacunar resorption (positive control). Statistical significance was determined by Mann-Whitney test or one-way ANOVA, using GraphPad Prism (Version 6.03). P value < 0.05 were considered as statistically significant.

RESULTS

LIGHT induces RANKL-independent osteoclastogenesis from SF macrophages

In 14-d cultures of SF macrophages incubated with M-CSF and RANKL, numerous TRAP$^+$ and F-actin ring$^+$ multinucleated cells were generated (Figure 1); these cells were capable of lacunar resorption when cultured on dentine slices (Figure 1). Cultures of SF macrophages with LIGHT and M-CSF under similar conditions also resulted in the formation of comparable numbers of TRAP$^+$ and F-actin ring$^+$ multinucleated cells (Figure 1A) and a similar level of lacunar resorption on dentine slices (Figures 1B). The addition of excess molar concentrations of OPG (100 ng/mL) did not result in a decrease in lacunar resorption pit formation compared with cultures treated with LIGHT alone (Figures 1B and C), confirming that LIGHT-induced osteoclast formation did not occur *via* the RANKL pathway. Resorption pits formed in LIGHT-treated SF macrophage cultures were smaller and more often single than in RANKL-treated cultures where most resorption pits were compound or confluent with many long resorption tracks being produced (Figure 1B). The overall percentage area of the dentine slice resorbed in LIGHT-treated cultures was comparable to that seen in RANKL-treated cultures (Figure 1C).

LIGHT augments RANKL-induced osteoclastogenesis from SF macrophages

In 14-d cultures of RA SF macrophages on dentine slices incubated with M-CSF and RANKL, a significant increase in lacunar resorption was seen compared with RA SF macrophage cultures treated with RANKL alone (Figure 2).

LIGHT in RA and OA synovial fluid

The concentration of LIGHT was significantly elevated in the synovial fluid derived from inflammatory RA compared with non-inflammatory OA joints (Figure 3). The mean level of LIGHT detected in the synovial fluid of RA patients was 452.5 ± 70.5 pg/mL. This was significantly higher (P < 0.05) compared with that found in non-inflammatory OA patients (45.43 ± 19.8 pg/mL).

Expression of LIGHT in RA and OA synovium

Immunohistochemistry showed that there was little or no expression of LIGHT in OA synovium, but in RA synovium LIGHT was strongly expressed by synovial lining cells and subintimal macrophages (Figure 4). Endothelial cells were also weakly stained, but lymphoid cells and fibroblasts were negative.

DISCUSSION

The canonical RANKL/OPG axis is the main pathway of osteoclastogenesis and is believed to play a major role in pathological bone resorption in RA[30,31]. RANKL expression is upregulated by inflammatory cytokines found in RA joints such as IL-1, IL-6, IL-17 and tumor necrosis factors (TNF)-α[30-32]. RANKL is a member of the TNF superfamily and it has been shown that other TNF superfamily ligands can induce RANKL-independent osteoclastogenesis. In this study we show that one of these factors, LIGHT, can substitute for RANKL and induce osteoclast formation from SF macrophages derived from RA joints. LIGHT-induced osteoclast formation was not inhibited by OPG and resulted in comparable levels of lacunar resorption to that seen in RANKL-treated cultures. LIGHT also augmented RANKL-mediated lacunar resorption and was found to be highly expressed in RA synovium and SF.

LIGHT is a potent RANKL-independent osteoclastogenic factor that has been shown in several studies to play a role in RA[13]. An increase in LIGHT levels has been noted in the serum of RA patients[14]. LIGHT is upregulated on B-lymphocytes and monocytes in RA and it has been shown to induce the expression of pro-inflammatory cytokines and metalloproteinases in macrophages and synoviocytes[33,34]. RA synovial fibroblasts express the LIGHT receptors HVEM and LTβR; LIGHT activates synovial fibroblasts, resulting in an increase in cytokines and growth factors that promote inflammation and resorption; LIGHT also induces the proliferation of RA synovial fibroblasts through LTβR[35,36]. It has also been shown that CD14$^+$ monocytes interact with stromal cells in RA synovium to induce the formation of TRAP$^+$ MNCs and that LIGHT enhances the generation of MNCs that release metalloproteinases including MMP9 and MMP12, both of which are found at sites of joint erosion in RA[37].

Our data indicates that LIGHT is likely to play a

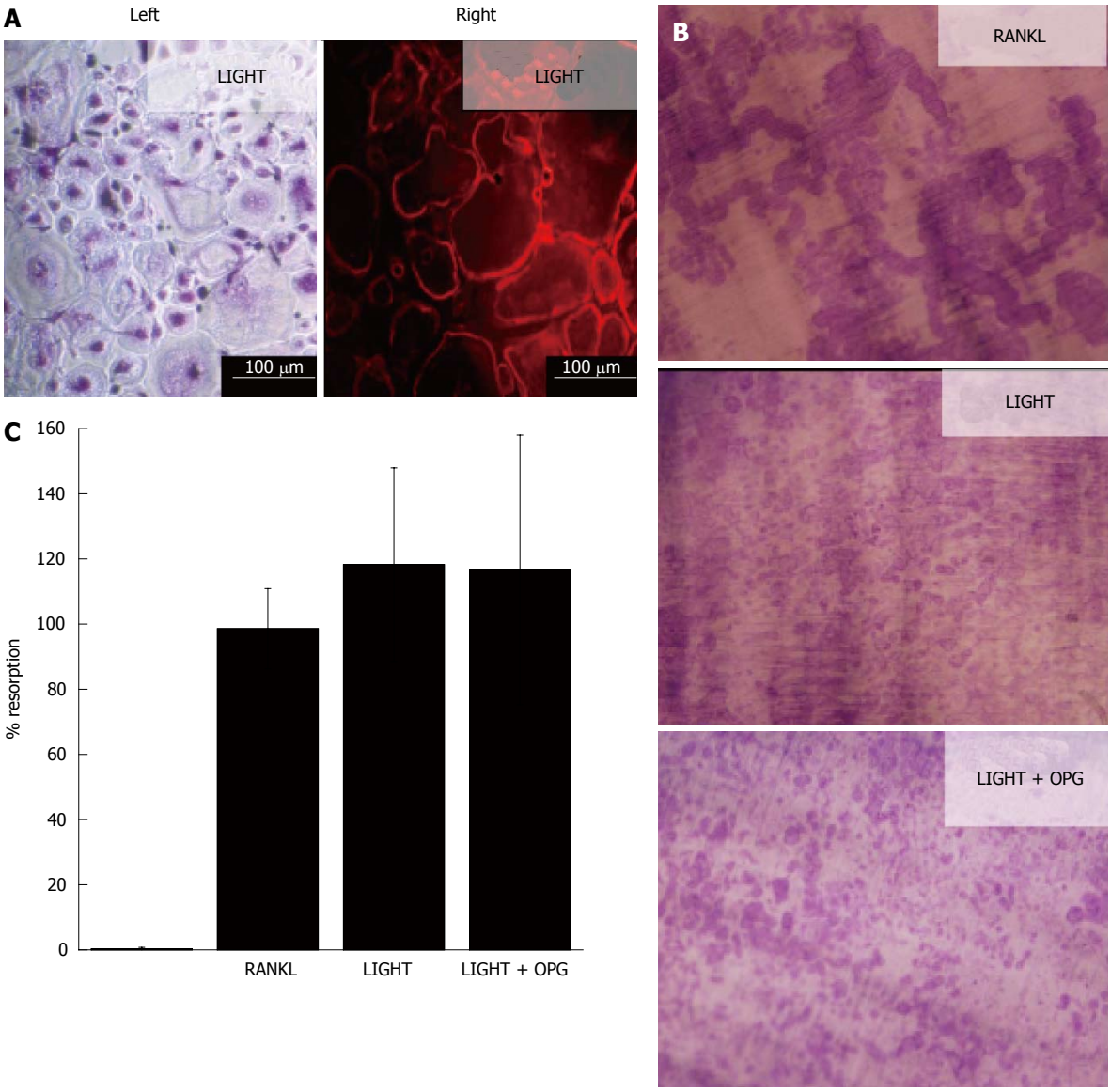

Figure 1 LIGHT induces receptor-activator nuclear factor kappa-B ligand-independent osteoclastogenesis from rheumatoid arthritis synovial fluid macrophages. A: Osteoclast differentiation in 14-d cultures of RA SF macrophages incubated with M-CSF and LIGHT showing (left) TRAP+ multinucleated osteoclasts and (right) F actin-ring formation; B: Dentine slices stained with Toluidine blue showing lacunar resorption in 14-d RA SF macrophage cultures treated with M-CSF and sRANKL, LIGHT or LIGHT ± OPG; C: Percentage surface area lacunar resorption on dentine slices in LIGHT- (± OPG) treated SF macrophage cultures relative to sRANKL - treated controls; data is expressed as mean ± SEM of three independent experiments where each condition was carried out in triplicate. RANKL: Receptor-activator nuclear factor kappa-B ligand; RA: Rheumatoid arthritis; SF: Synovial fluid; M-CSF: Macrophage-colony stimulating factor; OPG: Osteoprotegerin.

significant role in pathological bone resorption in RA. SF macrophages, like monocytes, are $CD14^+$ cells that are capable of differentiating into osteoclasts[28]. These cells are present in increased numbers in RA compared with OA joints and joint fluid, as are other inflammatory cells that are known to express LIGHT. This accords with our finding that the concentration of LIGHT is increased in RA compared with non-inflammatory OA joint fluid. There was little expression of LIGHT in OA synovium whereas it was strongly expressed in RA synovium mainly in synoviocytes and subintimal macrophages. The combination of an increased number of $CD14^+$ mononuclear phagocyte osteoclast precursors and high levels of LIGHT in the SF provides the conditions for LIGHT-induced osteoclastogenesis to exist in inflamed RA joints.

In keeping with previous studies[14], we found that LIGHT-induced osteoclast differentiation from RA SF macrophages occurred in the absence of RANKL. LIGHT-induced osteoclastogenesis was not inhibited by the RANKL inhibitor OPG and was comparable to that seen in RANKL-treated SF macrophage cultures in terms of formation of TRAP + MNC and lacunar resorption. We also found that LIGHT significantly augmented osteoclast formation in the presence of RANKL, in keeping with the findings of Ishida *et al*[37] who noted that LIGHT promotes RANKL-induced formation of TRAP + MNCs from $CD14^+$ precursors. Our findings indicate that LIGHT is therefore likely to play a role in both RANKL-dependent and RANKL-independent osteoclast formation and pathological bone

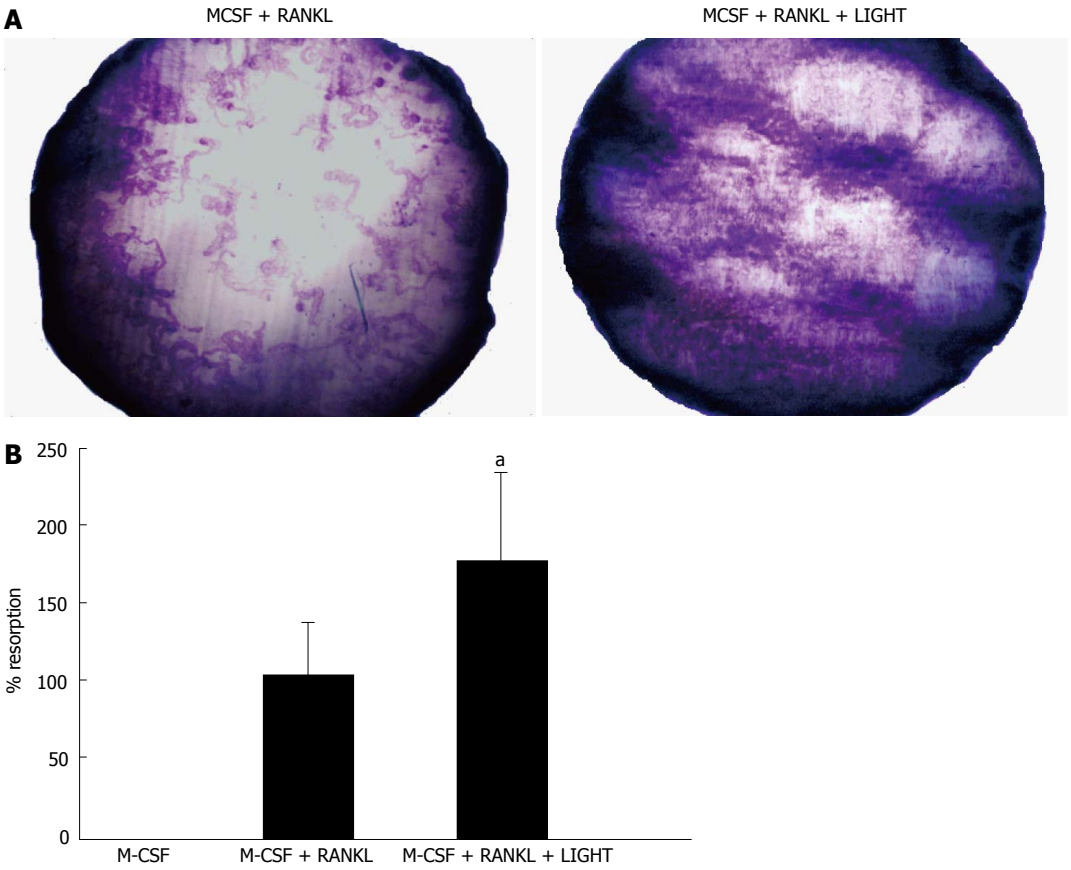

Figure 2 LIGHT augments receptor-activator nuclear factor kappa-B ligand-induced osteoclastogenesis from rheumatoid arthritis synovial fluid macrophages. A: Dentine slice stained with Toluidine blue showing lacunar resorption pits in 14-d RA SF macrophage cultures incubated with M-CSF and sRANKL ± LIGHT; B: Percentage surface area lacunar resorption on dentine slices in 14-d RA SF macrophage cultures incubated with M-CSF and sRANKL ± LIGHT. Data is expressed as mean ± SEM of three independent experiments where each condition was carried out in triplicate; $^{a}P < 0.05$. RANKL: Receptor-activator nuclear factor kappa-B ligand; RA: Rheumatoid arthritis; SF: Synovial fluid; M-CSF: Macrophage-colony stimulating factor.

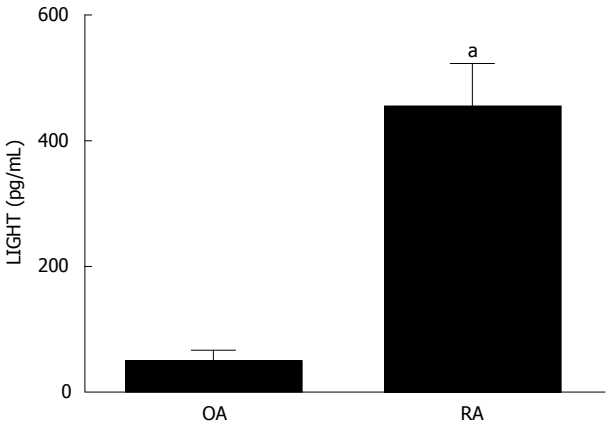

Figure 3 LIGHT levels are significantly higher in rheumatoid arthritis than osteoarthritis synovial fluid. LIGHT concentration is significantly increased in SF of RA patients compared with OA joints. Data is expressed as mean ± SEM of three independent experiments where each condition was carried out in triplicate; $^{a}P < 0.05$. RA: Rheumatoid arthritis; SF: Synovial fluid; OA: Osteoarthritis.

resorption in RA. In keeping with this conclusion, Fava *et al*[27] showed that prophylactic treatment with the LIGHT pathway inhibitor protein LTβR-Ig blocks induction of collagen-induced arthritis in mice and adjuvant arthritis in Lewis rats. There is likely to be a complex interplay between RANKL-independent and RANKL-dependent mechanisms in inflamed RA joints. The role that T cells and synovial fibroblasts play in RANKL/LIGHT-induced osteoclast formation is likely to be key given that these cells are known to express both RANKL and LIGHT. It has been shown the LIGHT contributes to the survival and activation of synovial fibroblasts in RA, resulting in pannus formation which promotes the generation of metalloproteinases, inflammatory cytokines and adhesion molecules[33-36].

RANKL-independent mechanisms of osteoclast formation induced by TNF superfamily members have been shown to play a role in the osteolysis associated with several neoplastic and non-neoplastic diseases of bone and joint, including giant cell tumour of bone, Ewing sarcoma, metastatic breast carcinoma, melanoma and myeloma[13,38]. LIGHT is the most potent TNF superfamily member identified to date that induces RA NKL-independent osteoclast formation. LIGHT is likely to represent a potentially useful therapeutic target in RA as inhibiting its action would not only reduce synovial inflammation but also LIGHT- and RANKL-mediated lacunar resorption, resulting in more complete healing of marginal erosions and preservation of periarticular bone in RA.

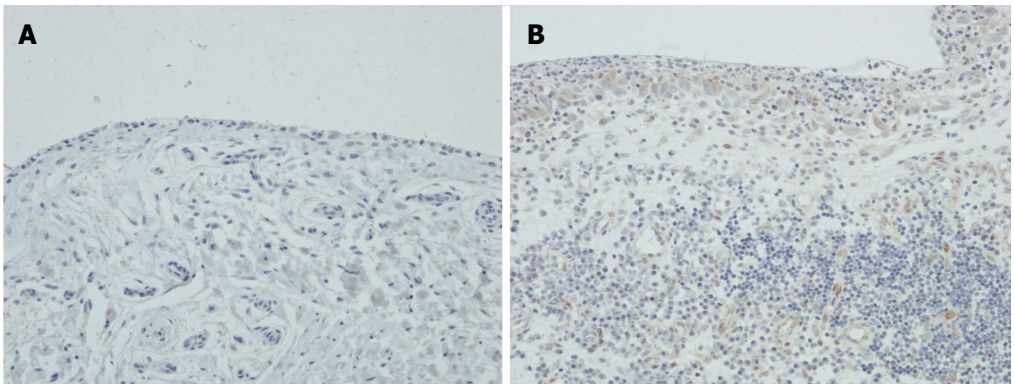

Figure 4 Expression of LIGHT in rheumatoid arthritis and osteoarthritis synovium. Immunohistochemical staining of (A) RA and (B) OA synovium, showing expression of LIGHT on synovial lining cells and subintimal macrophages in RA synovium with no staining for LIGHT in OA synovium. Magnification × 200. RA: Rheumatoid arthritis; OA: Osteoarthritis.

A number of studies have shown that Denosumab, a fully humanised antibody that specifically binds RANKL can be used to treat joint erosions and periarticular bone loss in RA[39-43]. Denosumab, however, unlike LIGHT, has no effect on joint inflammation[8,9,39]. It is well recognized that in the treatment of giant cell tumour of bone, withdrawal of Denosumab results in the re-emergence of osteoclasts with consequent osteolysis and regrowth of the tumour[44,45]. The use of Denosumab to treat bone loss in RA is also likely to encounter this problem as fibroblastic stromal cells that express RANKL would persist in the inflammatory environment. Following this therapy targeting LIGHT would therefore be useful in this context as it would not only inhibit the proliferation and activation of RANKL-expressing synovial fibroblasts but also reduce LIGHT- and RANKL-mediated bone resorption.

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COMMENTS

Background

Rheumatoid arthritis (RA) is characterized by a heavy lymphocyte and plasma cell infiltrate in the synovium, which leads to the formation of periarticular marginal erosions by specialized multinucleated cells, osteoclasts, which carry out lacunar bone resorption. Osteoclast formation and activation is controlled by a receptor-activator nuclear factor kappa-B ligand (RANKL)-OPG axis, in RA as well as RANKL-independent pathway. One of the RANKL-independent mediators of osteoclast activation is an activated T-cell product, known as LIGHT which is also known to be expressed by dendritic cells.

Research frontiers

Serum levels of LIGHT are increased in RA patients and blocking the action of LIGHT has been shown to reduce the severity of murine collagen-induced arthritis. It is uncertain whether LIGHT is involved in activation of RA synovial fluid macrophages to osteoclasts.

Innovations and breakthroughs

This is the first study evaluating the role of LIGHT in osteoclastogenesis of RA patients as well as demonstrating the increased LIGHT levels in synovial fluid of RA as compared to osteoarthritic patients.

Applications

The elevated levels of LIGHT in synovial fluid of RA patients and the ability of LIGHT to induce RANKL-mediated osteoclast activation indicate that this protein plays a significant role in pathogenesis of RA, which had not been previously reported.

Terminology

LIGHT is an abbreviation for lymphotoxin-like, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes) is the most potent RANKL (receptor activator for nuclear factor κB ligand) substitute identified to date, both of which are tumour necrosis family superfamily members.

Peer-review

The authors demonstrated the possible role of LIGHT in stimulating osteoclast resorption in synovial fluid macrophages. This type of activity was enhanced in combination of RANKL and macrophage-colony stimulating factor. Overall, the paper is logical and well-organized.

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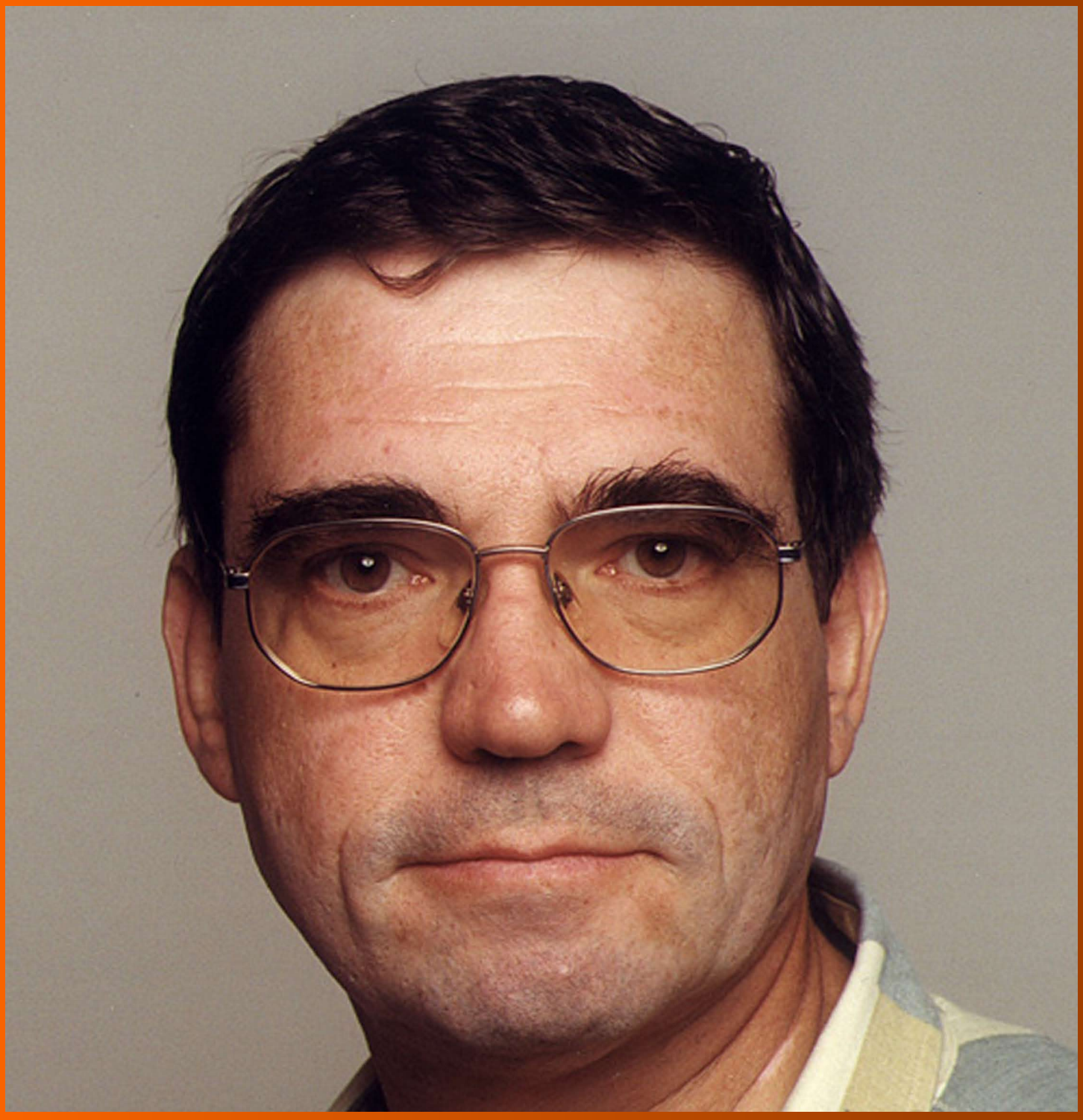

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REVIEW

Surgical and immune reconstitution murine models in bone marrow research: Potential for exploring mechanisms in sepsis, trauma and allergy

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Abstract

Bone marrow, the vital organ which maintains lifelong hemopoiesis, currently receives considerable attention, as a source of multiple cell types which may play important roles in repair at distant sites. This emerging function, distinct from, but closely related to, bone marrow roles in innate immunity and inflammation, has been characterized through a number of strategies. However, the use of surgical models in this endeavour has hitherto been limited. Surgical strategies allow the experimenter to predetermine the site, timing, severity and invasiveness of injury; to add or remove aggravating factors (such as infection and defects in immunity) in controlled ways; and to manipulate the context of repair, including reconstitution with selected immune cell subpopulations. This endows surgical models overall with great potential for exploring bone marrow responses to injury, inflammation and infection, and its roles in repair and regeneration. We review three different murine surgical models, which variously combine trauma with infection, antigenic stimulation, or immune reconstitution, thereby illuminating different aspects of the bone marrow response to systemic injury in sepsis, trauma and allergy. They are: (1) cecal ligation and puncture, a versatile model of polymicrobial sepsis; (2) egg white implant, an intriguing model of eosinophilia induced by a combination of trauma and sensitization to insoluble allergen; and (3) ectopic lung tissue transplantation, which allows us to dissect afferent and efferent mechanisms leading to accumulation of hemopoietic cells in the lungs. These models highlight the gain in analytical power provided by the association of surgical and immunological strategies.

Key words: Bone-marrow; Trauma; Repair; Transplantation; Surgery

Core tip: Bone-marrow generates multiple cell types which may play important roles in repair at distant sites. The use of surgical models in the characterization of this emerging function has hitherto been limited. Surgical strategies

allow nevertheless the experimenter to predetermine the site, timing, severity and invasiveness of injury; to add or remove aggravating factors (such as infection and defects in immunity) in controlled ways; and to manipulate the context of repair, including reconstitution with selected immune cell subpopulations. Here we review surgical models with great potential for exploring bone-marrow responses to injury, inflammation and infection.

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NEED TO EXPLORE THE EMERGING ROLES OF BONE MARROW IN RESPONSE TO SYSTEMIC INJURY

Bone marrow and repair of distant sites

Bone marrow is a vital organ, primarily because of its central role in adult hemopoiesis; accordingly, its failure requires lifesaving correction by hemopoietic cell transplantation[1-3]. In addition to maintaining steady-state hemopoiesis in healthy subjects, bone marrow supports emergency, or stress, hemopoiesis, *i.e.*, the lineage-selective expansion of hemopoietic cells to meet the exceptional demands of hemorrhage[4,5] or infection[6-9]. The cumulative evidence, however, highlights a third important function of bone marrow in whole-body homeostasis, namely its role in repair following injury of distant sites, especially the skin[10-13], Central Nervous System[14-22], eye[23-26], heart[27-32], lungs[33,34], liver[35-37], gastric mucosa[38,39], chronic wounds associated with diabetes and vasculopathy[40-46], oral mucosa and teeth[47-51], bone and cartilage[52-54] and skeletal muscle[55-59] among other structures.

This emerging role of bone marrow is discussed here as related, but not identical, to its better-known role in maintaining steady-state and emergency counts of blood cells and corpuscles. The following independent, but complementary, lines of evidence support a role for bone marrow in repair/regeneration of damaged extramedullary structures.

Developmental evidence: Bone marrow cells are capable of giving rise to a wide range of cell types which reconstitute parenchima of other organs, especially the brain and spinal cord[60-66], skeletal muscle[67,68], the heart[59-64], the liver[35,36] and skin[37-39].

Therapeutic evidence: Bone marrow cells have a beneficial effect on damaged organs in humans and in animal studies, including brain and spinal cord[14-17], heart[27-32] and skin[10-13]. The magnitude and duration of this benefit and the underlying cellular mechanisms, however, have shown important variations between studies, injured sites and experimental models, fueling sometimes long-standing controversies, such as that concerning the cellular mechanisms of therapeutic action in myocardial infarction[68-72].

Correlative evidence: Treatments which mobilize bone marrow cells in the context of bone marrow transplantation and emergency hemopoiesis[1-8], such as infusion of granulocyte colony-stimulating factor (G-CSF), have consistently beneficial effects on models of CNS[18-22], cardiovascular[73-78], and skin[79] injury, to name but a few, suggesting that the repair function of the bone marrow is integrated with its better understood roles in steady-state and emergency hemopoiesis.

Furthermore, sophisticated protocols have been developed to optimize the healing potential of bone marrow cells in some major incapacitating clinical conditions such as stroke[80] and diabetic chronic ulcers[40,46], lending further support to the view that bone marrow is indeed a source of highly diversified cell types for repair, capable of functional reconstitution (*i.e.*, of *regenerating* the injured tissue). Despite considerable advances made in this effort to improve over Nature, it remains unclear why, in the absence of these interventions, the reparative function of the bone marrow response to injury has such a limited impact on the functional recovery from stroke, myocardial infarction or chronic ulcers, all known to entail chronic disabilities to a variable degree.

Relationship of repair to immediate host defenses

An apparently less ambitious, but important, function of repair is to keep the host alive, even if total functional recovery through regeneration cannot be attained. This is particularly visible when a wound anywhere in the skin or mucosae creates an access into internal organs that poses a clear and present threat to survival, since blood can get out and germs can get in. Wound healing in previously healthy skin, such as typically is the case in surgery, begins with the vital process of blood clotting[81-86], and the clot is the primary organizing structure for wound healing[83,86]. Activation of the coagulation cascade is paralleled, when exposure to microbes or other triggers occurs, by activation of the complement cascade through the alternative pathway[87].

The variety of bone-marrow roles in repair

The neutrophils that clear the wound from invading bacteria[88] and the monocytes/macrophages that progressively transform that matrix into granulation tissue[89-91] are themselves bone marrow-derived. A great amount of evidence, however, further ascribes an important role in fibrotic (*i.e.*, permanent, as distinct from granulation tissue, which is transient) healing of wounds to cells that ultimately share a bone marrow origin: Blood-

borne fibrocytes[92,93] and myofibroblasts[94-96]. While this reinforces the view of bone marrow as exporter of vital parts for repair (and hopefully for regeneration), we understand but little of these complex processes. For instance, fibrocytes and myofibroblasts can also differentiate from resident cells[93,97], so the additional benefit provided by their circulating counterparts is not always obvious.

In addition to fibrosis, angiogenesis from bone marrow-derived endothelial progenitors in damaged tissues also contributes to nonregenerative repair in many contexts[98-100]. This is of conceptual interest because hemopoietic and angiogenic stem cells stem from an immediate common ancestor[101].

Indeed, angiogenesis may be intimately related to other events dependent on the bone marrow: Recent studies in humans and mice suggest that endothelial changes indicative of angiogenesis are among the earliest signs of immune damage to the lungs in the context of asthma, and even precede the arrival of eosinophils, which is one of the hallmarks of allergic inflammation; in addition, several lines of evidence suggest that production of the eosinophil-selective chemoattratant eotaxins by proangiogenic hematopoietic progenitor cells plays a major role in the subsequent development of TH2 polarization as well as in the accumulation of bone marrow-derived eosinophils in the lungs[102-105].

This hypothesis portrays angiogenesis and eosinophilia as distinct steps in the same sequence; furthermore, it suggests a close relationship between angiogenesis and extramedullary hemopoiesis, since the cell type which promotes angiogenesis is a specialized hemopoietic progenitor; finally, it deviates from the commonly held view that hemopoietic progenitor accumulation follows inflammation, advancing instead the view that hemopoietic progenitor accumulation promotes eosinophilic infiltration, which is part of allergic inflammation. Consistent with this view, colonization of the lungs by hemopoietic progenitors has also been shown in allergic disease models[106,107], suggesting that in situ production of some hemopoietic cell types from mobilized bone marrow progenitors participates in the systemic response to injury. This phenomenon, which accompanies immune-mediated local inflammatory responses, does not match the classical presentation of extramedullary hemopoiesis[108,109].

Although its biological significance remains incompletely understood (just as the role of proangiogenic hemopoietic progenitors, mentioned above, in the colonization) this phenomenon highlights the diversity of bone marrow reparative functions. Some studies attribute unique functions to these colonizing progenitors, including the maintenance of chronic inflammation in some experimental conditions[110]; it remains to be established, however, whether this duplicates the behaviour described above for proangiogenic hemopoietic progenitor cells[102-105], which encompasses the production of eosinophil-selective chemoattractants (eotaxins).

Specific immune responses promote both inflammation and repair through cytokines

As discussed below, in the context of surgical models associating surgery and allergic sensitization, hemopoietic progenitor colonization requires specific immune responses because these provide the required hemopoietic cytokines. This dependence of a chronic inflammatory process involving nonspecific mediators on a preexisting specific immune response is reminiscent of hypersensitivity granuloma formation, a type of cellular immune reaction that is long-lived in the tissues, and variably associated with angiogenesis, fibrosis and eosinophilia[111].

Relationship between the reparative and inflammatory aspects of bone marrow function in systemic injury

These distinct local processes (injury, blood clotting, activation of the complement cascade, acute inflammation with neutrophil infiltration, clearing of debris and apoptotic bodies by macrophages, granulation tissue formation and organization, fibrosis and epithelial regeneration) are often thought of as following each other smoothly in the ideal case of a sterile surgical wound. In these conditions, inflammatory mechanisms operate very effectively and for just as long as needed, so a surgical wound healing "by first intention" does not look very much like the textbook picture of inflammation as an unpleasant combination of redness, heat, swelling and pain, most often aggravated by some functional impairment.

This illustrates the paradox that if you notice inflammation, it is because it has not properly done its job of containing damage, preventing infection and preparing the regeneration of normal structure. Nevertheless, the cleanest of surgical wounds must still be handled with care, because it might reopen, bleed or get infected following mild mechanical trauma. Granulation tissue is well-known for its propensity for bleeding, which is at least in part accountable for by ongoing local angiogenesis[112]. Surgical wounds may also remain more sensitive to pain than normal tissue, long after surgery, which demonstrates the persistence of hyperalgesic mechanisms associated with long-term effects of transient exposure to inflammatory mediators such as prostaglandins, bradykinin and numerous cytokines[113-115].

All of this shows that, even in the absence of significant infection, trauma and damage inflicted to the tissue trigger low-grade (subclinical) inflammation, which eventually resolves, and ushers in epithelial and connective tissue repair. Bone marrow is an active participant throughout this long sequence, but its contribution varies over time, since it begins by supporting inflammatory mechanisms, and ends by helping repair and regenerative mechanisms. We will here focus on repair and regenerative mechanisms, discussing the inflammatory mechanisms only as factors impairing or promoting repair and regeneration, and highlighting

the usefulness of surgical models as experimental approaches to the reparative function of bone marrow in systemic injury.

SURGICAL MODELS AND THEIR VALUE FOR EXPLORING THE ROLE OF BONE MARROW RESPONSES TO INJURY

Defining a surgical model

Only a minority of the experimental approaches taken to probe the relationship of the bone marrow to repair at distant sites are surgical approaches, although it is virtually impossible to carry out surgery without causing some degree of injury, which in turn elicits repair. We define a surgical model, for the purposes of this review, as a systematic procedure using any combination of surgical techniques to study the contributions of bone marrow in the repair and/or regeneration of tissues distant from the bone marrow (*i.e.*, not contiguous to bone marrow, nor including any of it). Such definition therefore intentionally excludes healing processes to which bone marrow may contribute as part of a local response, such as may occur during repair of fractures in hemopoietically active bones.

An examination of the concrete example of bone healing sheds light on the reasons why these alternative scenarios are respectively converted or excluded by our definition. Bone marrow housed in axial skeleton of the adult[116], may contribute to the repair of distant bones which have no hemopoietically active marrow themselves, a description that fits most remaining bones.

This situation is covered by the definition, because it is assumed that no direct damage to the hemopoietically active bone marrow has occurred, and it must therefore have been called into action by some long-distance signal originating elsewhere. By contrast, in the situation where the hemopoietically active bone itself is damaged and its marrow has been involved to some extent, we no longer can distinguish between the effects of local factors that promote the adaptation and recovery from local injury, on the one hand, and the effects of systemically generated signals, on the other hand. This does not imply that no systemic signals or factors operate when a hemopoietically active bone is damaged and its marrow is involved; it implies, however, that this situation does not provide a useful surgical model for the role of bone marrow in systemic injury, since the model's value is linked to its ability to unambiguously dissect mechanisms.

Conceptual structure of a surgical model for the study of bone-marrow function in repair

Hence, in a useful surgical model afferent signals can be defined and controlled by the experimenter independently of the central organ (bone marrow) that responds to them; such signals originate in widely different structures, but uniformly elicit adaptations at the central organ which ultimately result in an output that is biologically meaningful (repair-promoting cells, for instance) and presumably delivered at the source of the signal. In the exploration of a surgical model, the location of the source of the afferent signal is less relevant than the nature and properties of this afferent signal, and the reactions of the central organ to it.

By placing an emphasis on afferent signals to which the central organ adapts by generating a biologically meaningful output (support at distance for repair), and by closing a loop in which the target of this beneficial response is the *same* bodily structure (the source of afferent signals) that conveyed the need for bone marrow help in the first place, the definition highlights the problem-solving value of surgical models for dissecting general mechanisms.

This value not only stems from the ability of the experimenter to unambiguously determine the site of injury which acts as a source of afferent signals; it is also reinforced by the experimenter's ability to collect and examine the output of the bone marrow on its way to this very site (which is, by definition, always known and always accessible). The latter feature allows the experimenter to examine the composition, properties and migration patterns of these multiple bone marrow-derived cell populations, which may shed light on their possible roles in repair and/or regeneration.

Below we will outline a variety of modifications of preexisting surgical protocols from our own and from other groups, aiming at the separate study of these aspects. In all but one of these models, the object of interest is bone marrow itself, not any the solid organs that can appeal to the bone marrow and benefit from its response.

Mice: Essential for immunological studies, underestimated for surgical models

Because bone marrow is easily studied in laboratory mice[117], this overcomes one of the usual limitations of experimental surgery, which is the need to work with experimental animals large enough to allow for handling of live solid organs *in vivo.* Mice offer undisputed advantages for immunological studies, including the availability of numerous conventional inbred strains and genetically modified or mutant strains, as well as of reagents which can be used to probe the roles of cytokines, receptors, mediators and leukocyte populations in bone marrow responses[118,119]. Furthermore, mice can be housed in small units, making experiments with many distinct treatment groups of genetically homogeneous animals routinely feasible in standard animal facilities at an affordable cost. All of the models discussed below are surprisingly simple, and do not require above-average surgical skills, which makes them accessible to most laboratories.

NONSPECIFIC AND SPECIFIC SIGNALS INFLUENCING BONE MARROW RESPONSE TO SYSTEMIC INJURY - WHAT IS KNOWN AND WHAT SHOULD BE KNOWN

The importance of matching output to demand

The conceptual sequence of afferent signal - central

adaptation - output matched to demand, has a number of aspects which have been insufficiently explored. For instance: Are the afferent signal and the export process totally unrelated, or, to the contrary, are the exported cells guided by the same kind of afferent signals that elicited their production in the first place? This is an apparently simple question, and the answer might be important, because matching the efferent product (the output) to the afferent stimulus would provide a simple and attractive mechanism of delivery.

Inflammation and repair as distinct phases in an evolving scenario

Inflammation and repair are different phases in the same continuum. While neither has a predetermined duration, inflammation precedes repair. Since injury is followed by inflammation (either sterile or compounded by infection), and inflammatory mechanisms, when successful, clear infection, remove debris and prepare the setting for repair, one might advance a simple hypothesis: Bone marrow continuously produces leukocytes, both polymorphonuclear and mononuclear, which find their way into injured sites very effectively[88]; other bone marrow-derived cell populations, with reparative or regenerative potential, just follow at latter times the trail of leukocytes into injured sites (for instance, by responding to the same chemoattractant signals). The prediction of this hypothesis would be that where there is inflammation, bone marrow will naturally deliver cells useful in repair.

However, inflammation and infection severely impair healing, as shown in many clinical and experimental settings[120,121]; in addition, clinical and surgical observations show that inflammation is usually on the way out before repair steps in[88,90,91,122-124]. This paradox prompts us to reject the hypothesis as originally formulated, and to revise it as follows: Ongoing inflammation and established infection severely impair healing, so that resolution of inflammation and elimination of pathogens must precede healing. The prediction of the revised hypothesis is that where inflammation has resolved, bone marrow can deliver cells useful in repair. Accordingly, signals originating in resolving inflammation, as distinct from signals originating in ongoing inflammation, should be relevant to bone marrow function in repair.

Resolution of inflammation and systemically active, nonspecific signals

One example of systemic signal known to be associated with resolution of inflammation that has a strong effect on bone marrow is G-CSF, which is believed to couple the rate of neutrophil death in inflammatory sites to the rate of neutrophil production in bone marrow[125]. G-CSF is one of the most effective mobilizers of cells in a variety of *in vivo* models of repair[18-22,73-79].

In addition, other nonspecific factors may provide afferent signals to request bone marrow support following systemic injury. Although they do not necessarily convey information that uniquely identifies the location of the source of the afferent signal, they might be proportionate to the magnitude or severity of injury (such as the area of a skin burn, or the volume of infarcted tissue, or to the degree of invasiveness, as defined by the rupture of internal barriers, and involvement of internal structures).

In addition to cytokines[125-130], adrenal glucocorticoids which promote hyperglycemia and insulin resistance[131-135], small polypeptide fragments of the activation of the complement cascade[87], and soluble intracellular molecules released during cell death, which are capable of activating a variety of receptors for damage-associated molecular patterns, or DAMPs[136,137] might play such roles. In the case of wounds exposed to a contaminated environment, in the skin or mucosae, chemical signals generated by receptors for pathogen-associated molecular patterns, or PAMPs[126,138], would possibly compound those arising in damage unrelated to infection. Chemokines are especially interesting because they attract many different cell types with high selectivity[88,123,139,140]. Chemokine gradients ensure a diffusible afferent signal that also identifies, as long as the gradient is maintained, the source of this signal, enabling the biologically relevant cell types exported by the bone marrow to reach this source and provide some benefit. Much information exists already about a sophisticated chemokine axis, which is known to control migration of stem cells in different physiological contexts, independently of tissue injury[139,140]. Whether a comparable mechanism underlies the reparative function of bone marrow is, therefore, an important issue for which there is no definitive answer yet, as discussed below.

Nonspecific signals are usually thought of as unrelated to adaptive (acquired) immune responses, and therefore lacking specificity and memory in the immunological sense. However, specific immune responses triggered by antigen or allergen involve release of cytokines[141,142]; while the stimulus is highly specific and amplified by memory, the output lacks both specificity and memory. The effects of specific immune responses are seldom discussed in the context of bone marrow function in surgical injury and wound repair. Nevertheless, specific immune responses may profoundly and durably imprint granulocyte production in bone marrow[143], suggesting the possibility of immunoregulatory influences on wound healing through bone marrow effects.

Open issues which need to be addressed

The many studies mentioned[10-59], concerning bone marrow contribution to repair and regeneration at distant sites, may suggest all important questions have already been answered, and there is nothing left to investigate. However, several basic aspects remain incompletely understood: (1) afferent signaling: How does the bone marrow detect damage outside the bone marrow? (2) selectivity: How does bone marrow adapt to meet specific demands related to the particular time, location, severity and type of injury by exporting the right cell type(s)? (3) delivery: How do the right cell types ex-

ported by the bone marrow get to the right place? (4) usefulness: How do these cell types help in repair and regeneration at the injured sites in a natural situation? (5) redundance *vs* complementarity: To what extent the bone marrow response duplicates or complements the repair and regeneration mechanisms that are intrinsic to each injured site? And (6) limits: What are the natural limits of bone marrow response in repair and regeneration and how can complex strategies help it overcome these limitations?

Most of the above issues (1-5) can be addressed experimentally, while the last one (6) is admittedly of a more philosophical nature. The three first issues (1-3) are discussed below in detail, because they can be effectively analyzed using in surgical models.

CECAL LIGATION AND PUNCTURE AS A SURGICAL MODEL AMENABLE TO MODULAR CONSTRUCTION AND ANALYSIS

The meaning of "model" and which variables matter in a surgical model

The term "model" is used here because we believe it reproduces in the laboratory a situation existing in real life. Cutting and suturing the skin of a mouse is, in this sense, a "model" of a moderate-severity surgical intervention in the skin, as often occurs in a variety of real-life situations. In this case, however, the focus in the model is on how this cutting and suturing, which is a basic common feature shared by all these real-life situations, affects the bone marrow and benefits from its help. This focus on an common denominator makes it irrelevant, for the experimental reasoning, where in the skin the wound was made (*i.e.*, the location or origin of the signal), while how much tissue was injured and to which depth (*i.e.*, the intensity or magnitude of the signal) remain clearly relevant.

One good illustration of these differences is provided by the immunoneuroendocrine response to trauma, a major factor intrinsic to surgical wounds at all sites. This response is not only stereotyped across a variety of sites, but is very similar to those elicited by a wide variety of physical and psychological stressors. This is characterized by increased circulating levels of adrenal glucocorticoids[131,133]. While much of the current literature on glucocorticoids tends to emphasize their anti-inflammatory and immunosuppressive effects, there is evidence that the stress response is an adaptive physiological response and that both it boosts immunity and stimulates repair processes[144-147]. Importantly, glucocorticoids have been for a long time discussed as having a deleterious effect on wound healing and fibrosis[148-150], although this effect is highly dependent on the clinical context and the timing of exposures[150,151]; an important correlate of this effect on wound healing is the strong evidence that glucocorticoids can trigger ulceration of the digestive mucosa, and probably contribute to the pathogenesis of stress ulcers[152]. If taken at face value, this would suggest the paradox that injury elicits an immunoneuroendocrine response which through glucocorticoid-dependent or glucocorticoid-mediated effects makes healing more, not less, difficult[153,154].

Metamorphoses of a "model"

Even though models begin as experimental systems designed to mimic a real-life situation, they soon become enriched by secondary aspects that no longer aim to reproduce anything in real life, but to help dissection of the mechanisms involved. A surgical "model" in which the skin is cut and sutured, but in which drugs or cells are injected, is no longer the simple imitation of dermatological surgery, but a controlled setting for testing hypotheses on the mechanisms mobilized by dermatological surgery, through the observations of the superimposed effects of pharmacological intervention or of selected regulatory cell subpopulations. Because this response is independent of the location and even of the type of injury, it is possible to evaluate it, in a separate set of experiments (which we term a thematic module) in a surgical model. Within this module, the possibility that at least some of the actions of glucocorticoid hormones promote repair and/or regeneration, thereby modifying the negative effects that have been classically identified, presents an interesting opportunity for research.

Modules help us organize the thinking about a surgical model, as shown in the following situations: The first open issue in our list, for example, concerns the nature of one or more afferent signals that trigger an adaptive response in bone marrow. These signals are likely to be generated as a consequence of injury, and play a role in alerting the organism as a whole about the damage inflicted on one of its parts. Many molecules with this general alarm function have been described, including several cytokines[125-131], along with products of the activation of the coagulation and complement cascades[87]. The issue is therefore not whether diffusible alarm signals connect injured sites to systemic responses, but rather whether one of the known molecules endowed with this function connects injured sites to stimulation of a bone marrow response promoting repair, in addition to inducing other well-characterized, coordinate effects on the central and peripheral nervous system, endocrine glands, liver and adipose tissue[125-131]. One important aspect of afferent signaling is that it represents an adaptation to injury, and is likely to cease once injury has been compensated by the local and systemic mechanisms it mobilizes. As such, the duration of afferent signals are a major (but not the only) determinant of the duration of the systemic response. In this respect, very little is known about the duration of bone marrow responses to injury at distant sites.

The second point in our list of open issues, that of the selectivity of response, is more complex, because it involves several distinct aspects of the response: Diversity, proportionality, context and invasiveness. Unlike the liver, which has a coordinate but stereotyped acute phase response to inflammatory cytokines, especially IL-6[155], bone marrow has a variety of ways to provide for the needs of injured sites at distance, so diversity of response is a central issue. To illustrate this issue with one concrete example out of many possibilities, it is unclear to what extent bone marrow responses to brain injury, on the one hand, resemble those elicited by damage to the skin, on the other hand. Along the same lines, it is also unclear whether distinct types of skin injury (exemplified by the clinically relevant cases and clearly distinct cases of sterile surgical wounds *vs* contaminated burns) elicit comparable responses from bone marrow[10-13,155]. In addition, even within a single type of skin damage, the invasiveness of the lesion may differ greatly, raising the germane issue of proportionality of the response to the severity of injury. Surgery, of course, offers a major experimental approach to the issue of proportionality, because the timing, location, size and depth of a surgical wound can be precisely controlled by the experimenter. The issue of context relates not to the bone marrow response *per se*, but to the background to which this response will be directed. Surgical wounds of comparable invasiveness can be inflicted to different interfaces of the organism with the environment, as exemplified by skin and oral mucosa. These have different structures, compositions, functions and immunological defenses, but share the features of being colonized by potentially harmful microorganisms and being subject to frequent mechanical injury. Most injuries to the skin and to the oral mucosa in subjects without an underlying disease heal within a short time, which testifies to the effectiveness of innate immunity as well as repair mechanisms at both locations, but tells us little about the relationship between immunity and repair at either site. Does the bone marrow response discriminate between surgical injuries inflicted upon the skin and the oral mucosa, to give a concrete example[47]? Simple as the question may seem, it has no clear-cut answer at this time, although it certainly deserves attention.

Surgery further provides an excellent approach to the issue of invasiveness within a single context (for instance, surgical access from the skin into underlying structures) since this involves qualitative as well as quantitative shifts. Sterile surgical opening of the skin can be the first step in invasion of internal spaces, such as the peritoneal cavity. Roughly speaking, this progression is a matter of quantitative increase in damage, only up to the point where the internal barrier presented by the peritoneal membrane is violated, thereby marking a quantal leap in periculosity as access to vital organs is obtained. In this case, invasiveness *per se*, *i.e.*, in the absence of infection, is the variable of interest. Does a deeper surgical wound, which provides access to the viscera, elicit a bone marrow response qualitatively distinct from that observed with a deep cut to the skin alone, or is it just a quantitative change? Even though this is a very straightforward issue, we do not have a clear-cut answer on that.

Cecal ligation and puncture is one surgical model which addresses a wide panel of variables in discrete modules

By taking this reasoning a little bit deeper, surgical injuries in this internal space - the peritoneal cavity - may compound the issue of invasiveness with that of life-threatening infection. Indeed, one of the most widely used models for studying sepsis in animals is cecal ligation and puncture (CLP)[156,157], a combination of invasive surgery exposing abdominal viscera, on the one hand, and direct mechanical attack on the intestinal containment structures (which are punctured at specific sites after cecal ligation), on the other hand (Figure 1 for a graphic summary of the procedure).

Interestingly enough, even this brutal invasion of a central space in the organism (which despite its crudity accurately reflects critical phases in the real-life situation of polymicrobial peritonitis resulting from a perforating wound to the abdomen) admits of degrees of severity, allowing us to distinguish between a sublethal procedure with a high rate of spontaneous recovery, and a so-called lethal procedure, which involves a higher microbial load in the peritoneal cavity but can nevertheless be successfully treated with aggressive antimicrobial therapy (Figure 1). By varying the number of puncture holes (Figure 1), the gauge of the needles used, and by providing antibiotic therapy, one generates distinct outcomes, ranging from full recovery to a uniformly lethal sepsis. Even more interestingly, the traumatic and the infectious components of the CLP procedure can be distinguished by injecting a controlled amount of cecal slurry in the peritoneal cavity, thereby bypassing the trauma of invasive surgery[158]. Although this modified protocol is proposed as a better alternative to CLP, it actually provides a very convenient alternative for the study of responses to trauma as opposed to responses to infection, which can itself be included as part of the modular structure of the CLP model.

CLP, therefore, is a versatile surgical procedure which provides many opportunities to study the impact of each these variables - anesthesia, external trauma, invasion of the cavity, manipulation of the intestine, perforation of the intestines, polymicrobial peritonitis and antibiotic treatment. Thanks to moderate severity and/or antibiotic treatment, CLP even provides a window on the "day after" when infection has apparently been eliminated and the organism is expected to go back to business as usual.

CLP followed by immune reconstitution provides an approach to long-lasting immunosuppressive mechanisms

Interestingly, many studies suggest that a protracted immunosuppressed state overshadows subjects surviving

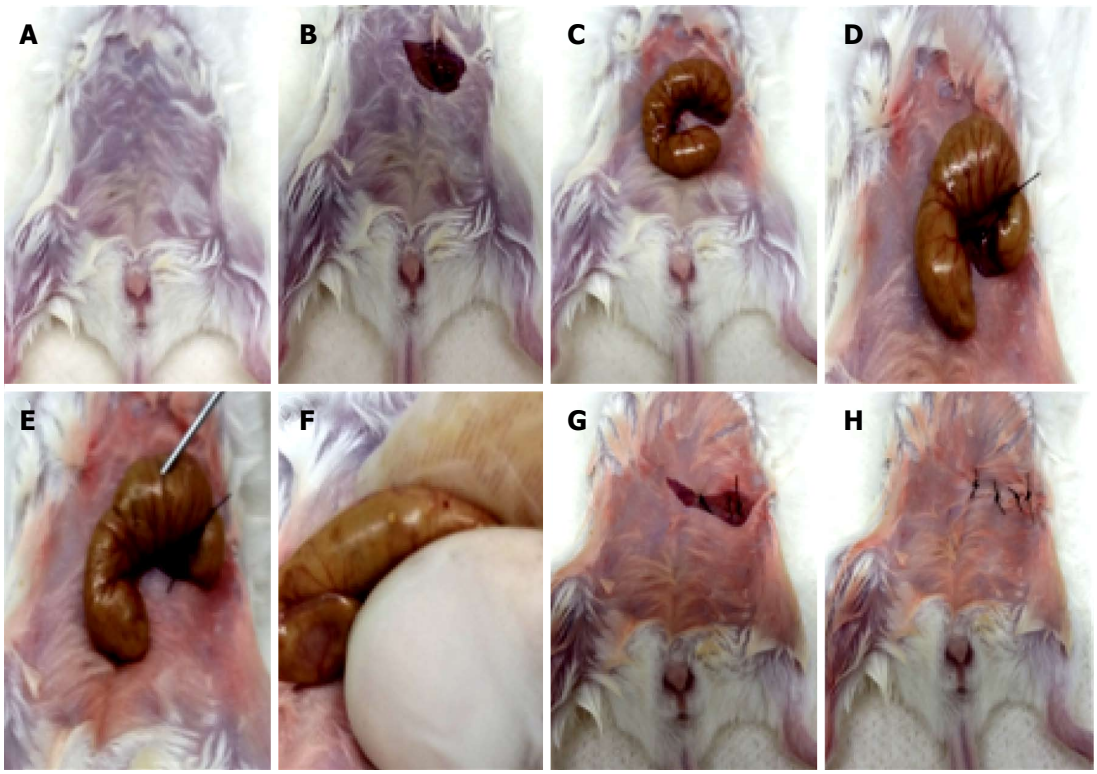

Figure 1 Main steps of the cecal ligation and puncture procedure. A: Mice are anesthetized with *i.p.* administration of ketamine (100 mg/kg) and xylazine (12 mg/kg); B: After local asepsis, a cut is made in the skin and in the peritoneal membrane, providing access to the peritoneal cavity; C: The caecum is externalized through the incision and handled outside the peritoneal cavity; D: The caecum is ligated with suture thread right underneath the ileocaecal junction, but not so tightly that intestinal obstruction will ensue; E: The caecum is perforated with a needle, either on the proximal wall only [sublethal cecal ligation and puncture (CLP)] or completely transfixing (lethal CLP); F: The caecal contents are squeezed through the single (sublethal CLP) or double (lethal CLP) perforations; G: The caecum is repositioned inside the peritoneal cavity in its original location; H: The peritoneal membrane and the skin are sutured and the animals are undergo recovery from anesthesia protected from hypothermia and corneal damage or exposure to direct light. For sham-operated controls, steps D-G are omitted.

sepsis[158-160]. To what extent this immunosuppression may reflect long-term adaptations in bone marrow function - which is essential for appropriate defenses against infection - remains to be established, but is undoubtedly a relevant, open issue. It is clear, however, that bone marrow-derived cells, especially neutrophils, play a key role in the immunological deficits associated with sepsis[161-163]. Here, again, it is important to distinguish between the effects of sepsis as a whole[161] and the effects of the trauma component[162], even though the cellular target is the same (neutrophil). Of course, the observation of long-term immunosuppression in the sepsis-survivors, and the known fact that neutrophils are short-lived in the circulation and thereby replaced through fast and intense neutropoiesis in the bone marrow[8,125,163,164] prompts the hypothesis that a bone marrow adaptation to the context of sepsis might contribute to this vulnerable state. Strategies of immune reconstitution, involving, for instance, myeloablation followed by bone marrow transplantation or adoptive transfer of neutrophils from normal syngeneic donor mice might therefore enrich what is already a very interesting surgical model.

Therefore, infection and the associated immunological dysfunction that overshadows the aftermath of sepsis play privileged parts in this surgical model (CLP). It is important to point out, in this respect, that infection and immunity are known to affect the bone marrow in many respects, but very little is known about how either affects the ability of bone marrow to support a systemic response to injury (as distinct from a systemic response to infection).

Modular structure of CLP-based models

This highlights the importance of adapting current surgical models such as CLP to separately focus on each of these aspects - local injury, systemic trauma, invasion, infection, immunity - through a more elaborate design. The multiplicity of variables to be studied can only be managed rationally by isolating each one of them in a thematic module, which is embodied in the appropriate experimental and control groups. A surgical sepsis model, such as CLP, can therefore unfold as a large modular construct (Figure 2). Due to the number of groups involved and the amount of work it brings, this may present a formidable challenge to the experimenter; all the same, it remains a fascinating challenge. Although we use CLP here as particularly suitable example of a complex surgical model that allows us to separately dissect important variables in discrete modules, this reasoning can easily be adapted to other surgical models, such as those discussed at later sections.

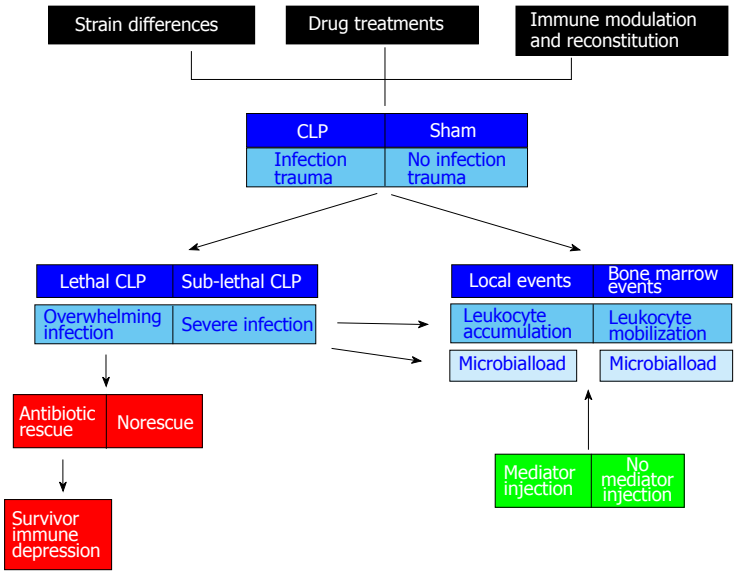

Figure 2 Modular distribution of multiple variables of interest in the cecal ligation and puncture surgical model. The core parts of a cecal ligation and puncture (CLP) experiment (blue boxes) can be subdivided in modules that deal with trauma plus infection (CLP) or trauma without infection (sham). In both cases, events at the site of surgical injury (local events), *i.e.*, the peritoneal cavity, and at a distant site (bone marrow events) can be observed in the same animals at any given time. Observation allows the experimenter to monitor progress of the host reaction (leukocyte accumulation in the peritoneal cavity; leukocyte mobilization from the bone marrow) as well as the dissemination of infectious pathogens (microbial load) at both sites. To this core, addition of preoperative modules (black boxes) involving a choice of different wild-type and mutant mouse strains (strain differences), a variety of prophylactic agents (drug treatments) and immunological interventions (immune modulation and reconstitution) considerably enriches the model in experimental possibilities. The issues of severity of infection (overwhelming infection vs severe infection, blue boxes at the left) and of the long-standing immune depression following recovery in antibiotic-rescued mice (red boxes at the left) are treated as separate modules of the trauma plus infection sector. Addition of postoperative modules (green boxes at the right) allows us to analyze the curative effect of mediators which restore mobilization of leukocytes from bone marrow into the peritoneal cavity, in mutants lacking 5-lipoxygenase (5-LO), or in wild-type mice preoperatively given inhibitors of the 5-LO pathway. In such a surgical model, every animal undergoes surgery, but the addition of genetical, pharmacological and immunological variables greatly enriches the model in its investigative power.

CLP provides novel insights of the bone marrow response to systemic injury

Recent observations in our group have shown how versatile and interesting the CLP model is for the study of bone marrow function in systemic injury. While CLP is one of the most intensively studied models of sepsis worldwide, little of the research focuses on the bone marrow events. We have been able to detect three major events in sepsis (Xavier-Elsas *et al*, manuscript in preparation) by looking at murine bone marrow in sham-operated (trauma and invasion of the peritoneal cavity, but no perforation) and CLP mice (all of the preceding, plus perforation and polimicrobial peritonitis): (1) A decrease in bone marrow neutrophil counts, which is accompanied by an increase in peritoneal exudate neutrophil counts, during the first 24 h following surgery; (2) The lack of a significant decrease in bone marrow neutrophil counts, and a significant decrease in peritoneal exudate neutrophil counts, in the same period of observation, when the CLP mice lack functional 5-lipoxygenase (5-LO), the indispensable enzyme in the synthesis of leukotrienes[165-167]; (3) The correction of the defective response of 5-LO-deficient mice, both with respect to decrease in bone marrow neutrophils and increase in peritoneal neutrophils, by *i.p.* administration of leukotriene B_4, a powerful neutrophil chemoattractant generated through the 5-LO pathway; and (4) The presence of bacteria in bone marrow of 5-LO-deficient mice 24 h after CLP, but not in bone marrow of wild-type CLP controls.

Bone marrow is therefore intensely involved and deeply affected in surgical sepsis models. Much of what we see is a response to infection, not to trauma, because the appropriate (sham-operated) controls, contained in a separate module that isolates on the surgical trauma component (Figure 2), show no significant decrease in bone marrow neutrophil counts, and only a minor neutrophil accumulation in the peritoneal exudate.

These observations suggest that the decrease in neutrophil counts in bone marrow is due to a rapid mobilization of mature neutrophils to blood and ultimately to infected sites, especially the primary focus of infection, inside the peritoneal cavity. The main argument for this hypothesis is that neutrophils in peritoneal exudate are increased over the same period, although the numbers of neutrophils lost from bone marrow are somewhat higher than the numbers of neutrophils acquired by the peritoneal exudate. An additional argument is that both events are prevented by a single change in the system, namely the inactivation of 5-LO. Finally, this is reinforced by restoration of both events by a single procedure, namely the administration of exogenous LTB_4 to 5-LO-deficient mice, which lack endogenous production of LTB_4. Overall, the evidence is that bone marrow releases neutrophils in large numbers during the initial 24 h of CLP-induced sepsis, which for the most part enter the

initial focus of infection and successfully fight it (the observations were done with a sublethal CLP protocol, in which survival is the rule). Importantly, this critical mobilization function is highly dependent on 5-LO, and the key 5-LO product was shown to be LTB_4.

In addition to the mobilizing cytokines such as G-CSF, a wide variety of neutrophil chemoattractants exist, which are expected to be generated in the context of sepsis, including C5a from activation of the Complement system through the alternative pathway[168,169], cytokines such as TNF-α[170,171], and chemokines, including MIP-1α and MCP-1[172,173]. In addition, the CXCL12 (SDF-1)-CXCR4 chemokine axis, which plays an essential role in homeostatic maintenance of the hemopoietic niche in bone marrow[174], and in the phenomena of stem cell/progenitor mobilization and homing to injured tissues[175], may also be important for the large-scale mobilization of neutrophils from the bone marrow reserve pool into peripheral blood, in experimental sepsis[176]. With so many apparently redundant systems, it is rather unexpected that a specialized, nonredundant, role is played by 5-LO in mobilization of neutrophils from bone marrow in the CLP model.

An equally unexpected finding is the detection of bacteria inside bone marrow *in vivo,* in mice lacking appropriate mobilization, since this shows that timely mobilization effectively protects this vital structure in an early phase of sepsis. It also raises the issue of whether bacterial invasion of bone marrow is more than a biomarker of severity - is it a factor that prevents further mobilization, and possibly further dysregulates host defenses? The thoughtful exploration of the CLP model and its multiple variants might shed some light on this important problem.

EWI MODEL ALLOWS US TO DISTINGUISH BETWEEN NEUROENDOCRINE AND IMMUNOLOGICAL FACTORS IN BONE MARROW RESPONSE TO TRAUMA AND ALLERGY

Egg white implants induce eosinophilic inflammation through antigen-specific mechanisms

Intense and chronic eosinophilia induced by subcutaneous heat-coagulated egg white implants (EWI, for short) was first described by Professor Mario Mariano and his associates[177-179]. It remains a most interesting phenomenon, although much of the underlying mechanisms remains incompletely understood. EWI proved very effective as a means of sensitizing to ovalbumin, as shown by vigorous eosinophilia in lung interstitium and in bronchoalveolar lavage fluid of mice receiving EWI in the dorsum and challenged with purified ovalbumin by the respiratory route[178]. These morphological changes were accompanied by the functional abnormalities common to murine asthma models, including airway hyperreactivity[178]. The cellular composition and kinetics of the inflammatory infiltrates at the ovalbumin challenge site resemble those of the late phase in type Ⅰ hypersensitivity reactions, so the authors proposed it would be a good experimental model for the late phase reaction[177]. EWI induces ovalbumin-specific cytophilic IgG1 and IgE antibodies, but the latter become detectable only after ovalbumin challenge[177,178]; to our knowledge, a role for mast cells in the development of the eosinophilia has not been established. Eosinophilia at the challenge site (lungs) and in the bone marrow was shown, paralleled by measurements of eosinophil peroxidase activity in the tissues[177,178]. By contrast, a role of cellular immunity in the phenomenon has been demonstrated by adoptive transfer of lymph node lymphocytes, which induce eosinophilia following ovalbumin challenge in the recipients[177]. Whether these are IL-5-secreting TH2 lymphocytes has not been formally established, to the best of our knowledge. Importantly, the eosinophilia in the lungs and bone marrow was sensitive to oral tolerance induction[179]. This procedure targets T and B cells and decreases specific antibody titers in the EWI model, especially IgE titers[179]; by contrast, in conventional sensitization/challenge protocols, the effect of oral tolerance induction on specific IgE and IgE titers was modest, while adoptive transfer protocols showed a major impact on cell-mediated specific immunity[180].

Open issues in the EWI model

Further characterization of the EWI model might nevertheless prove informative, since two distinct variables are relevant here: (1) the nature of the allergen (ovalbumin); and (2) the physical state of the allergen (an insoluble pellet with heat-denatured protein). Recognition of allergen epitopes during peritoneal (or airway) challenge with native ovalbumin by cells sensitized by heat-denatured allergen following EWI points to T cells as the critical factor promoting eosinophilia, as T cell epitopes, unlike those recognized by serum antibody, are preserved even after partial proteolysis and heat denaturation of protein antigens[141].

EWI is also of interest in a discussion of surgical models because it necessarily involves moderate-severity surgical trauma in the absence of infection (Figure 3)[181]. Even in this comparatively simple context, distinct modules allow us to dissect the role of trauma and the role of allergen. Several independent lines of evidence support the view that adrenal glucocorticoid hormones surge in the first 24 h after surgery, both in sham-implanted controls (full surgery but no allergen) and EWI recipients (full surgery and allergen implant)[181]. This is accompanied by significant bone marrow eosinophilia, showing that increased glucocorticoids, rather than killing eosinophils inside bone marrow, stimulate their production. Glucocorticoid surge (but not baseline) levels, are required for the eosinophilia of bone marrow in this model, both in sham-implanted controls and EWI-recipients, as shown by three independent approaches. However, as specific

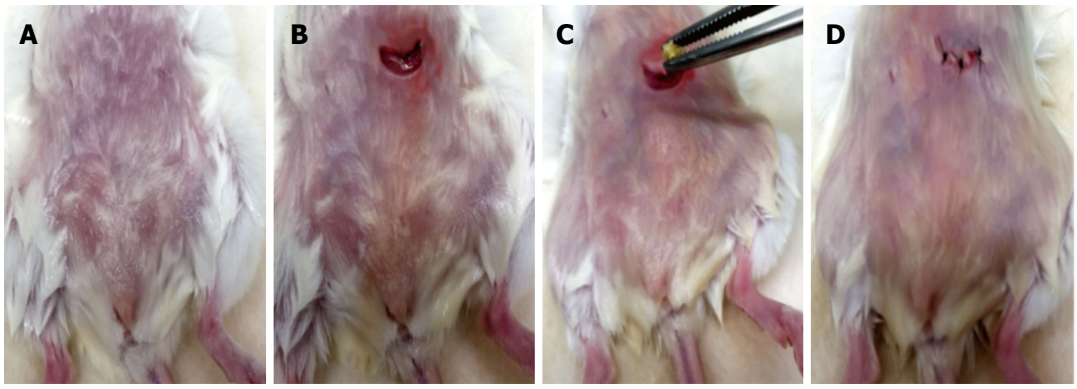

Figure 3 Main steps of the intraperitoneal modification of the egg white implant procedure. A: Mice are anesthetized with *i.p.* administration of ketamine (100 mg/kg) and xylazine (12 mg/kg); B: After local asepsis, a cut is made in the skin and in the peritoneal membrane, providing access to the peritoneal cavity; C: A pellet of heat-coagulated egg white is placed through the opening in the peritoneal cavity; D: The peritoneal membrane and the skin are sutured and the animals are placed to recover from anesthesia with care to avoid hypothermia and corneal dryness or undue exposure to direct light. For sham-implanted controls, step C is omitted.

sensitization to the allergen pellet progresses over the first two weeks, eosinophilia subsides in the sham-implanted controls but persists and increases in the EWI-recipients, showing that, in the latter, it is driven by specific immunity[181].

Interestingly, the nonspecific bone marrow eosinophilic response is demonstrable in the sham-implanted controls up to two weeks after surgery; such a period is considerably longer than the duration of the glucocorticoid surge. This raises the possibility that transient rises in glucocorticoid levels due to the surgical trauma induce persistent effects on bone marrow cells, through reversible modifications in chromatin structure ("epigenetic" effects), similar to those described by other studies of trauma and stress[182,183].

Immune reconstitution strategies might also enrich the multiple possibilities of the EWI model. The effects of glucocorticoids on bone marrow eosinophilia *in vivo* (which are directly relevant to the EWI model) are variable among strains. A systematic screening showed that while wild-type C57BL/6 controls (B6) respond to glucocorticoid administration with bone marrow eosinophilia (thereby mimicking the effect of surgical trauma alone in the EWI model), perforin-deficient knockout mice from the same background lack this response. Reconstitution of the glucocorticoid-induced eosinophilic response is achieved through transfer of splenic T lymphocytes from wild-type (but not from perforin-deficient) donors to perforin-deficient recipients[184]. Other strains, not restricted to the B6 background, were also shown to lack an eosinophilic response to exogenous and/or endogenous glucocorticoid exposure in the bone marrow (manuscript in preparation). To our knowledge, none of these unresponsive strains has been studied using the EWI model, but it is of obvious interest to study a response to insoluble allergen pellets, previously shown (in wild-type mice) to be driven by an acute glucocorticoid surge, in mutant mice lacking this eosinophilic response to glucocorticoids, especially if reconstitution of both the bone marrow response and the eosinophilia at the implant site can be achieved by adoptive transfer of immunoregulatory lymphocyte populations.

Another open issue is whether eosinophilia in the EWI model is accompanied by fibrosis. Studies from many groups suggest a relationship between eosinophils and eosinophilia, on the one hand, and fibrosis resulting from a wide variety of pathological processes[185-192], on the other hand. This relationship has been proposed for eosinophilic esophagitis[188], toxoplasmosis[191], schistosomiasis[111], and the extensive remodelling of the airways associated with the chronic phase of asthma[189,190]. Airway remodelling, not easily reversed, involves many different pathobiological components, including angiogenesis, thickening of basal membrane, hyperplasia of mucus-secreting ("goblet") cells, smooth muscle cell proliferation, increased collagen deposition, among others[191,193]. Hence, its fibrotic component, which is part of a much more complex scenario, is consistent with the view of airway remodelling as a misguided repair process. Because it develops in the presence of chronically infiltrating eosinophils, but is abolished by eosinophil depletion[191], eosinophils would appear to promote fibrosis, at least in these experimental conditions. It should be noted that eotaxin, the eosinophil-selective chemoattractant[194] and enhancer of eosinopoiesis in the bone marrow[195], is strongly involved in fibroblast-eosinophil interactions[192], although its main contribution may lie in recruiting eosinophils, rather than in activating fibroblasts. Eotaxin promotes eosinophil production in the bone marrow indirectly, through secondary production of cysteinyl-leukotrienes, a potent proallergic series of 5-lipoxygenase derivatives[195]. These lipid mediators were shown to induce the gp130-signaling cytokines[190,193,196], IL-6 and IL-11, which have strong profibrotic actions of their own[188]. So far, the evidence that eosinophils are associated with a tissue composition that evolves into fibrosis is strong; by contrast, definitive evidence that they are a major driving force in fibrotic processes is lacking.

Addressing the cellular mechanisms of eosinophilia and fibrosis in the original version of the EWI model would be difficult because it involves introduction of the allergen under the skin, which leads to accumulation of infiltrating eosinophils in solid tissue; as a consequence,

laborious and expensive tissue excision/dissociation and cell separation techniques are required to isolate the eosinophils from the lesion and to study their properties, as well as their relationship to fibroblasts, fibrocytes and myofibroblasts isolated from the same site.

How mutations and reconstitution strategies targeting immunological factors increase the analytical power of the EWI model

A minor modification of the original EWI protocol - namely introducing the allergen pellet in the peritoneal cavity (Figure 2) - has recently allowed us to recover eosinophils and other infiltrating cell types from the site of the implant by a simple peritoneal lavage, which is fast and quantitative. This modification allows us to study the properties of these eosinophils and their ability to promote fibrosis. It also facilitates the characterization of other cell types present at the same site. In this respect, a further modification of the EWI model has allowed us to precisely define the specificity of the T cells responding to the insoluble allergen, since EWI induces eosinophilia in DO11 transgenic mice of the BALB/c background, which have an essentially monoclonal T cell response to a peptide of ovalbumin associated with an autologous Class Ⅱ molecule (I-A^{d})[197]. It is very convenient that in wild-type mice as well as in DO11 transgenic mice the eosinophilia induced by ovalbumin sensitization is abolished by oral tolerance induction, thus providing a further control for the specificity of the eosinophilia in the modified EWI models. A third minor modification of the original protocol - keeping the original implant site (subcutaneous) and attracting eosinophils to the peritoneal cavity by local challenge with ovalbumin - has already allowed us to study the mechanisms of their accumulation[198] in response to allergen, and provides an obvious alternative setting for comparison, which should be informative on the issue how much the physical state of the allergen influences the outcome.

In principle, EWI can be studied in the absence of eosinophilia as well. In this case, dblGATA-1 mutant mice, which lack eosinophils, can be studied following EWI, since eosinophilia is not expected, but inflammation of other sorts is likely to the develop as a result of ovalbumin sensitization. Immune reconstitution of dblGATA-1 mice with purified eosinophils from normal donors can help us understand which features of the model are dependent on eosinophilic inflammation.

ECTOPIC LUNG TISSUE TRANSPLANTATION PROVIDES NOVEL INSIGHTS OF BONE MARROW FUNCTION IN LUNG DISEASE

Hemopoietic cell colonization of the lungs: A puzzling response to allergen challenge

The colonization of the lungs by hemopoietic progenitors committed to the eosinophil lineage, following allergen exposure of sensitized subjects, which parallels the accumulation of mature eosinophils in the same organ, has been described in human and animal studies[106,107,110,198,199]. It is a less conspicuous result of the allergic reaction, not only because progenitors in the challenged lungs are largely outnumbered by mature infiltrating eosinophils, but because progenitors are defined by their developmental potential, rather than by a unique morphology or surface phenotype. A progenitor, independently of its hemopoietic lineage, is a relatively rare cell type in bone marrow or peripheral blood, phenotypically distinct from a stem cell[200], which in the presence of the appropriate hemopoietic cytokine environment gives rise to a clonal growth in semisolid media[143,195]; this amplification potential was, for a long time the main reason why progenitor colonization of the lungs has received so much attention[110,201]. More recently, however, this was reinforced by evidence that these progenitors may be important in ways unrelated to proliferation, such as a strong proinflammatory activity due to secretion of cytokines and other mediators[202].

Ectopic lung tissue transplantation as a highly creative surgical model

We next summarize what has been learned about the underlying mechanisms using a specific surgical model. This model - ectopic tissue transplantation in the peritoneal cavity[199] - is somewhat more challenging than CLP or EWI, not because it requires greater surgical ability, but because it involves tissue transplantation, hence a particular donor-recipient combination, established through a surgical procedure. Of course, clinical lung transplantation substitutes presumably healthy whole lungs for diseased ones, in the anatomically correct (orthotopic) site, and this is a challenge for the surgeon in many respects, as the ultimate goal is to restore as much as possible normal respiratory function and correct the secondary cardiovascular and hematological abnormalities, such as pulmonary hypertension and polycytemia. In this surgical model, however, none of these complexities is involved, because the recipient's lungs remain untouched; instead, a piece of lung tissue is placed into an anatomically incorrect (ectopic) cavity (peritoneal rather than thoracic) and no effort is made to make it function as a respiratory organ. So, it this "model" does not mimic a meaningul situation in clinical lung transplantation, why should we even mention it?

The answer is that the use of ectopic lung tissue transplantation to explore bone marrow roles in systemic injury is not intended to reproduce clinical lung transplantation; instead, it provides important insights of little-understood allergic processes. Allergic processes associated with transplantation have consistently been reported in humans, both in the context of bone marrow and hemopoietic cell transplantation and of solid organ transplantation, especially of liver, but also of heart, pancreas and lungs[203-208]. Such observations suggest that transmission of an asthma-like experimental disease of the lungs through lung tissue transplantation can be achieved. Ectopic transplantation of lung tissue was

conceived as a rather crude, but effective, experimental approach to the hypothesis that lung releases some "asthma-inducing" mediator(s). Similar strategies were successfully used in the functional characterization of thymus (which is transplantable under the kidney capsule) as well as various endocrine glands; this success reflects the fact that these structures export cells or molecules to the general circulation, not necessarily restricted by a precise anatomical connection to a particular outlet.

Ectopic lung tissue transplantation is easy to perform because the lower lobe of the right lung is anatomically accessible and can be handled individually; the lung lobe remains viable for the duration of the experiment and releases a number of mediators, including the cytokines, IL-5 and eotaxin, in the peritoneal lavage fluid[199]. In many respects the transplanted tissue behaves as a sponge imbibed into a soup of mediators; of course, the procedure does not mimic a meaningful situation in lung transplantation, because we are implanting damaged tissue into a recipient which has perfectly healthy lungs in the right place.

Despite its artificialy, the ectopic lung tissue transplantation model allows us to analyze the entire procedure as a sequence in which separate modules address variables which: (1) operate in the donor alone; (2) operate in the recipient alone; and (3) originate in the surgical procedure. The outcome of interest (accumulation of eosinophil progenitors in the recipient's own lungs) is dependent on both donor-related and recipient-related variables, but can only be detected through the surgical procedure. It is observed only when lung tissue from sensitized and airway-challenged donor mice is surgically implanted into the peritoneal cavity of histocompatible recipient mice which have been sensitized but not challenged[199]. Hence the outcome requires events of all three classes: (1) those operating in the donor alone (sensitization and challenge); (2) those operating in the recipient alone (sensitization without challenge); and (3) those that bring together the two preceding contexts, through surgery, thereby adding trauma, anesthesia and other factors to an already complex scenario.

This outcome is very unexpected, and prompts us to reexamine a number of assumptions. The issue here is how matching of output to demand in the responses of bone marrow to systemic injury is achieved; in other words, which mechanisms underlie an effective delivery of bone marrow-derived cells at the injured *site* among many uninjured sites in the same tissue or organ. The ectopic lung tissue transplantation model is therefore concerned with location of the source of afferent signals and with its relationship to the output from bone-marrow.

There is published evidence of repair of lung by bone marrow-derived cells[209,210]; logically, this demonstration requires that the target organ has been somehow damaged. By contrast, the entry of bone marrow cells (eosinophil progenitors) into uninjured lungs, as evidenced in the ectopic lung tissue transplantation model is not expected, and is likely to be missed by the experimenter, if the experimental design does not address this possibility. The observation that an injured piece of lung tissue, placed inside the peritoneal cavity, somehow promotes the colonization of healthy lung tissue by eosinophil progenitors suggests that signals emanating from injured lung tissue promote the mobilization of eosinophil progenitors from bone marrow, but that these colonize lung tissue that is untouched by both surgery and allergy. Perhaps this is made possible by a constitutive process of lung colonization by progenitors that occurs in the absence of damage[211]; if so, these progenitors are unlikely to call anyone's attention by their proinflammatory actions[202]. At any rate, the observation suggests that matching delivery of bone marrow cells to the exact site that was injured is only one of several possibilities, and that some bone marrow cells may be mobilized and ultimately recruited into healthy tissues as well, provided injured tissue releases an afferent signal.

INVITATION TO EXPLORE A HIGHLY CREATIVE FIELD

Surgical models have just arrived at an intersection of many exciting aspects of immunology, experimental pathology and pharmacology, and they contribute something that has received comparatively little attention in these highly competitive fields of research - namely, a focus on simple experiments on living animals, with the goal of dissecting variables that affect the entire body.

Surgical models combine the advantage of little competition with the thrill of creativity in experimentation. A surgical model can be rich enough in itself, as is the case of CLP; or look more like a curiosity, as is the case of EWI; or even appear as something exotic, bordering on the esoteric, as ectopic lung tissue transplantation. What makes these all three surgical models interesting and potentially useful is their power of adaptation to research in immunology, experimental pathology and pharmacology.

This adaptation is accomplished by expanding each model through the inclusion of novel variables (such as, to name but a few, sensitization and challenge; drug administration; transfer of immunologically relevant cell subpopulations; mutations affecting the immune response), which can be studied separately as the subjects of experiments-within-the-experiment (our, as we prefer to call them, thematic modules). Because our group, coming from a long-term commitment to bone-marrow research, has been pleasantly surprised by the convenience of these three models to approach complex issues in a simple way, we hope this summary of our experience will encourage others to pursue the exploration of surgical models in their own specialized fields of interest.

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MINIREVIEWS

Multifunctional biomimetic spinal cord: New approach to repair spinal cord injuries

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Abstract

The incidence of spinal cord injury (SCI) has been gradually increasing, and the treatment has troubled the medical field all the time. Primary and secondary injuries ultimately lead to nerve impulse conduction block. Microglia and astrocytes excessively accumulate and proliferate to form the glial scar. At present, to reduce the effect of glial scar on nerve regeneration is a hot spot in the research on the treatment of SCI. According to the preliminary experiments, we would like to provide a new bionic spinal cord to reduce the negative effect of glial scar on nerve regeneration. In this hypothesis we designed a new scaffold that combine the common advantage of acellular scaffold of spinal cord and thermosensitive gel, which could continue to release exogenous basic fibroblast growth factor (BFGF) in the spinal lesion area on the basis of BFGF modified thermosensitive gel. Meanwhile, the porosity, pore size and material of the gray matter and white matter regions were distinguished by an isolation layer, so as to induce the directed differentiation of cells into the defect site and promote regeneration of spinal cord tissue.

Key words: Spinal cord injuries; Glial scar; Hydrogel materials; Basic fibroblast growth factor; Acellular scaffold

Core tip: Traumatic spinal cord injury often leads to serious consequences and also adds great burden to families and society. Usually people believe that the regeneration of lost tissue is limited after central nervous system injury. Due to these reasons, we would like to provide a new bionic spinal cord to reduce the negative effect of glial scar on nerve regeneration. We design biomimetic spinal

cord by the combination of basic fibroblast growth factor modified thermosensitive hydrogel and acellular spinal cord scaffold, which is conducive to the designation of a three-dimensional composite scaffold more suitable for cell growth, and corresponding mechanical properties and biodegradability more close to the structure of normal spinal cord.

Liu Y, Li Q, Zhang B, Ban DX, Feng SQ. Multifunctional biomimetic spinal cord: New approach to repair spinal cord injuries. *World J Exp Med* 2017; 7(3): 78-83 Available from: URL: http://www.wjgnet.com/2220-315X/full/v7/i3/78.htm DOI: http://dx.doi.org/10.5493/wjem.v7.i3.78

INTRODUCTION

Spinal cord injury (SCI) is a central nervous system disease that is mainly manifested in sensory-motor dysfunction, incontinence and sexual dysfunction below the plane of SCI[1]. Clinically, trauma-caused SCI is common. Rehabilitation of SCI is an unsolved medical problem, in that the regeneration ability of the human central nervous system is extremely low, and a variety of pathophysiological activities and metabolites are involved in the changes in the microenvironment at the injured site, which is not conducive to axonal regeneration.

Repair of SCI with nerve tissue engineering aims at repairing the injured nerve by loading seed cells into the injured site with scaffold as carrier or implanting new tissue[2]. But it is difficult to repair SCI by tissue engineering, possible reason may be that its regenerative capacity is much lower than that of peripheral nerves, the structure of the spinal cord is complex at the same time[3]. Due to the complexity of the structure and composition of the human spinal cord, traditional single scaffold for spinal cord cannot completely simulate the macro and micro structure of the spinal cord; therefore, the development of bionic spinal cord has become a hot research topic.

HYPOTHESIS

It is difficult to repair SCI by routine tissue engineering scaffolds because of the spinal cord's low regeneration ability and its complex structure, and the traditional single spinal cord cannot simulate the macro and micro structure of the spinal cord. For the above reasons and the basis of the present work, a tissue-engineered spinal cord was designed in this hypothesis by combining the common advantage of acellular scaffold of spinal cord and thermosensitive gel, which could continue to release exogenous basic fibroblast growth factor (BFGF) in the spinal lesion area on the basis of BFGF modified thermosensitive gel, meanwhile, the porosity, pore size and material of the gray matter and white matter regions were distinguished by an isolation layer (Figure 1), so as to induce the directed differentiation of cells into the defect site and promote regeneration of spinal cord tissue.

EVALUATION OF THE HYPOTHESIS

Glial scar and inhibitory molecules

Mechanical violence in acute SCI includes traction and compression. Direct compression is caused by spinal fracture and dislocation, intervertebral disc and ligament injury, leading to vascular damage, axonal degeneration and disintegration, the apoptosis of neurons, astrocytes and oligodendrocytes, *etc*[4]. Slight bleeding occurs in grey matter within several minutes after injury; within a few hours, the injury rapidly spreads to the upper and lower segments of the injured spinal cord along the axial direction. Several minutes after injury, when spinal cord swelling constricts the central canal and the pressure in the spinal cord exceeds the pressure in blood vessels, local secondary ischemia occurs. Moreover, neurogenic shock after the injury aggravates spinal cord ischemia, which further causes hypoxia and leads tissues to produce and release toxic products, resulting in a series of effects of cascade and amplification damage. There are some important cellular responses after SCI. For example, astrocytes divide and proliferate to "scar-like" astrocytes; the myelin sheath splits into fragments; precursor cells of microglias and oligodendrocytes proliferate and migrate to the site of injury. Therefore, gliacytes, astrocytes, oligodendrocytes, oligodendroglia, precursor cells and microglias are detected at the site of injury. In addition, these cells have an inhibitory effect on axonal regeneration. Mature oligodendrocytes produce nogo and MAG, and the precursor cells of oligodendrocytes produce proteoglycans and NG2, which are all inhibitory molecules[5,6]. Astrocytes may promote axon growth in non-injured CNS and immediately after the injury; however, several days after the injury, they begin to produce a series of inhibitory proteoglycans. Generally, microglias play a role in the promotion of axonal regeneration, but produce various toxins to kill neurons and damaged axons after stimulation[7]. Due to considerable inhibitory molecules, the application of the therapy with all these molecules neutralized is quite difficult.

Structure of spinal cord

The internal structure of the spinal cord is composed of gray matter and white matter. Located in the center of the spinal cord, the gray matter is shaped as a symmetrical butterfly seen in cross-section, composed of various neural cells. The gray matter can be divided into anterior, lateral and posterior horns. There are a large number of motor neurons in the anterior horn. The lateral horn contains sympathetic nerve cells and the posterior horn contains sensory nerve cells. Composed of longitudinal nerve fibers for conduction, the white matter is located around the gray matter. These nerve fibers are mainly composed of corticospinal

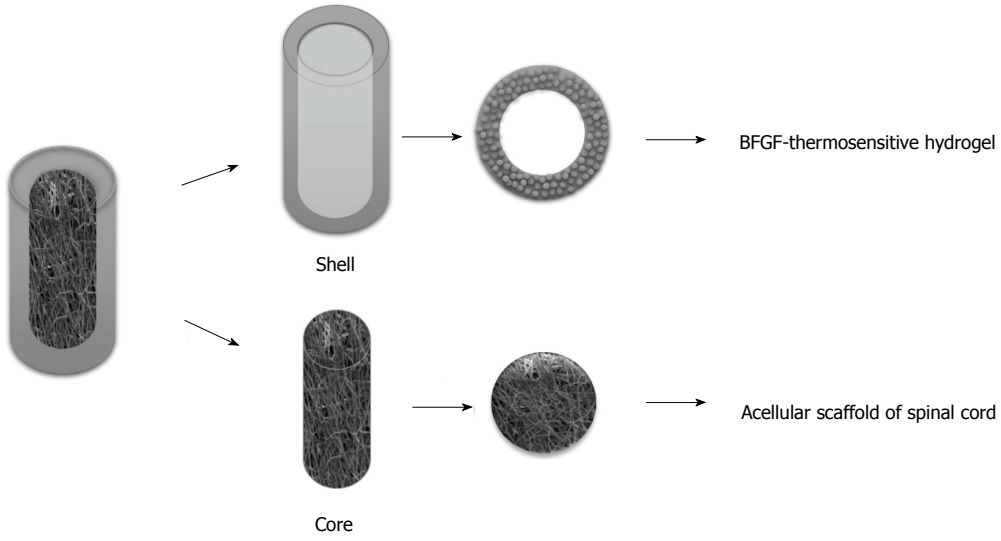

Figure 1 Construction of biomimetic spinal cord. BFGF: Basic fibroblast growth factor.

tracts, namely the motor nerve fibers for the conduction from the brain to the spinal cord, and thalamic tracts, namely the sensory nerve fibers for the conduction from the spinal cord to the brain. The design idea of the partition-type artificial spinal cord is to correctly guide the regeneration and extending of the main descending fiber tracts according to the original position of the spinal cord[8-10]. And the idea also intends to adjust the deacetylation degrees of chitosan in the outer wall of the catheter and of partition chitosan between the partitions in the catheter in the chitosan production, in order to make the partition chitosan degrade in a short time after the beginning of spinal cord regeneration and facilitate the regenerated spinal cord to horizontally form a neural network. And the outer wall of the catheter should degrade after the spinal cord regeneration to block the invasion of foreign non-nerve tissues.

Hydrogel materials

Hydrogel materials are characterized by high water content and similar mechanical properties to collagen in the spinal cord, which is the major structural protein of human. As an important component of extracellular matrix, collagen in the spinal cord has a gene sequence of arginine-glycine-aspartic acid with cell adhesion signal, which promotes the adhesion of seed cells to scaffold, and the differentiation and migration of seed cells. The axons of the organism are favorable for the attachment to the collagen scaffold, and thereby promoting the regeneration of axons[11]. In the site of SCI, collagen can also carry growth factors to regulate the local microenvironment and reduce scar formation, which is conducive to the recovery of the injury. So hydrogel materials are often used in the implantation of scaffold into the spinal cord. However, these regenerated nerve fibers are disorganized, and collagen scaffold cannot lead regenerated nerve fibers to caudal tissue through the injured site to form complete neural pathway[12]. It has also been reported that an overly high collagen concentration in the injured site inhibits the growth of axons.

BFGF

As a neuropeptide substance, BFGF plays an important role in embryonic development, angiogenesis, wound healing, and the growth and development of nervous system in the organism, and is a novel neurotrophic factor that has been frequently studied in recent years[13]. Moreover, BFGF not only has a nutritional effect on a variety of neurons cultured *in vitro*, but also can promote the regeneration of injured peripheral nerve *in vivo*, which has been evidenced by studies. Research has demonstrated that the expression of c-fos mRNA in spinal cord neurons increases, while BFGF inhibits the expression of *c-fos* gene after SCI, suggesting that BFGF may have a protective effect on nerve in SCI. Haenzi *et al*[14] have found that after SCI, early continuous administration of exogenous BFGF may play an important role in the protection of the area of SCI, promoting the recovery of spinal cord function. Furthermore, research has demonstrated that after SCI, early continuous administration of exogenous BFGF may significantly protect the area of SCI, significantly decrease calcium accumulation and edema in the injured area, decrease magnesium ion loss and its degeneration, obviously alleviate SCI, and enhance the recovery of spinal cord function.

Acellular scaffold of spinal cord

Acellular allogenic grafts is a tissue scaffolds produced by artificial extraction and decellularization, *etc.* It is widely used to substitute natural biomaterial scaffold in the studies of tissue repair[15]. The protein and other substances in the tissue were removed by chemical method. Then the antigen-free acellular tissue scaffold was obtained. This scaffold has the advantages of good

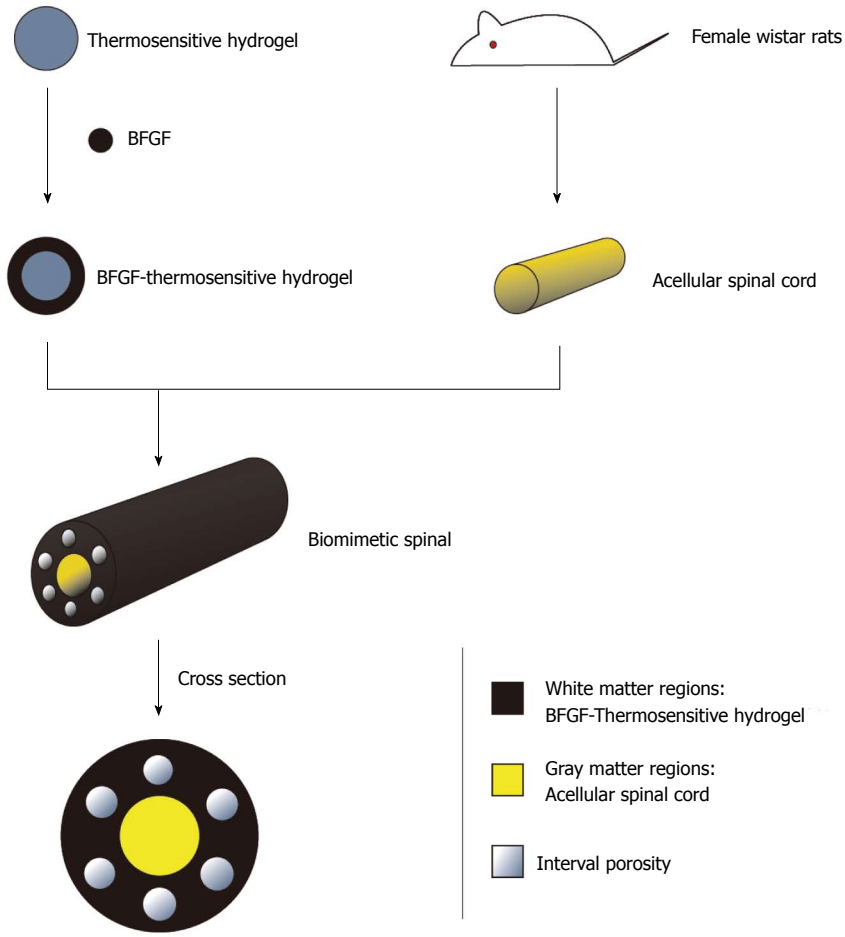

Figure 2 Technology road mapping. BFGF: Basic fibroblast growth factor.

biocompatibility, low immunogenicity, and it is convenient to manufacture. When implanted into the body, it can provide seed cells with the growth space similar to their *in vivo* niche. Fresh sciatic nerve was removed, then the cells and other parts of the sciatic nerve tissue was taken off by Triton X-100 and sodium deoxycholate through chemical extraction, and the fibrous skeleton as well as the basement membrane have been left. The loose three-dimensional porous structure left by the nerve cells can be viewed under the electron microscopy. This scaffold was transplanted into the body, and 20 d later, compared with the control group without extraction, the extracted groups contained more microvessels and nerve axons through the injury area. The motor function has been greatly improved in the extracted groups[16]. Hudson *et al*[17] and Rovak *et al*[18] subsequently demonstrated this scaffold causes little immunological rejection after transplantation in a large number of acellular nerve allografts in rodent. Hu *et al*[19] used the bone marrow stromal cells of acellular allogenic nerve grafts to repair long-segment ulnar nerve defects of a primate. The repair effect is similar to autologous transplantation in 6 mo after surgery[19]. Ban *et al*[20] frozen and thawed the spinal cord tissue, then prepared acellular spinal tissue scaffold by modified chemical extraction. The appearance of the scaffold is comparable to that of the normal spinal cord. It is in a translucent villous shape, and the axons of the tissue scaffold and the auxiliary cells are successfully removed, leaving the loose three-dimensional porous structure. Its flat structure is constituted by the different sized gaps which are longitudinally parallel or irregularly arranged in a channel-like way and are connected to each other with a high degree of emulation. These structures can provide a natural guide for the regeneration of the axon. Regenerated axons can effectively pass through the lesion area, so to provide the conditions for the coupling of regenerated nerve and terminal nerve tissue. Moreover, co-culture with neuronal cells has proved its excellent biocompatibility[20].

Although there are many advantages, acellular scaffold of spinal cord is difficult to undertake the second modification process. A variety of measures have been taken to try to regenerate the spinal cord nerve fibers, however, the result is that this kind of regeneration is a disordered growth or extension, and the repair effect is not ideal. Therefore, it is necessary to correctly guide the orderly extension of the regenerated nerve fibers in the specific division of the original fiber bundle so as to achieve better repair purposes[21]. Different configurations of scaffolds for tissue engineering affect the effect of nerve regeneration to a great extent, including the upstream and downstream fiber bundles

on the macroscopic and microscopic axonal growth. Nevertheless, the scaffold material has pores, even single or multiple conduits at present, the location of these holes or catheters is random relative to the structure of the spinal cord, and not consistent with the histological structure of the gray and white matter of spinal cord, not to mention the correspondence with major fiber tracts in white matter[22]. In this regard, upstream and downstream bundles, which are distributed in the white matter of the spinal cord, are regenerated in the scaffold material, they can only grow in mismatched or even misplaced pipes or micropores, in this way, the regenerated nerve fibers can still not grow and extend regularly in the corresponding region, but grow in disorder, the upstream and downstream regenerated fibers are hence twisted into a group to affect the extension of other fibers or the migration of neurons, greatly affecting the recovery effect. Thus, the design and construction of configuration of the artificial scaffold material consistent with the gray and white matter of the spinal cord, as well as upstream and downstream fiber bundles of the white matter is one of the prerequisites for tissue engineering to repair SCI and also a key problem to be solved urgently, which may improve the repair effect of SCI significantly.

CONCLUSION

A single scaffold material is often difficult to have the ideal characteristics of spinal tissue scaffold material at the same time, the study of composite biomaterials made of two or more than two kinds of materials has hence become a hot topic in the research of spinal cord tissue engineering. Composite biomaterials can make up for the deficiency of single material and retain the characteristics of raw materials, which is conducive to the designation of a three-dimensional composite scaffold more suitable for cell growth, and corresponding mechanical properties and biodegradability more close to the structure of normal spinal cord (Figure 2). This method will provide new ideas for clinical treatment of SCI.

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ORIGINAL ARTICLE

Basic Study

Treg/Th17 cell balance and phytohaemagglutinin activation of T lymphocytes in peripheral blood of systemic sclerosis patients

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Abstract

AIM

To investigate T-cell activation, the percentage of peripheral T regulatory cells (Tregs), Th17 cells and the circulating cytokine profile in systemic sclerosis (SSc).

METHODS

We enrolled a total of 24 SSc patients and 16 healthy controls in the study and divided the patients as having diffuse cutaneous SSc (dcSSc, n = 13) or limited cutaneous SSc (lcSSc, n = 11). We performed a further subdivision of the patients regarding the stage of the disease - early, intermediate or late. Peripheral venous blood samples were collected from all subjects. We performed flow cytometric analysis of the activation

capacity of T-lymphocytes upon stimulation with PHA-M and of the percentage of peripheral Tregs and Th17 cells in both patients and healthy controls. We used ELISA to quantitate serum levels of human interleukin (IL)-6, IL-10, tissue growth factor-β1 (TGF-β1), and IL-17A.

RESULTS

We identified a decreased percentage of CD3+CD69+ cells in PHA-stimulated samples from SSc patients in comparison with healthy controls (13.35% ± 2.90% *vs* 37.03% ± 2.33%, $P < 0.001$). However, we did not establish a correlation between the down-regulated CD3+CD69+ cells and the clinical subset, nor regarding the stage of the disease. The activated CD4+CD25+ peripheral lymphocytes were represented in decreased percentage in patients when compared to controls (6.30% ± 0.68% *vs* 9.36% ± 1.08%, $P = 0.016$). Regarding the forms of the disease, dcSSc patients demonstrated lower frequency of CD4+CD25+ T cells against healthy subjects (5.95% ± 0.89% *vs* 9.36% ± 1.08%, $P = 0.025$). With regard to Th17 cells, our patients demonstrated increased percentage in comparison with controls (18.13% ± 1.55% *vs* 13.73% ± 1.21%, $P = 0.031$). We detected up-regulated Th17 cells within the lcSSc subset against controls (20.46% ± 2.41% *vs* 13.73% ± 1.21%, $P = 0.025$), nevertheless no difference was found between dcSSc and lcSSc patients. Flow cytometric analysis revealed an increased percentage of CD4+CD25-Foxp3+ in dcSSc patients compared to controls (10.94% ± 1.65% *vs* 6.88% ± 0.91, $P = 0.032$). Regarding the peripheral cytokine profile, we detected raised levels of IL-6 [2.10 (1.05-4.60) pg/mL *vs* 0.00 pg/mL, $P < 0.001$], TGF-β1 (19.94 ± 3.35 ng/mL *vs* 10.03 ± 2.25 ng/mL, $P = 0.02$), IL-10 (2.83 ± 0.44 pg/mL *vs* 0.68 ± 0.51 pg/mL, $P = 0.008$), and IL-17A [6.30 (2.50-15.60) pg/mL *vs* 0 (0.00-0.05) pg/mL, $P < 0.001$] in patients when compared to healthy controls. Furthermore, we found increased circulating IL-10, TGF-β, IL-6 and IL-17A in the lcSSc subset *vs* control subjects, as it follows: IL-10 (3.32 ± 0.59 pg/mL *vs* 0.68 ± 0.51 pg/mL, $P = 0.003$), TGF-β1 (22.82 ± 4.99 ng/mL *vs* 10.03 ± 2.25 ng/mL, $P = 0.031$), IL-6 [2.08 (1.51-4.69) pg/mL *vs* 0.00 pg/mL, $P < 0.001$], and IL-17A [14.50 (8.55-41.65) pg/mL *vs* 0.00 (0.00-0.05) pg/mL, $P < 0.001$]. Furthermore, circulating IL-17A was higher in lcSSc as opposed to dcSSc subset (31.99 ± 13.29 pg/mL *vs* 7.14 ± 3.01 pg/mL, $P = 0.008$). Within the dcSSc subset, raised levels of IL-17A and IL-6 were detected *vs* healthy controls: IL-17A [2.60 (0.45-9.80) pg/mL *vs* 0.00 (0.00-0.05) pg/mL, $P < 0.001$], IL-6 [2.80 (1.03-7.23) pg/mL *vs* 0.00 pg/mL, $P < 0.001$]. Regarding the stages of the disease, TGF-β1 serum levels were increased in early stage against late stage, independently from the SSc phenotype (30.03 ± 4.59 ng/mL *vs* 13.08 ± 4.50 ng/mL, $P = 0.017$).

CONCLUSION

It is likely that the altered percentage of Th17 and CD4+CD25-FoxP3+ cells along with the peripheral cytokine profile in patients with SSc may play a key role in the pathogenesis of the disease.

Key words: Systemic sclerosis; T-cell activation; Th17; Tregs; CD4+CD25-Foxp3+ cells; Interleukin-17; Tissue growth factor-β; Interleukin-10; Interleukin-6

Core tip: Systemic sclerosis (SSc) is a devastating autoimmune disorder, which can be subclassified into limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) based on the skin manifestations. One of the original contributions of our study has demonstrated a decreased capacity for PHA-induced peripheral T-cell activation in patients with SSc. For the first time, our research group has identified an up-regulated percentage of CD4+CD25-FoxP3+ cells in the dcSSc subset. Regarding the peripheral cytokine profile in SSc, the serum levels of interleukin (IL)-17A have been increased in lcSSc as opposed to the dcSSc subset. The rest of our data, concerning the elevated circulating IL-6, IL-10, and TGF-β in SSc patients, has confirmed literature-based results.

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INTRODUCTION

Systemic sclerosis (SSc) is a generalized debilitating connective tissue disease affecting the skin and internal organs characterized by vasculopathy, fibrosis, and autoimmune alterations[1]. SSc is subclassified into two major clinical subsets, namely diffuse cutaneous (dcSSc) and limited cutaneous (lcSSc) form depending on the spread of the skin sclerosis[2]. Each of these subtypes has three stages - early, intermediate and late[2,3]. The dcSSc form distinguishes by rapidly progressive fibrosis of the skin and internal organs, which is a major cause of morbidity and mortality of the patients[4]. The lcSSc form is marked by vascular injury with milder skin and visceral fibrosis and generally, has a low progression rate[2,3].

The autoimmune dysregulation in SSc comprises lymphocyte activation that leads to the generation of autoantibodies, abnormal production of cytokines and chemokines, and impairment of the innate immunity[5-7]. Over the last decade, the accumulating data has shown the central role of T lymphocytes in the pathogenesis of SSc[8,9].

It is thought that the cytokine production by T cells influences the function of fibroblasts and endothelial cells, thereby playing a central role in vascular disease and fibrosis development[1,5]. Therefore, many efforts have been made to identify the T cell derived cytokine patterns in SSc and the subsets of T helpers involved. Most studies performed in SSc patients have examined

the characteristics of T cells isolated from peripheral blood.

There is a strong evidence in literature for altered T-cell activation[10-12] and T helper cells abnormalities in SSc[8,9]. Several authors have reported higher frequency of Th17 lymphocytes in the peripheral blood of SSc patients and have pointed out the role of these cells as a factor engaged in the pathogenesis of the disease[13-15]. Th17 cells, firstly described in 2005, produce interleukin (IL)-17A, IL-17F, IL-21, IL-22, and IL-26 and play a key role in host defense against extracellular bacteria and fungi[16]. Recent data has revealed their implication in the pathogenesis of several inflammatory and autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, investigating their animal models - experimental autoimmune encephalomyelitis and collagen-induced arthritis[17]. IL-17 is an inductor of the surface expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) by endothelial cells, and foreskin fibroblasts and induces the production of IL-1 and IL-6[18-20]. IL-17 also increases the production of pro-inflammatory cytokines such as chemokine (C-C motif) ligand 2 (CCL2), IL-6, IL-8 by synoviocytes and fibroblasts from both human skin and lungs[19,21]. Regarding the fibrotic process in SSc, IL-17 inhibits type Ⅰ and type Ⅲ collagen deposition[18,22] and reduces the connective tissue growth factor (CTGF) production *via* up-regulation of miR-129-5p in dermal fibroblasts[23]. Animal models of SSc have demonstrated the involvement of IL-17 in the bleomycin-induced lung and skin fibrosis[24-26]. Meanwhile, human studies have reported inverse correlation between the number of IL-17+ cells in the skin of SSc patients and the extent of skin sclerosis[27].

Not only Th17 cells, but also Tregs (CD4+FoxP3+) are involved in pathogenesis of SSc and there is a controversial data concerning their functional and numerical alterations. Some authors have found markedly up-regulated Tregs in all SSc phenotypes[10,28] particularly in active and severe disease[29]. Tregs from SSc patients demonstrated a diminished ability to control CD4 effector T cells and this defective function seemed to correlate with lower expression of CD69 and tissue growth factor-β (TGF-β) levels[10]. One study did not found Treg alterations in SSc patients compared to control groups[15]. Finally, several studies demonstrated a decreased frequency/impaired function of Tregs in SSc[30-32].

The CD4+Foxp3+ T cells produce anti-inflammatory cytokines including TGF-β and IL-10 and Tregs are mandatory to establish immune tolerance. TGF-β is a master regulator of the fibrotic process and alterations in TGF-β signaling are well described in SSc[1]. TGF-β promotes the fibrosis by both stimulating the synthesis, and suppressing the degradation of extracellular matrix[1]. TGF-β is involved in the generation of peripheral Tregs as well[33]. Accordingly, the same cytokine, TGF-β, is implicated in the generation of two functionally opposite T cell subsets, effectors - Th17 and Tregs, and the co-presence or not of pro-inflammatory cytokines, such as IL-6 and IL-1, determines the fate of TGF-β-exposed T cells[30]. Thus, the concomitance of TGF-β and IL-6 in SSc skin infiltrates could favor the generation of effector Th17 cells at the expense of Tregs, leading to complete alteration of the homeostatic equilibrium. Regarding IL-10, it has been reported to be increased in the serum of SSc patients[34]. Moreover, one paper has revealed that the raised serum levels of IL-10, and IL-6 correlated positively with the interstitial lung disease and the modified Rodnan skin score (MRSS) of patients[35].

Based on all the aforementioned data, we decided to evaluate the activation capacity of T cells in the peripheral blood of SSc patients and healthy controls, using phytochaemagglutinin (PHA-M). Our next aim was to determine both the percentage of the effector (Th17) and regulatory (Treg) cell subsets in the peripheral blood of the patients and the controls. We also investigated the serum levels of the peripheral cytokine milieu in both SSc patients and controls, scilicet, IL-6, IL-10, TGF-β1 and IL-17A.

It is laborious to obtain reliable incidence and prevalence estimates of SSc since the disease rarely occurs. Up to now, no studies have been carried out on the SSc incidence and prevalence in Bulgarian population. However, several epidemiological studies have been performed in Southeastern Europe. For instance, the incidence of SSc in Greece (North West) was 11 cases/million per year and the prevalence - 7.7 cases/million (1981-2002[36]. Respectively, the estimated prevalence of SSc in Croatia (Split-Dalmatia) based on 2008 data was 15 cases/million[37]. Although our study included a relatively small cohort of SSc patients, it could be assessed quite representative for our population if compared to the existing epidemiological data.

MATERIALS AND METHODS

Ethical committee statement

Informed written consent was obtained from all the subjects, enrolled in our study after approval of the Ethics Committee at the University Hospital St. Ivan Rilski, Sofia. All experiments carried out complied with the Declaration of Helsinki.

Population studied

Twenty-four patients, who attended the Clinic of Rheumatology of Department of Internal Medicine, Medical University of Sofia, were enrolled in this study. The mean age of the patients (male - 1, female - 23) was 47.1 ± 13.2 years. All the patients fulfilled the 2013 ACR/EULAR Criteria for the classification of SSc[38] and were divided as having dcSSc or lcSSc depending on the extent of skin sclerosis[2]. A further subdivision of the patients was performed in the groups based on the years from diagnosis[3]. Patients with dcSSc were divided in three groups: Early dcSSc (< 3 years' duration), intermediate (3-6 years) and late (6+ years). In the

Table 1 Clinical data of patients with systemic sclerosis, enrolled in the study

Patient No.	Gender	Age	Form	Stage	Active SSc	Visceral damage	Autoantibodies[1]	Treatment regimen
1	M	50	dcSSc	Intermediate	Yes	No	Speckled	PMP
2	F	49	dcSSc	Late	No	E	Anti-Scl70	MTX
3	F	55	dcSSc	Intermediate	No	E	Speckled	MP
4	F	58	dcSSc	Late	Yes	PF	Anti-Scl70	PMP, PCYP
5	F	44	dcSSc	Early	Yes	PF	Anti-Scl70	DPA, MP
6	F	27	lcSSc	Early	Yes	No	Anti-Scl70	DPA, MP, TCZ
7	F	48	dcSSc	Early	Yes	PF	Anti-Scl70	DPA, MP
8	F	37	lcSSc	Early	Yes	No	Anti-Ro52	CHQ
9	F	65	dcSSc	Late	No	No	Anti-CENP-A, Anti-CENP-B	MP, CHQ
10	F	36	lcSSc	Intermediate	Yes	No	Speckled	PMP, PCYP, DPA
11	F	47	dcSSc	Early	Yes	SRC	Anti-Scl70	PMP, PCYP
12	F	32	lcSSc	Early	Yes	PF	Speckled	MP, TCZ
13	F	62	dcSSc	Early	Yes	No	Speckled	PMP, PCYP
14	F	27	lcSSc	Late	Yes	No	Anti-PM/Scl-100	MTX
15	F	73	lcSSc	Intermediate	No	PF	Speckled	MP, MTX
16	F	32	dcSSc	Late	Yes	PF	Anti-Scl70, Anti-PM/Scl-75	PMP, PCYP
17	F	60	dcSSc	Late	Yes	No	Speckled	PMP, PCYP
18	F	34	dcSSc	Early	Yes	No	Anti-Scl70	MP, MTX
19	F	56	lcSSc	Late	No	E	Anti-CENP-B	MP
20	F	53	lcSSc	Early	Yes	No	Anti-PM/Scl-75	MP, AZA
21	F	30	lcSSc	Late	Yes	No	Speckled	MP, DPA
22	F	61	dcSSc	Late	No	E, PF, PH	Anti-Scl70	MP
23	F	39	lcSSc	Early	No	No	Anti-CENP-B	MTX
24	F	56	lcSSc	Intermediate	No	E	Speckled	MP, MTX

[1]In cases where no SSc specific autoantibody was detected, the staining pattern of patient's serum on indirect immunofluorescence is shown. F: Female; M: Male; E: Esophageal dysmotility; PF: Pulmonary fibrosis; PH: Pulmonary hypertension; SRC: Scleroderma renal crisis; MP: Methylprednisolone; PMP: Pulse MP; MTX: Methotrexate; CYP: Cyclophosphamide; PCYP: Pulse CYP; DPA: D-penicillamin; CHQ: Chloroquine; TCZ: Tocilizumab; AZA: Azathioprine.

lcSSc group, the following subdivision was performed: Early lcSSc (< 5 years' duration), intermediate (5-10 years) and late (10+ years) stages. The disease activity was assessed according to the Preliminarily Revised EUROSTAR Activity Index[39]. Sixteen age and gender-matched healthy individuals served as controls. Patients' clinical data as well as treatment regimens are shown in Table 1.

Activation capacity of T-lymphocytes in response to PHA-M stimulation of in patients with SSc

Heparinized whole venous blood, 2 mL was collected (LH 68 IU BD-Plymouth, United Kingdom, 5 mL) from each subject and was separated equally into two tubes - control tube and a PHA-M stimulated sample. To the stimulated test tube 20 μg/mL PHA-M (Roche Diagnostics GmbH, Germany) was added and the two samples were incubated for 4 h at 37 ℃, 5% CO_2. The samples were gently shacked, at regular intervals, on a multispeed vortex (MSV-3500 BioSan LV). Afterwards, 100 μL blood from each tube was labeled with monoclonal anti-CD3 FITC (for determination of the T lymphocytes) and anti-CD69 PE, an early activation marker for T cells (BD Biosciences, United States) and incubated for 30 min, at room temperature (RT) in the dark. Followed a lysis of erythrocytes (BD FACS Lysing Solution, BD Biosciences, United States), then centrifuging at 1300 rpm for 10 min and double washing (CellWash, BD Biosciences, United States). Subsequently, cells were fixed with 200 μL CellFIX (BD Biosciences, United States) and were analyzed with BD FACSCalibur flowcytometer using the Cell Quest software for data acquisition and analysis. Then 20000 lymphocytes were counted and analyzed for expression of CD69. The results obtained for each patient and healthy subject were analyzed for PHA-stimulated and unstimulated lymphocytes.

Flow-cytometric analysis of Th17 cells in SSc patients

Peripheral whole venous blood, 1 mL was collected (K2E BD-Plymouth, United Kingdom, 5 mL) from each subject. Monoclonal anti-CD3 FITC, anti-CD161-PE, anti-CD4-PerCP and anti-CD196-Alexa Flour 647 antibodies (BD Biosciences, United States) were added to the blood samples and incubated for 30 min, at RT in the dark. Followed a lysis of erythrocytes with a lysing solution (BD FACS Lysing Solution, BD Biosciences, United States) and after double washing in a CellWash solution (BD Biosciences, United States) the cells were fixed (CellFIX, BD Biosciences, United States). The specific fluorescent labeling was analyzed with BD FACSCalibur flowcytometer and 10000 lymphocytes were counted and analyzed using the Cell Quest software program of the same company.

Flow-cytometric analysis of Tregs in SSc patients

Peripheral venous blood, 1 mL was collected (K2E BD Vacutainer, BD-Plymouth, United Kingdom, 5 mL) from each individual. Monoclonal anti-CD25 FITC and anti-CD4-PE (BD Biosciences, United States) were added to the blood samples and incubated for 30 min, at RT in the dark. Followed a lysis of erythrocytes with a lysing solution (BD FACS Lysing Solution, BD Biosciences, United States) and after double washing in a CellWash

Table 2 T helper subsets in systemic sclerosis patients and healthy controls

T cell subpopulation (%)	SSc patients	Healthy controls	*P* value
CD4+Foxp3+	14.24 ± 1.39 (5.68-28.73)	11.04 ± 1.22 (3.55-20.84)	
CD4+CD25-Foxp3+	10.22 ± 1.21 (2.09-23.09)	6.88 ± 0.91 (1.42-12.79)	0.052
CD4+CD25+Foxp3+	4.02 ± 0.52 (0.71-10.77)	4.16 ± 0.53 (2.08-8.05)	
CD4+CD25+	6.30 ± 0.68 (1.40-13.36)	9.36 ± 1.08 (2.84-19.60)	0.016
Th17	18.13 ± 1.55 (9.18-32.64)	13.73 ± 1.21 (4.30-20.99)	0.031

Data are expressed as means ± SE. SSc: Systemic sclerosis.

solution (BD Biosciences, United States) a Human FoxP3 Buffer set (BD Biosciences, United States) was used for permeabilization of the cell membranes, as described by the manufacturer's instructions. Afterwards, a monoclonal antibody against intracellular expression of FoxP3 was used (anti-FoxP3 PE). After double washing, the cells were re-suspended in a wash buffer and analyzed immediately with BD FACSCalibur flowcytometer. At least 20000 CD4 positive lymphocytes were acquired using the Cell Quest software program.

Evaluation of serum soluble cytokines

Serum from each subject, 5 mL was collected using serum separator tubes (Vacutainer BD-Plymouth, United Kingdom, 5 mL). Circulating cytokine levels (serum IL-6, IL-10, IL-17A, TGF-β1) were measured using Diaclone Human ELISA kits (Diaclone SAS, France) according to the manufacturer's instructions and every sample was tested in duplicates.

Statistical analysis

For the analysis of the data's distribution, the Kolmogorov-Smirnov test was used. In cases of normal distribution, we determined mean ± SE, minimum, and maximum values and used a two-sample *t*-test and ANOVA for further statistical evaluation of the experimental data. In cases of non-normal distribution, median, interquartile range (IQR), minimum, and maximum values, were calculated and the Mann-Whitney test was applied. The strength of linear relationship between two continuous variables was examined using Pearson's correlation coefficient. Differences were considered as significant at $P < 0.05$. All statistical analyses were performed using IBM SPSS Statistics (IBM® SPSS® Statistics, Version 19).

RESULTS

PHA-activation of peripheral blood lymphocytes

We found no significant differences in the frequency of early activated T cells (CD3+CD69+) in unstimulated peripheral blood samples (control test tube) between healthy control subjects and SSc patients. However CD4+CD25+ lymphocytes, which are considered to be activated cells, were represented in decreased percentage in patients when compared to controls ($P = 0.016$, Table 2). Regarding the disease phenotype, dcSSc patients demonstrated lower frequency of CD4+CD25+ T cells against healthy subjects (5.95% ± 0.89% *vs* 9.36% ± 1.08%, respectively, $P = 0.025$).

In the PHA-stimulated samples, CD3+CD69+ cells were represented in decreased percentage in patients when compared to controls (13.35% ± 2.90% *vs* 37.03% ± 2.33%, respectively, $P < 0.001$) (Figure 1). As regards the lcSSc and dcSSc, there was no difference between the two phenotypes and in comparison with the healthy subjects.

Th17 cells

With regard to the Th17 cells, we found an up-regulated percentage in patients as opposed to controls ($P = 0.031$; Table 2). Accordingly, an increased percentage of Th17 cells was detected within the lcSSc subset *vs* controls (20.46% ± 2.41% *vs* 13.73% ± 1.21%, respectively, $P = 0.025$) (Figure 2). We detected no difference regarding the percentage of Th17 cells between the dcSSc and lcSSc phenotypes nor, when compared to controls.

Treg cells

There was no difference between patients and healthy individuals regarding CD4+Foxp3+ cells. We detected a certain trend toward increased percentage of these cells within the dcSSc subgroup as opposed to controls (14.73% ± 1.71% *vs* 11.04% ± 1.22%, respectively, $P = 0.083$). There was also no difference between patients and healthy individuals, regarding CD4+CD25+Foxp3+ T cells, nor within the distinct subtypes of SSc (Table 2). The percentage of CD4+CD25-Foxp3+ was marginally higher in patients ($P = 0.052$; Table 2) compared to controls. Although their percentage was increased in dcSSc *vs* controls (10.94% ± 1.65% *vs* 6.88% ± 0.91%, respectively, $P = 0.032$) (Figure 3). Still, we did not found differences between the dcSSc and lcSSc subsets.

Circulating cytokines

Regarding the peripheral cytokine profile, we detected increased levels of IL-6 [2.10 (1.05-4.60) pg/mL *vs* 0.00 pg/mL, $P < 0.001$], TGF-β1 (19.94 ± 3.35 ng/mL *vs* 10.03 ± 2.25 ng/mL, $P = 0.02$), IL-10 (2.83 ± 0.44 pg/mL *vs* 0.68 ± 0.51 pg/mL, $P = 0.008$), and IL-17A [6.30 (2.50-15.60) pg/mL *vs* 0 (0.00-0.05) pg/mL, $P < 0.001$] in patients when compared to healthy controls (Table 3). Furthermore, we found increased circulating IL-10, TGF-β, IL-6 and IL-17A in the lcSSc subset *vs* control subjects, as it follows: IL-10 (3.32 ± 0.59 pg/mL *vs* 0.68 ± 0.51 pg/mL, $P = 0.003$), TGF-β1 (22.82 ± 4.99 ng/mL *vs* 10.03 ± 2.25 ng/mL, $P = 0.031$), IL-6 [2.08 (1.51-4.69) pg/mL *vs* 0.00 pg/mL, $P < 0.001$], and IL-17A [14.50 (8.55-41.65) pg/mL *vs* 0.00 (0.00-0.05) pg/mL, $P < 0.001$]. Furthermore, circulating IL-17A was higher in lcSSc as opposed to

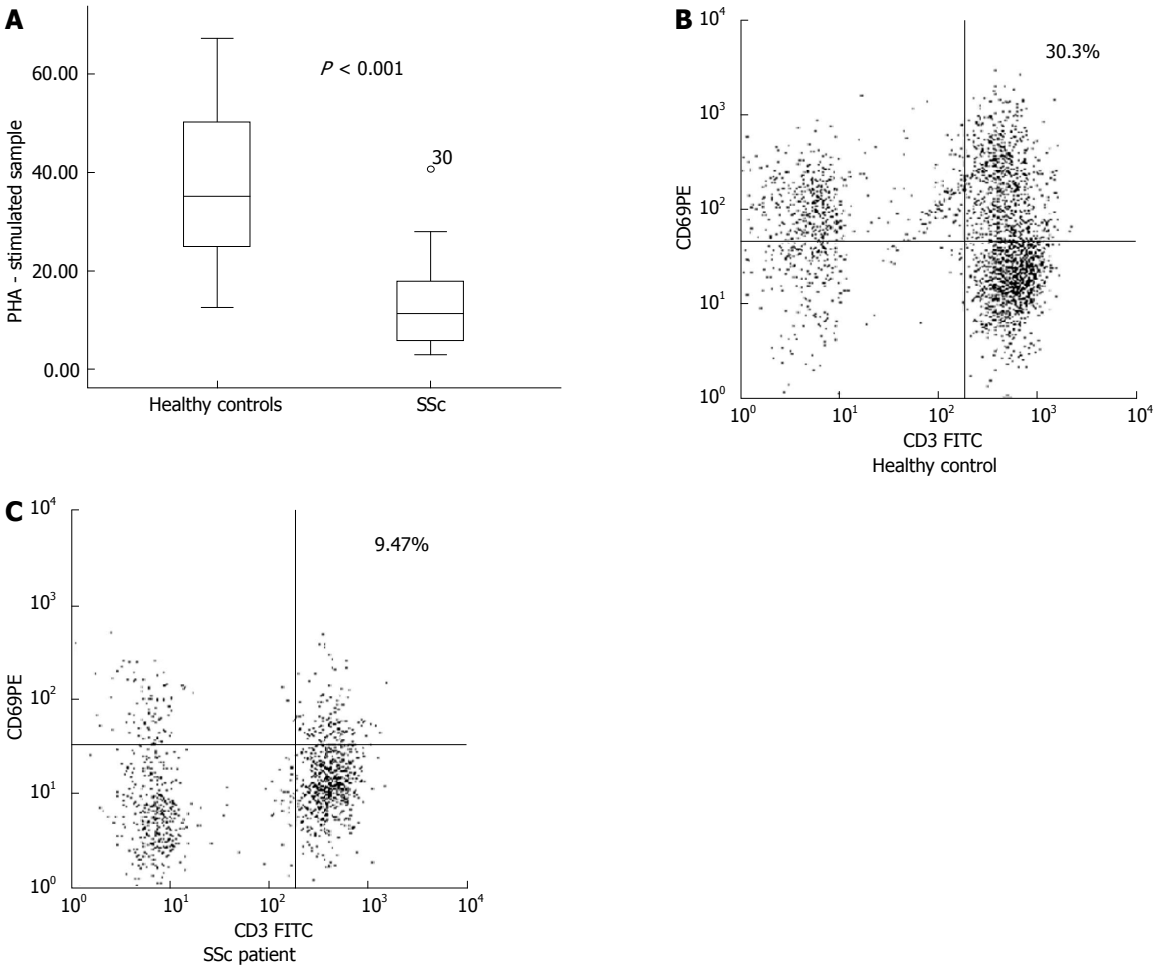

Figure 1 Decreased percentage of CD3+CD69+ cells upon PHA stimulation in the circulation of patients with systemic sclerosis as opposed to healthy controls. A: Percentage of CD3+CD69+ cells in PHA-stimulated samples from SSc patients (n = 24) and healthy controls (n = 16), as it follows: 13.35% ± 2.90% *vs* 37.03% ± 2.33%, P < 0.001. The boxplots represent mean ± SD; B and C: PHA stimulated sample of one representative subject from each group is shown. The percentage of CD69+ cells in the whole T cell pool (CD3+ cells) is depicted. SSc: Systemic sclerosis.

dcSSc subset (31.99 ± 13.29 pg/mL *vs* 7.14 ± 3.01 pg/mL, P = 0.008). Within the dcSSc subset, raised levels of IL-17A and IL-6 were detected *vs* healthy controls: IL-17A [2.60 (0.45-9.80) pg/mL *vs* 0.00 (0.00-0.05) pg/mL, P < 0.001], IL-6 [2.80 (1.03-7.23) pg/mL *vs* 0.00 pg/mL, P < 0.001].

The findings on circulating cytokines regarding the comparison of the two SSc phenotypes and *vs* healthy controls are depicted in Figure 4.

Relationship between activity, stage of SSc, presence of visceral organ involvement and the investigated immune parameters

Patients were divided in two groups depending on the disease activity. Sixteen patients had active disease, while eight patients were with stable/inactive SSc (Table 1). We identified no differences between the two groups, regarding Tregs, Th17 cells and levels of the serum soluble cytokines.

The distribution of the patients according to the stage of SSc was as follows: Early SSc, n = 10, intermediate SSc, n = 5, and late SSc n = 9 (Table 1). The stage of the disease did not influence the percentage of Tregs, nor the frequency of Th 17 cells in patients' peripheral blood. As regards to the circulating cytokines, only TGF-β1 serum levels were increased in early stage against late stage, independently from the SSc phenotype (30.03 ± 4.59 ng/mL *vs* 13.08 ± 4.50 ng/mL, P = 0.017).

Twelve patients enrolled in the study had visceral organ involvement, the distribution was as follows: Pulmonary arterial hypertension (PAH), n = 1; pulmonary fibrosis (PF), n = 7; esophageal dysmotility (E), n = 5; scleroderma renal crisis (SRC), n = 1 (Table 1). No differences were observed regarding the peripheral immune parameters in cases of presence of visceral organ involvement.

DISCUSSION

For the purposes of our study, we used PHA-M to activate resting T cells. PHA is a classical mitogen leading to selective nonspecific T-cell activation and proliferation[40]. In the mid-1970s, it was found that T-cell proliferation induced by PHA requires the presence of monocytes. Ceuppens *et al*[41] confirmed this statement and identified that the addition of purified human IL-6,

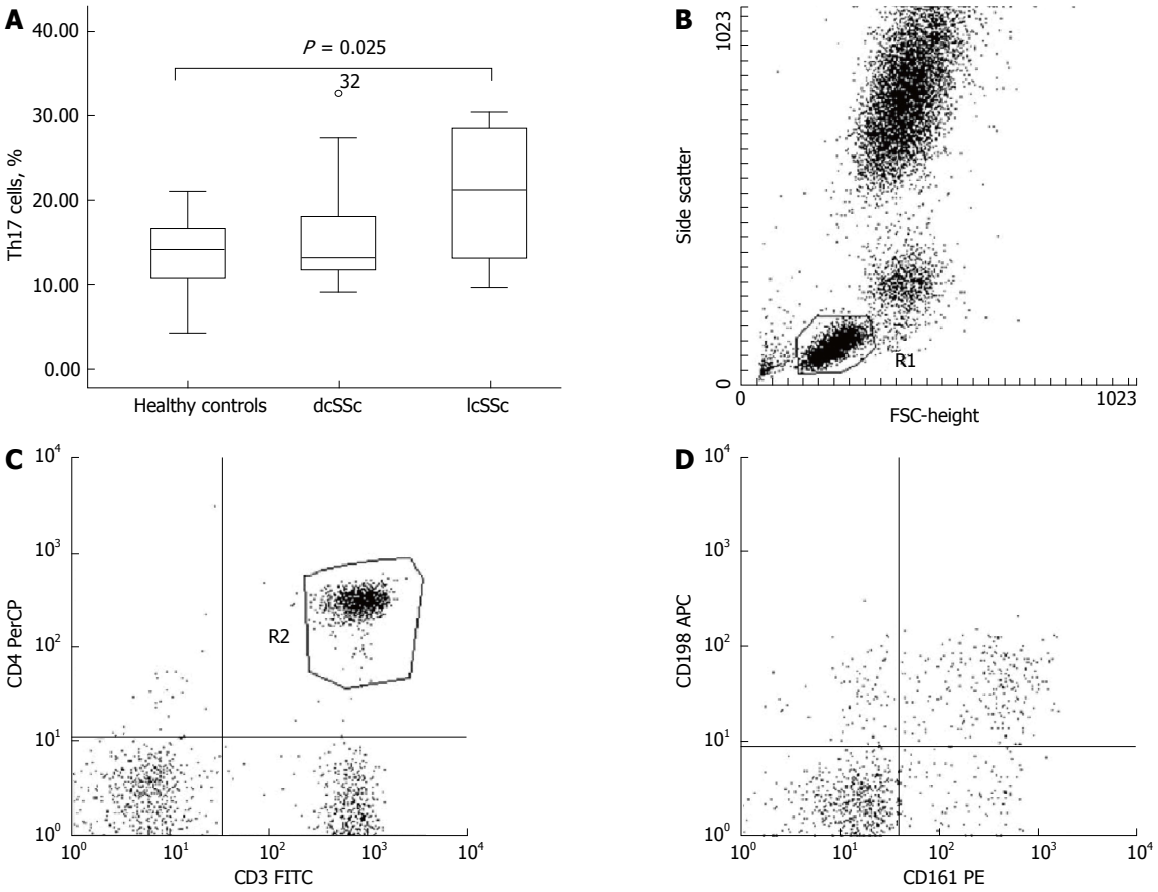

Figure 2 Increased percentage of Th17 cells within limited cutaneous systemic sclerosis subset *vs* healthy controls. A: Percentage of Th17 cells for lcSSc (*n* = 11), dcSSc (*n* = 13), and healthy controls (*n* = 16) is presented. Increased percentage of Th17 cells within lcSSc patients as opposed to controls, respectively, 20.46% ± 2.41% *vs* 13.73% ± 1.21%, *P* = 0.025. Boxplots are expressed as means ± SD; B-D: Panel B depicts the flow cytometric analysis of Th17 cells. A representative patient with lcSSc phenotype is shown. The lymphocytes were gated according to their physical characteristics (FSC and SSC) in R1; afterwards T helper cells (CD3+CD4+) were gated in R2. T helpers, which were detected double positive for CD161 and CD196 surface expression (R3, upper right quadrant) were defined as Th17 cells. lcSSc: Limited cutaneous systemic sclerosis.

along with monocytic supernatant, to PHA-stimulated cell cultures has led to effective T-cell activation and proliferation.

Aiming to approach our study to the conditions *in vivo*, we used heparinized venous blood samples. Moreover, we identified increased serum levels of IL-6 in our SSc patients, which as previously mentioned, is a factor involved in T-cell activation. In the PHA - stimulated samples, we detected a decreased percentage of CD3+CD69+ cells in patients when compared to healthy controls.

The circulating cytokine profile in our SSc patients might relate to the decreased ability of T cells to be activated. Our data has revealed increased levels of IL-10, TGF-β, and IL-6 in peripheral blood of SSc patients and all these cytokines are engaged directly or not in the process of suppression of T-cell activation.

IL-10 is a pleiotropic cytokine with important anti-inflammatory and immunoregulatory functions, which inhibits the activity of Th1 cells[42,43]. Along with the tolerogenic dendritic cells and Treg subsets, other immunocompetent cells secreting IL-10 has been studied, including B cells, NK cells, neutrophils, and macrophages. The role of Th2 cells that produce IL-10 is also well-established[44]. However, recent data have paradoxically demonstrated that Th1 and Th17 cells are also able to secrete IL-10. It is thought that these "double-natured" T cell subsets use the secretion of IL-10 to suppress their own proinflammatory activity, directly, or in concert with tolerogenic antigen-presenting cells[43]. Some studies suggest that IL-10 (alone or in combination with IFN-γ) also has an inhibitory function regarding the fibrotic process in SSc[45]. Based on the literature, IL-6 inhibits the differentiation of monocytes in dendritic cells alone or through induced autocrine secretion of IL-10[46,47]. Likewise, both IL-6 and IL-10 restrain the antigen-presenting function of dendritic cells, which ultimately results in a formation of immature tolerogenic myeloid cells secreting IL-10 and their antigen-presenting capacity results in T lymphocytes' anergy[48]. Along with IL-10, TGF-β also exerts an inhibitory action on T cells. TGF-β inhibits the IL-2 promoter/enhancer activity, which results in a block of *IL-2* gene expression in T cells[49].

TGF-β inhibitory effect on T cells may be mediated through up-regulation of cyclin-dependent kinase inhibitors p15, p21, and p27 expression[50] and down-regulation of C-myc, cyclin D2, and cyclin E expression, too[51]. The concept for the suppressive role of the

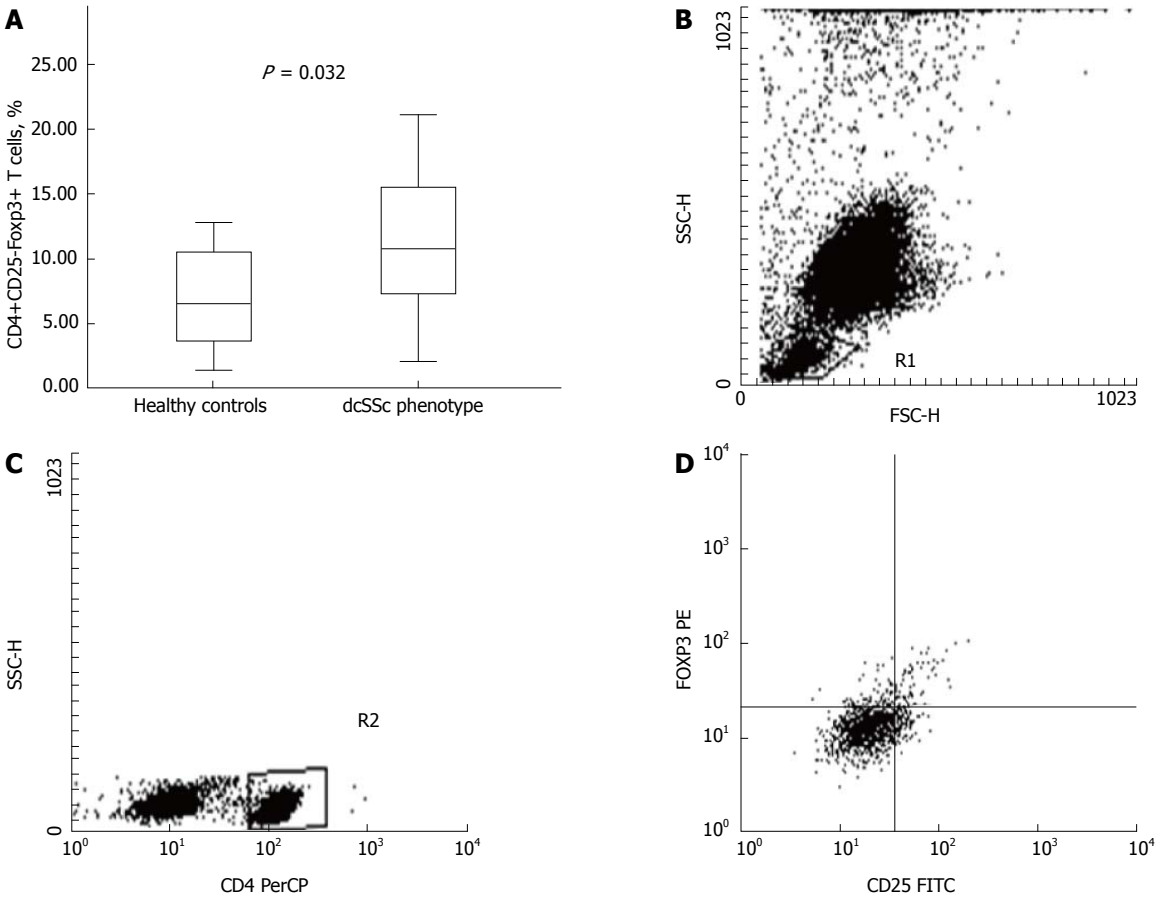

Figure 3 Increased percentage of CD4+CD25-Foxp3+ T cells within diffuse cutaneous systemic sclerosis phenotype *vs* healthy controls. A: Increased percentage of CD4+CD25-Foxp3+ T cells within dcSSc patients (n = 13) as opposed to controls (n = 16), respectively, 10.94% ± 1.65% *vs* 6.88% ± 0.91%, P = 0.032. Boxplots are expressed as means ± SD; B-D: Panel B depicts the flow cytometric analysis of CD4+CD25-Foxp3+ T cells. A representative patient with dcSSc phenotype is shown the lymphocytes were gated according to their physical characteristics (FSC and SSC) in R1; afterwards T helper cells were gated in R2. T helpers, which were found negative for CD25 surface expression and positive for Foxp3 intracellular expression (R3, upper left quadrant) were defined as CD4+CD25-Foxp3+ T cells. dcSSc: Diffuse cutaneous systemic sclerosis.

Table 3 Circulating cytokines in systemic sclerosis patients and healthy controls

Cytokine	SSc patients	Healthy controls	*P* value
IL-10, pg/mL	2.83 ± 0.44 (0.10-6.90)	0.68 ± 0.51 (0.00-5.20)	0.008
IL-17A, pg/mL	6.30 [2.50-15.60] (0.20-124.90)	0.00 [0.00-0.05] (0.00-1.36)	< 0.001
TGF-β1, ng/mL	19.94 ± 3.35 (0-52.80)	10.03 ± 2.25 (1.16-21.80)	0.02
IL-6, pg/mL	2.10 [1.05-4.60] (0.45-198.10)	0 (0.00-0.27)	< 0.001

Data represents means ± SE (range) or medians [IQR] (range). SSc: Systemic sclerosis; IL: Interleukin; TGF: Tissue growth factor.

circulating cytokine milieu in SSc, regarding the T-cell activation, is in agreement with data reporting inhibited activation of Tregs from healthy donors or SSc by SSc plasma[10].

On the other hand, the peripheral T cell anergy upon PHA-stimulation in our SSc patients may be due to the immunosuppressive therapy administered. Most of the patients enrolled in the study were under treatment with glucocorticoids (GCs) (Table 1).

Normally, stimulation of T cells by cross-linking of both T-cell receptor TCR/CD3 and CD28 up-regulates both the nuclear factor of activated T cells (NFAT) and activating protein 1 (AP-1) transcription factors, resulting in increased transcription of the interleukin-2 (IL-2) gene and activation[52]. One of the important genomic mechanisms of GC action includes the interaction of activated cytosolic GC receptor (cGCR) monomers with transcription factors. The GC/cGCR complex modulates the activity of AP-1, NFAT, and NF-κB (nuclear factor-kB)[53]. The inhibition of their nuclear translocation and function leads to blockage of the expression of many proinflammatory cytokines, *e.g.*, IL-2, IL-6, TNF-α[54]. This genomic mechanism of GC action may explain the decreased percentage of peripheral CD4+CD25+ cells in our SSc patients compared to healthy subjects, bearing in mind that CD25 along with a marker for T cell activation is an IL-2 receptor alpha chain as well. Moreover, we found decreased peripheral CD4+CD25+ cells in dcSSc patients, all of which had been under treatment with methylprednisolone.

Based on our results, we are not able to answer unconditionally to the question who exactly is responsible

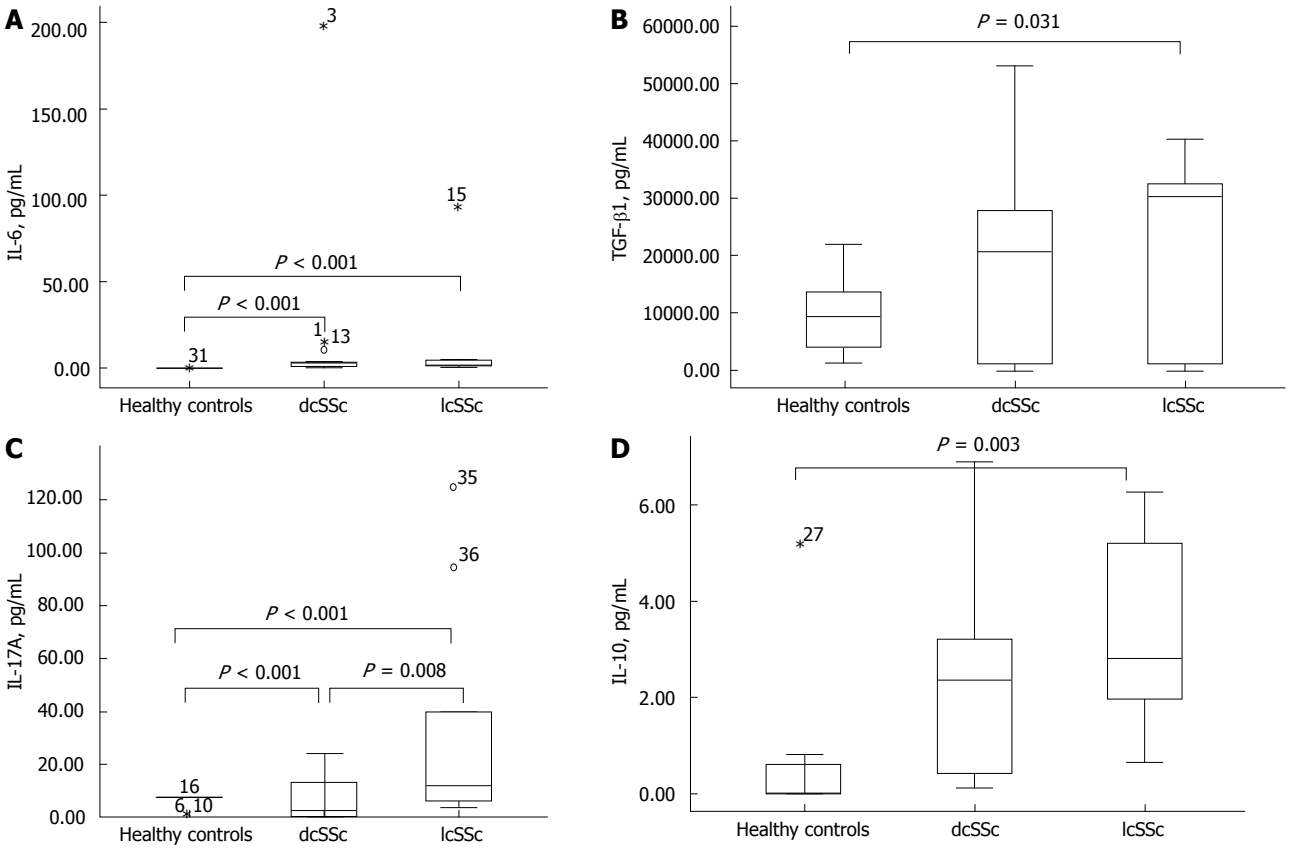

Figure 4 Elevated serum levels within the limited cutaneous systemic sclerosis (*n* = 11) and diffuse cutaneous systemic sclerosis (*n* = 13) phenotypes *vs* healthy controls (*n* = 16). Boxplots are expressed as means ± SD. A: Increased serum levels of IL-6 in lcSSc phenotype *vs* controls, $P < 0.001$. Raised serum levels of IL-6 in dcSSc patients *vs* controls, $P < 0.001$; B: Raised serum levels of TGF-β1 in lcSSc patients *vs* controls, $P = 0.031$; C: Elevated serum levels of IL-17 A in lcSSc phenotype *vs* dcSSc patients, $P = 0.008$, and *vs* controls, $P < 0.001$. The IL-17A were also increased in dcSSc patients *vs* controls, $P < 0.001$; D: Raised serum levels of IL-10 in lcSSc patients *vs* controls, $P = 0.003$. lcSSc: Limited cutaneous systemic sclerosis; dcSSc: Diffuse cutaneous systemic sclerosis; TGF-β1: Tissue growth factor-β1; IL: Interleukin.

for the decreased activation capacity of T cells in SSc patients - the therapeutic regiment, cytokines, both of them or perhaps, additional factors get involved.

One of the most considerable findings of our study is the increased percentage of Th17 cells and the elevated serum levels of their respective cytokine, IL-17.

Many papers have reported a higher frequency of Th17 cells in the peripheral blood of SSc patients as opposed to healthy controls[13-15,30] which corresponds to our results.

Overproduction of IL-17 by T cells in the peripheral blood and in both skin and lung Kurasawa *et al*[18] have described overproduction of IL-17 by T cells in the peripheral blood and in both skin and lung lesions from SSc patients. These results suggest the central role that IL-17 overproduction plays in the pathogenesis of SSc, especially in the early stages of the disease, by enhancing the fibroblast proliferation and the production of IL-1 and the expression of adhesion molecules on endothelial cells[18]. Our data have not revealed any difference in the level of serum IL-17, regarding the stage of SSc. However, we describe for the very first time elevated serum levels of IL-17 in patients with lcSSc when compared to the dcSSc phenotype.

Even though IL-17 enhances the fibroblast proliferation, this cytokine does not induce collagen production in dermal fibroblasts, but rather decreases the ability of TGF-β to activate them. Furthermore, the number of IL-17 positive cells in SSc skin has been reported to correlate inversely with the extent of global skin thickness[27]. Thus, in humans IL-17 may instead act as an antifibrotic inflammatory mediator. It is worth mentioning that prostanoids currently used to treat SSc vasculopathy, including prostaglandin I2, increase *in vivo* the number of Th17 cells[55]. Therefore they could be beneficial to the vascular compartment, particularly to endothelial cells, and might be crucial for the modulation of the inflammatory response.

Whether Th17 cells and IL-17 might have indirect pro-fibrotic effects *via* interaction with endothelial/epithelial cells or *via* the enhanced production of pro-angiogenic factors, such as IL-8, CCL-2, remains to be investigated. Similarly, the role of Th17 cells in autoantibody generation in SSc has not been investigated so far. However, in animal studies IL-17 has been shown to promote autoantibody generation in BXD2 mice by orchestrating the spontaneous formation of autoreactive germinal centers[56].

Recent data has revealed that IL-6 plays an important role in the regulation of the balance between

IL-17-producing Th17 cells and Tregs[30,35]. Our results demonstrate increased serum levels of IL-6 in both of lcSSc and dcSSc patients compared to controls with no difference between the two clinical subsets. IL-6 in concert with TGF-β induces the expression of RORγt in naïve T cells, transforming them in Th17 cells; in contrast, IL-6 inhibits TGF-β-induced Treg differentiation[57].

Even though Th17 cells are crucial in the modulation of the inflammatory response, the Treg subset might also play a central role in the pathogenesis of SSc. Our results demonstrate nonsignificant increase in CD4+Foxp3+ Tregs in SSc patients when compared to controls and no difference between patients and healthy individuals regarding the percentage of CD4+CD25+FoxP3+ Tregs. There is controversial data in literature concerning the Treg numerical and functional alterations in SSc. Some of the papers have announced elevated circulating CD4+CD25+Foxp3+ Treg cells[10,28] particularly in active and severe disease[29]. Besides the up-regulation, Tregs from SSc patients demonstrate a defective suppressive capacity, which has been reported to correlate with a diminished CD69 expression and TGF-β levels[10]. One study has not detected any differences between SSc patients and control groups[15]. Finally, several studies have demonstrated a decreased frequency/impaired function of Tregs in SSc[30-32].

However, our data reveals an increased percentage of CD4+CD25-Foxp3+ cells in our dcSSc patients in comparison with the healthy controls. Recent studies have reported up-regulated CD25 negative CD4+Foxp3+ cells in the peripheral blood of patients with systemic lupus erythematosus (SLE)[58-60]. Both CD4+CD25-Foxp3+ T cells and CD4+CD25+ Foxp3+ Treg cells from SLE patients have demonstrated a similar pattern regarding the expression of CD62L, CD95, GITR, CD127, and CTLA-4, which are typical markers for the Treg phenotype[61]. A considerable suppressive activity of CD4+CD25-Foxp3+ cells, comparable to the suppressive capacity exerted by the classical Tregs (CD4+CD25+Foxp3+ cells) has been reported[62]. According to another hypothesis, CD4+CD25-Foxp3+ T cells subset could represent a peripheral reservoir of the CD4+CD25+ Foxp3+ Treg cell subset[61]. In case of autoimmune reactivation, such as in SLE patients, CD25 negative Foxp3+ T cells could regain the expression of CD25, trying to reverse a homeostatic imbalance shift to more aggressive expansion of autoreactive T cells and B cells[61]. However, another paper have considered CD4+CD25-Foxp3+ cells as functionally incompetent in SLE[63].

The GC treatment of our dcSSc patients could also unravel the up-regulated peripheral CD4+CD25-Foxp3+ cells that we have found. The CD4+CD25-Foxp3+cell subset has been reported increased in patients with rheumatoid arthritis treated with GCs and have correlated inversely with the disease parameters[64]. GC-treated patient carriers of the high IL-10 genotype demonstrated higher levels of CD4+CD25-Foxp3+ cells, which finding corresponds to our results.

In conclusion, our study demonstrates a decreased capacity for PHA-induced peripheral T-cells activation in patients with SSc. We also describe for the first time an up-regulated percentage of CD4+CD25-FoxP3+ cells in patients with dcSSc. Regarding the circulating cytokine profile in SSc, we originally identify increased serum levels of IL-17 in lcSSc as opposed to patients with dcSSc phenotype. The rest of our data, concerning the elevated circulating IL-6, IL-10, and TGF in SSc, confirms literature-based results.

COMMENTS

Background

Systemic sclerosis (SSc) is a generalized debilitating connective tissue disease affecting the skin and internal organs characterized by vasculopathy, fibrosis, and autoimmune alterations. The autoimmune dysregulation in SSc comprises lymphocyte activation that leads to the generation of autoantibodies, abnormal production of cytokines and chemokines, and impairment of the innate immunity. Over the last decade, the accumulating data has shown the central role of T lymphocytes in the pathogenesis of SSc. There is strong evidence in literature for altered T-cell activation and T helper cells abnormalities in SSc.

Research frontiers

There is accumulating data for numerical and functional alterations of Tregs and Th17 cells in patients with SSc. However, a functional heterogeneity exists between the T lymphocytes in the peripheral blood of patients with SSc and the corresponding T cell subsets in skin lesions or internal organs. The cytokine production by T cells affects the function of fibroblasts and endothelial cells, thereby influencing the vascular disease progression and the fibrosis development. Many efforts have been made to identify the cytokine patterns in SSc. Nevertheless, important issues remain unresolved, among them, identification of the trigger of the autoimmune response in SSc and the immunological differences between the dcSSc and lcSSc.

Innovations and breakthroughs

This is the first study demonstrating an up-regulated percentage of CD4+CD25-FoxP3+ cells in patients with dcSSc as compared to healthy subjects. Another of the original contributions of this research demonstrates a decreased capacity for PHA-induced peripheral T-cells activation in patients with SSc. Regarding the peripheral cytokine profile in SSc, this research group describes for the first time elevated serum levels of IL-17A in the lcSSc as opposed to the dcSSc subset of the disease.

Applications

It is likely that the altered percentage of Th17 and CD4+CD25-FoxP3+ cells may play a key role in the disease progression along with the peripheral cytokine profile in SSc patients.

Terminology

SSc is an abbreviation for systemic sclerosis as well as lcSSc and dcSSc are abbreviations for the limited cutaneous and the diffuse cutaneous subsets of the disease. Tregs represent the T regulatory lymphocytes (CD4+FoxP3+ cells), a T helper cell subset which is crucial for the establishment of immunological self-tolerance and for the prevention of autoimmunity.

Peer-review

The study represents an interesting continuum to the research series towards unveiling the immunological profile in SSc.

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