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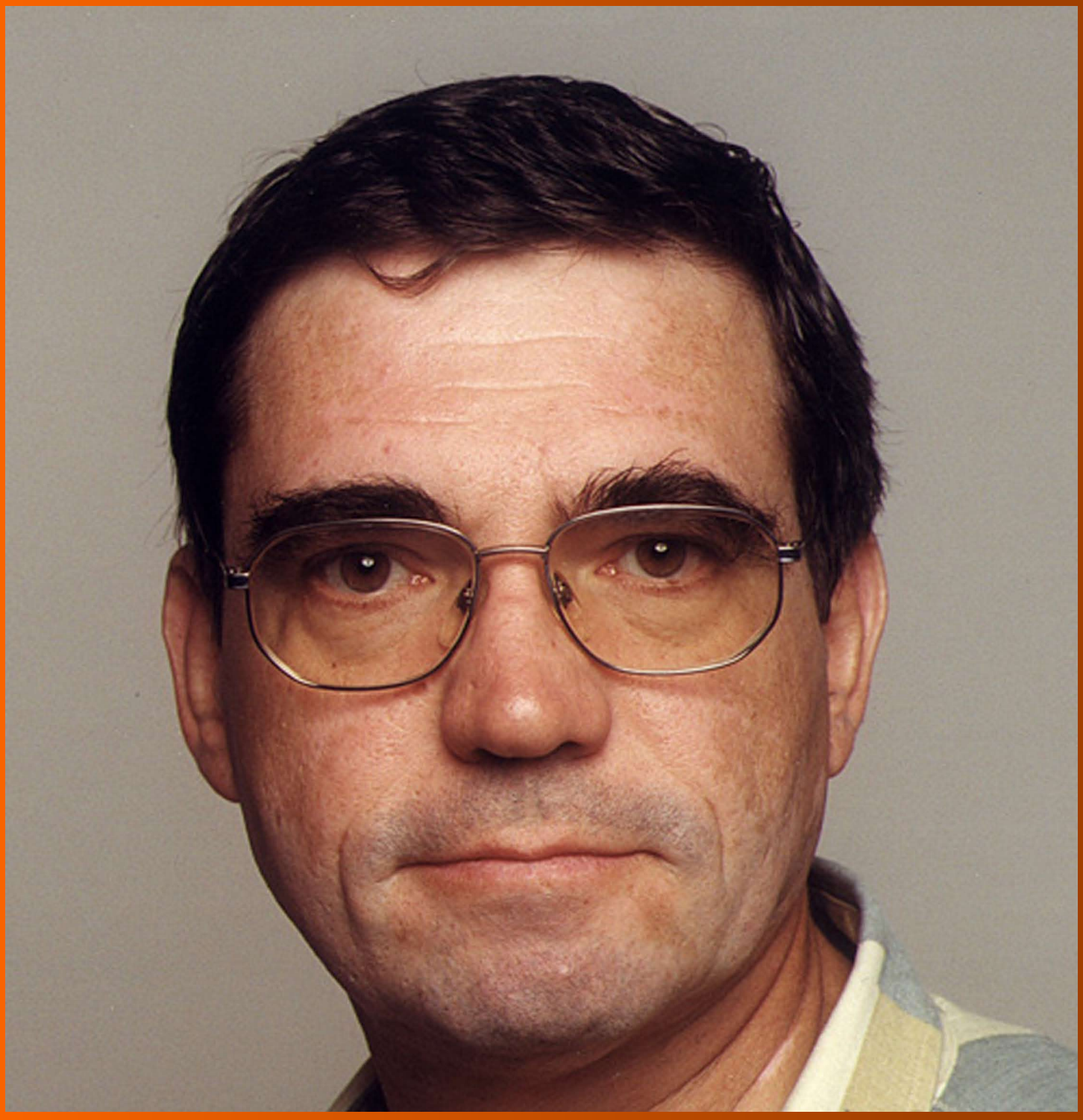

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REVIEW

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

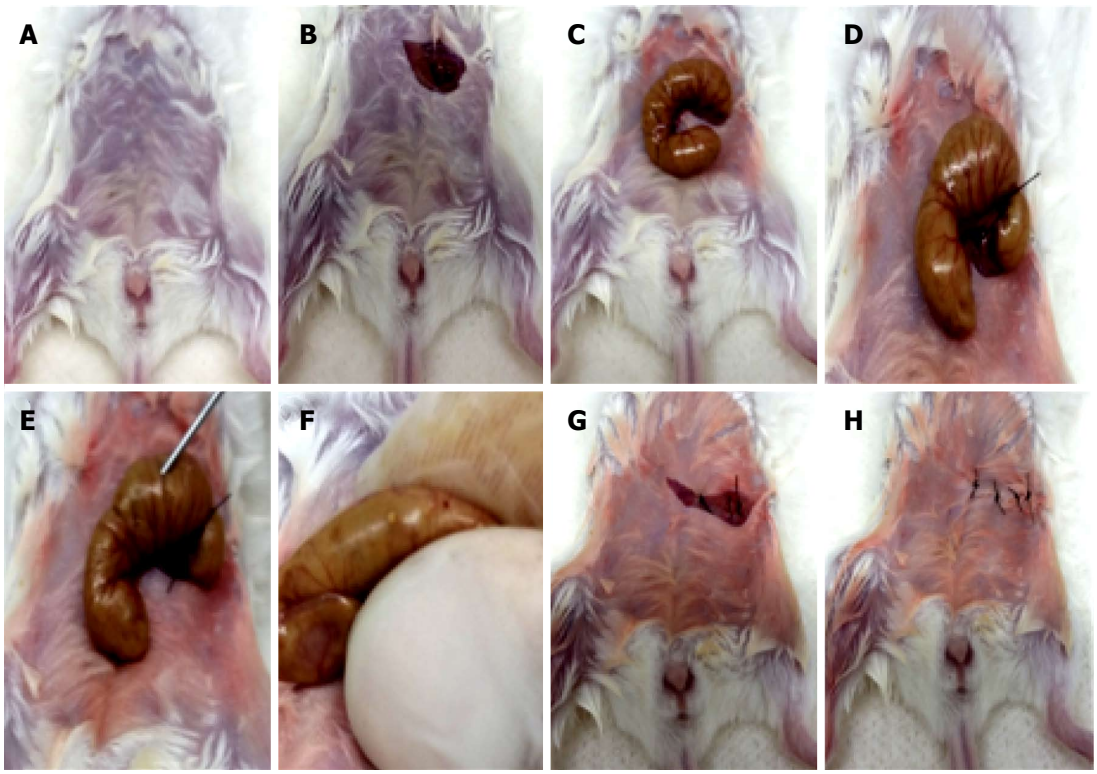

Figure 1 Main steps of the cecal ligation and puncture procedure. A: Mice are anesthetized with *i.p.* administration of ketamine (100 mg/kg) and xylazine (12 mg/kg); B: After local asepsis, a cut is made in the skin and in the peritoneal membrane, providing access to the peritoneal cavity; C: The caecum 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.

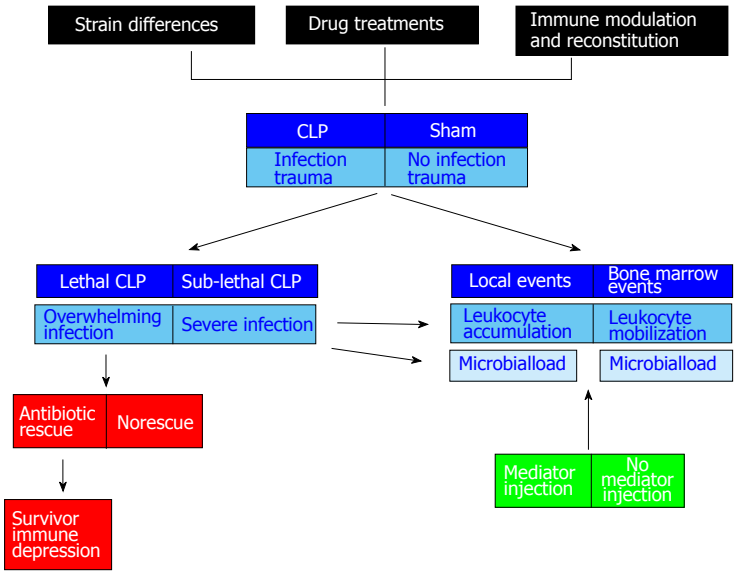

Figure 2 Modular distribution of multiple variables of interest in the cecal ligation and puncture surgical model. The core parts of a cecal ligation and puncture (CLP) experiment (blue boxes) can be subdivided in modules that deal with trauma plus infection (CLP) or trauma without infection (sham). 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

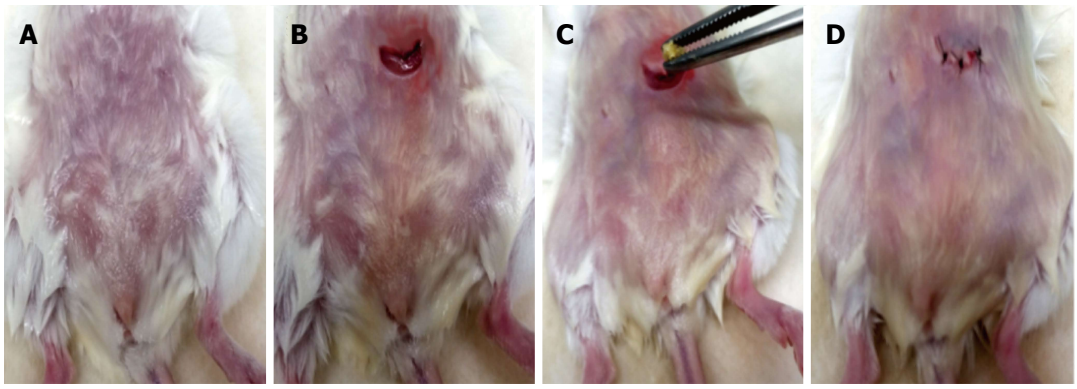

Figure 3 Main steps of the intraperitoneal modification of the egg white implant procedure. A: Mice are anesthetized with *i.p.* administration of ketamine (100 mg/kg) and xylazine (12 mg/kg); B: After local asepsis, a cut is made in the skin and in the peritoneal membrane, providing access to the peritoneal 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

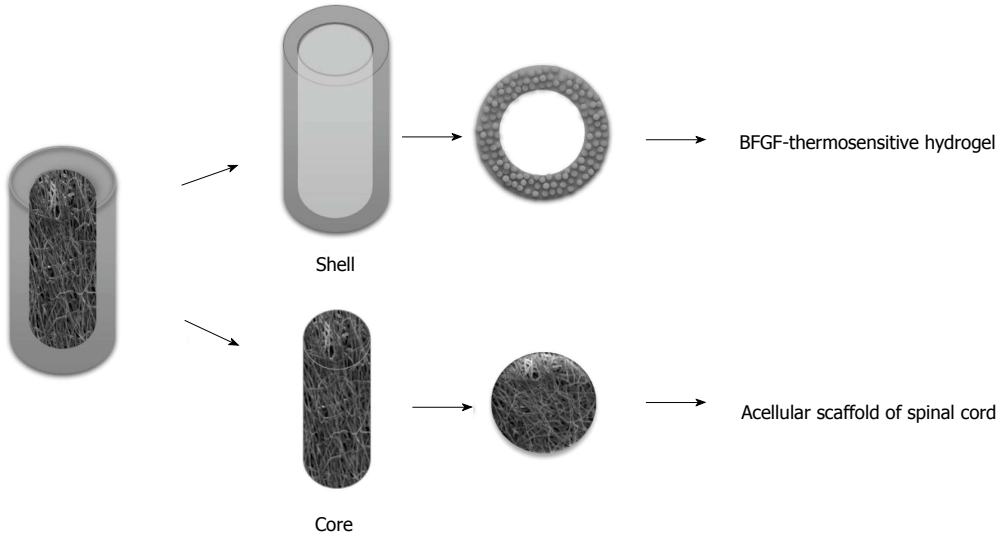

Figure 1 Construction of biomimetic spinal cord. BFGF: Basic fibroblast growth factor.

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

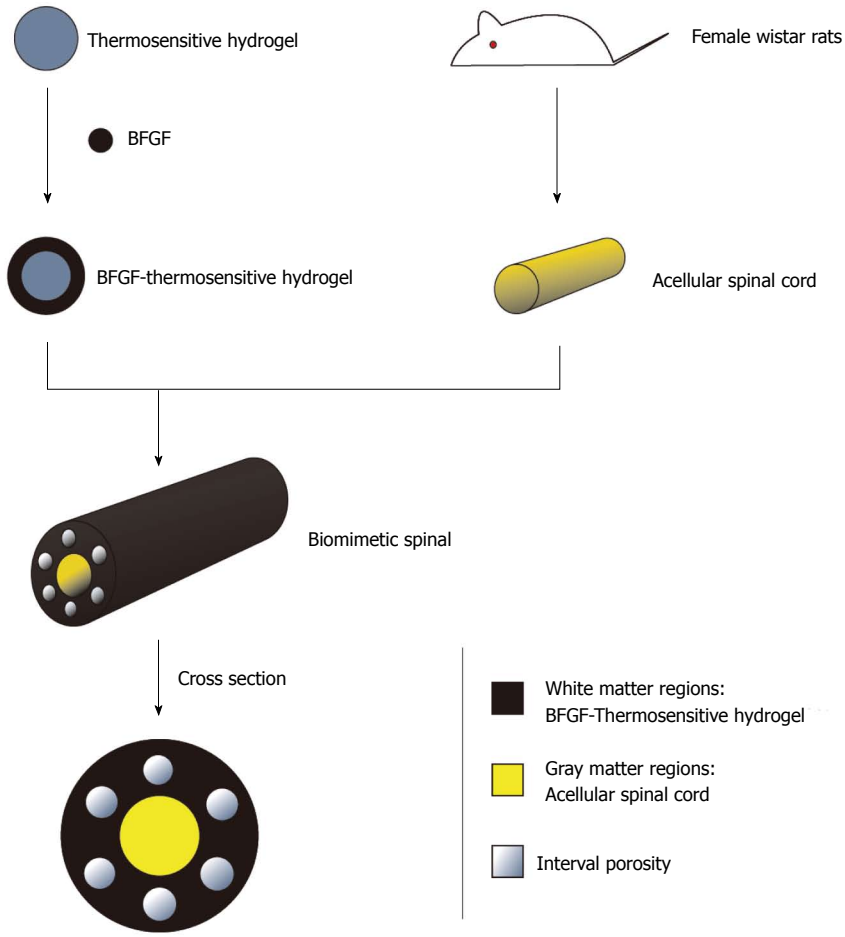

Figure 2 Technology road mapping. BFGF: Basic fibroblast growth factor.

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.

Krasimirova E, Velikova T, Ivanova-Todorova E, Tumangelova-Yuzeir K, Kalinova D, Boyadzhieva V, Stoilov N, Yoneva T, Rashkov R, Kyurkchiev D. Treg/Th17 cell balance and phyto-haemagglutinin activation of T lymphocytes in peripheral blood of systemic sclerosis patients. *World J Exp Med* 2017; 7(3): 84-96 Available from: URL: http://www.wjgnet.com/2220-315X/full/v7/i3/84.htm DOI: http://dx.doi.org/10.5493/wjem.v7.i3.84

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

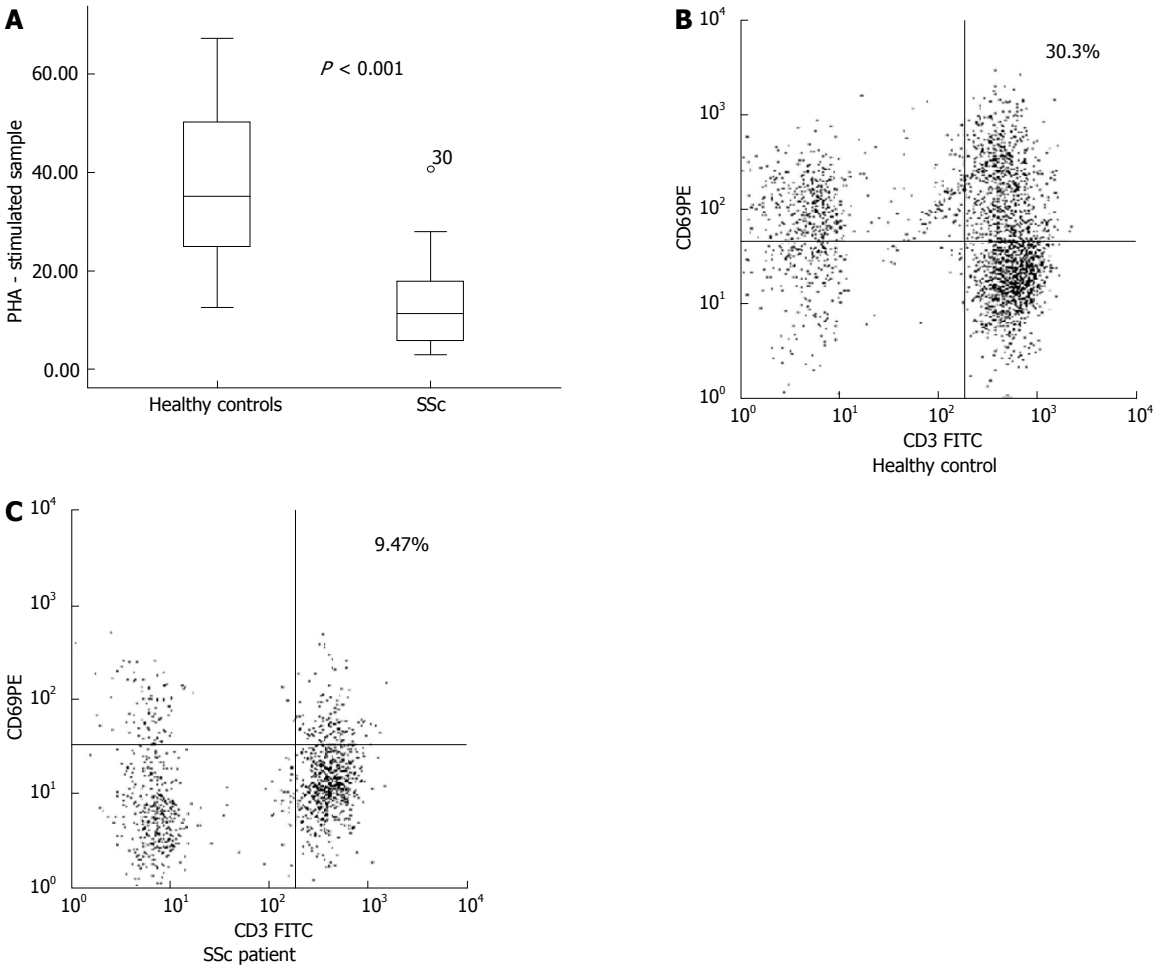

Figure 1 Decreased percentage of CD3+CD69+ cells upon PHA stimulation in the circulation of patients with systemic sclerosis as opposed to healthy controls. A: Percentage of CD3+CD69+ cells in PHA-stimulated samples from SSc patients (n = 24) and healthy controls (n = 16), as it follows: 13.35% ± 2.90% *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,

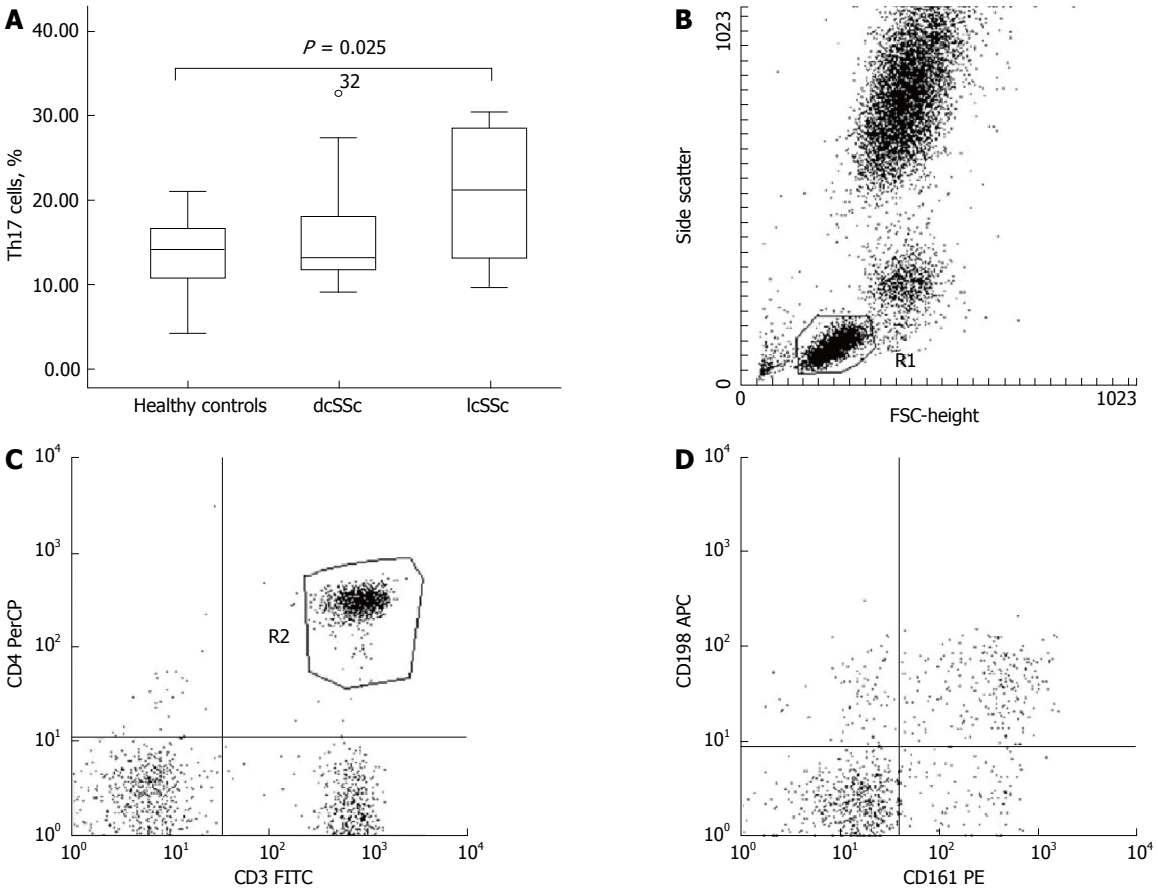

Figure 2 Increased percentage of Th17 cells within limited cutaneous systemic sclerosis subset *vs* healthy controls. A: Percentage of Th17 cells for lcSSc (*n* = 11), dcSSc (*n* = 13), and healthy controls (*n* = 16) is presented. Increased percentage of Th17 cells within lcSSc patients as opposed to 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

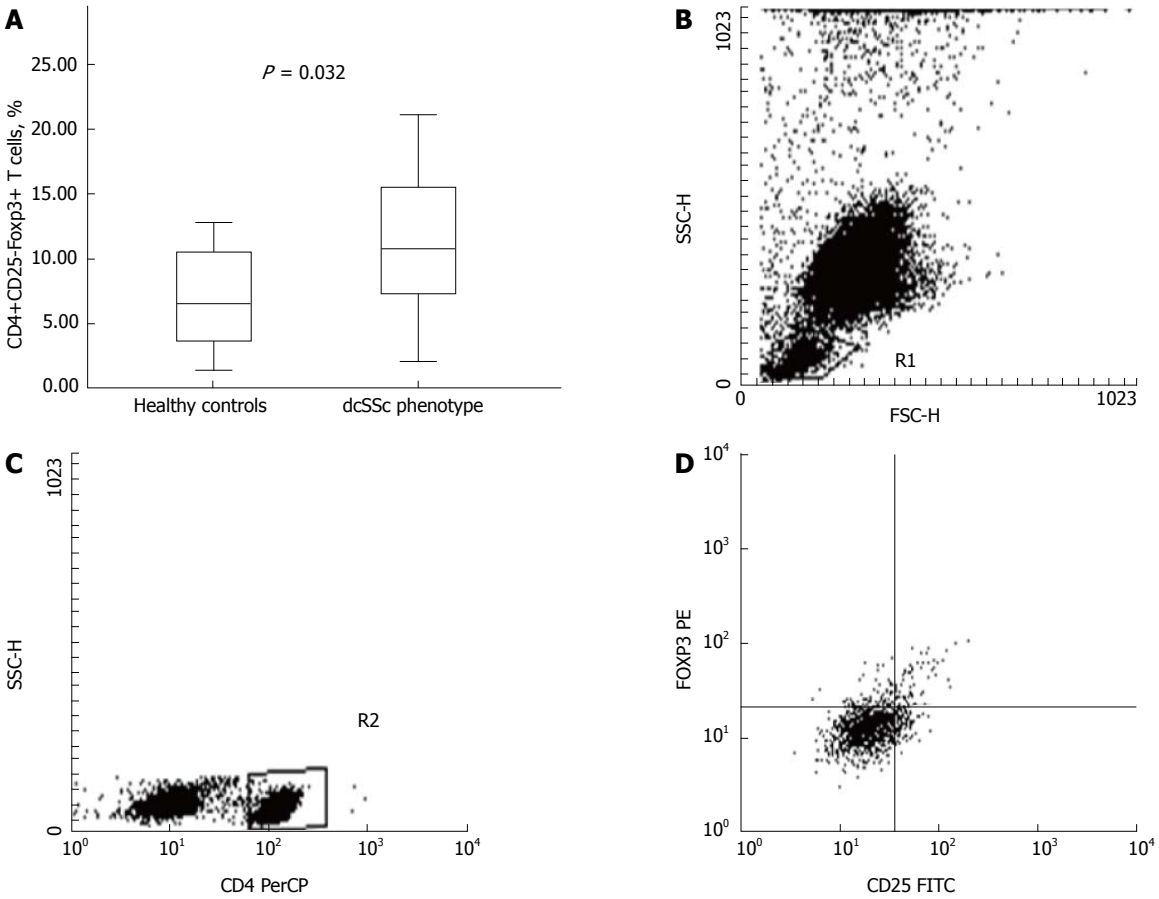

Figure 3 Increased percentage of CD4+CD25-Foxp3+ T cells within diffuse cutaneous systemic sclerosis phenotype *vs* healthy controls. A: Increased percentage of CD4+CD25-Foxp3+ T cells within dcSSc patients (n = 13) as opposed to controls (n = 16), respectively, 10.94% ± 1.65% *vs* 6.88% ± 0.91%, P = 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

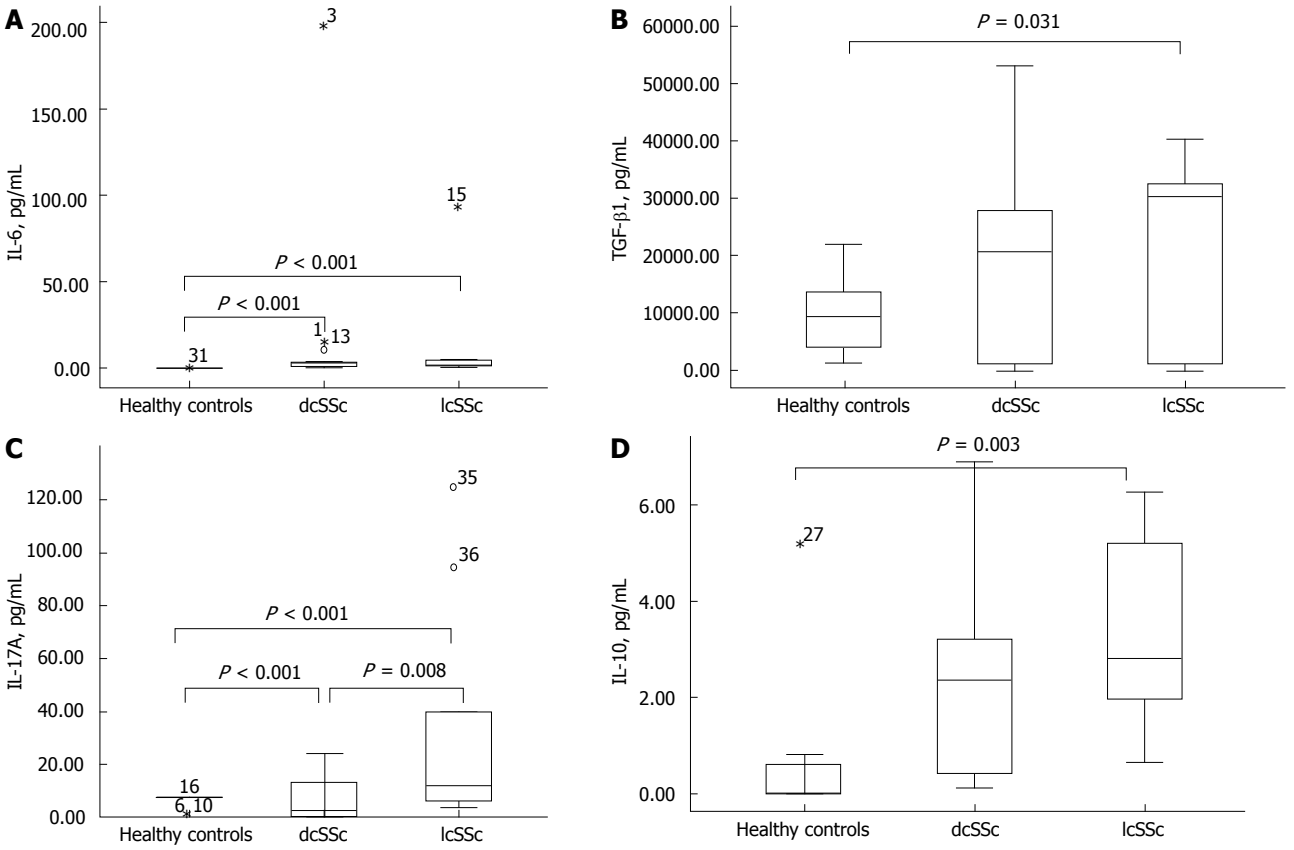

Figure 4 Elevated serum levels within the limited cutaneous systemic sclerosis (*n* = 11) and diffuse cutaneous systemic sclerosis (*n* = 13) phenotypes *vs* healthy controls (*n* = 16). Boxplots are expressed as means ± SD. A: Increased serum levels of IL-6 in lcSSc phenotype *vs* controls, $P < 0.001$. 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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