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Autologous Stromal Vascular Fraction Cells: A Tool for Facilitating Tolerance in Rheumatic Disease

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Abstract

Since the days of Medawar, the goal of therapeutic tolerogenesis has been a "Holy Grail" for immunologists. While knowledge of cellular and molecular mechanisms of this process has been increasing at an exponential rate, clinical progress has been minimal. To provide a mechanistic background of tolerogenesis, we overview common processes in the naturally occurring examples of: pregnancy, cancer, oral tolerance and anterior chamber associated immune deviation. The case is made that an easily accessible byproduct of plastic surgery, the adipose stromal vascular fraction, contains elements directly capable of promoting tolerogenesis such as T regulatory cells and inhibitory macrophages. The high content of mesenchymal and hematopoietic stem cells from this source provides the possibility of trophic/regenerative potential, which would augment tolerogenic processes by decreasing ongoing inflammation. We discuss the application of this autologous cell source in the context of rheumatoid arthritis, concluding with some practical examples of its applications.

1. Introduction:

The possibility of selectively inducing immunological non-responsiveness to specific antigens or tissues, while leaving other immune functions intact, would conceptually solve the problems of autoimmunity and transplant rejection. However, to date, while substantial progress has been made in our understanding of mechanisms of tolerance in animal models and limited clinical situations, translation to therapeutically viable solutions has not occurred. One possible explanation is the current regulatory and commercial pressures which maintain the paradigm of minimalistic dissection and interventions in specific biological processes which may yield some limited clinical benefits in isolation, while ignoring potent synergies that may be obtained by combination therapies based on a "systems" approach. While this paper is not a position paper on translational medicine, the current limitations of today's framework for development of therapeutics in the arena of immunotherapies may impose restrictions on contemplation of strategies that have a realistic possibility of practical implementation today.

In the current paper we use a somewhat unorthodox approach by attempting to globally synthesize common elements associated with immunological tolerance in several naturally occurring situations. The description of these common elements of tolerogenesis will serve as a background for our proposal of a novel, feasible, therapeutic procedure. Specifically, the procedure we will be describing involves extracting autologous adipose mononuclear cells, termed stromal vascular fraction (SVF), and subsequent systemic readministration. This has been used by us in over 160 patients with multiple sclerosis as part of a medical procedure. No adverse effects have been reported, and anecdotal reports of benefit have been published [1]. Additionally, autologous SVF administration has been used commercially in over 3000 race horses for post-injury acceleration of healing [2], with published efficacy data in a double-blind canine osteoarthritis trial [3]. Currently autologous SVF is in clinical trials for post infarct remodeling [4], ischemic heart failure [5], type I diabetes [6], and liver failure [7]. Previously the therapeutic rationale for this approach was based on high content of

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multipotent MSC in the SVF [8]. However in light of a recent publication demonstrating the large number and potent regulatory function of Treg in adipose tissue [9], we propose that alternative cell populations in this heterogeneous mixture may not only be regenerative, but also promote tolerance-. By decreasing ongoing inflammation alone, a re-equilibration of tolerogenic mechanisms may occur. Such an effect would hypothetically be amplified by components in the SVF, as well as by other therapeutic interventions that either failed, or performed poorly, as a monotherapy. We will base our discussion on the condition of rheumatoid arthritis as an autoimmune disease in which tolerance induction is sought, as well as the potential of the proposed approach to simultaneously induce tissue regeneration.

2. Four Examples of Immunological Tolerance

The concept of "immunological tolerance" dates back to the days of Medawar and observations that shared circulation during fetal development leads to selective immunological nonresponsiveness to genetically discordant fraternal party [10]. The word "tolerance" can mean numerous states and can be achieved by numerous pathways. Tolerance in its functional sense requires lack of immunological attack on the target antigen or tissue. There are two general, non-mutually exclusive, means in which this occurs: stimulation of Treg cells that actively suppress responses to the specific antigen or clonally inactivating the T cells that are responding to the specific antigen. However, in order to achieve a therapeutic response in a disease condition it is not strictly necessary to achieve "full tolerance" but in some situations immune modulation may be sufficient. For example, inhibition of Th17 responses or deviation from Th17 to Th2 may be sufficient to elicit a clinical effect. For the purposes of this discussion, we will use the word "tolerance" to include immune deviation.

Tolerance naturally occurs in several situations such as pregnancy, cancer, following oral ingestion of antigen, or administration of antigen into the anterior chamber of the eye. In animal studies, immune deviation in pregnancy was demonstrated by observations of selective immunological non-responsiveness in T cells recognizing fetally-expressed antigens [11]. Clinically, it is believed that a substantial number of pregnancy failures in the first trimester may be associated with immunological causes [12]. Immunological intervention such as allogeneic lymphocyte infusions, which are believed to inhibit production of inflammatory cytokines and increase Treg numbers [13, 14], have been demonstrated to inhibit spontaneous abortions in mice [15] and humans [16]. As an interesting aside, third party lymphocyte administration has been demonstrated to inhibit clinical RA in a small pilot trial [17]. In animal models of neoplasia, transgenic expression of defined antigens on tumors appears to lead to selective inhibition of systemic T cell responses to the specific antigens [18-20]. Clinically, the ability of tumors to inhibit peripheral T cell activity has been associated in numerous studies with poor prognosis [21-23]. Ingestion of antigen, including the putative RA autoantigen collagen II [24], has been shown to induce inhibition of both T and B cell responses in a specific manner [25, 26]. Remission of disease in animal models of RA [27], multiple sclerosis [28], and type I diabetes [29], has been reported by oral administration of autoantigens. Anterior chamber associated immune deviation (ACAID) is a phenomena in which local implantation of antigen results in a systemic immune modulation towards

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the antigen. Commonly this is demonstrated by antigen-specific suppression of DTH responses after intra-chamber administration of antigen [30]. Induction of ACAID has been used therapeutically in treatment of a mouse model of pulmonary inflammation: pretreatment with anterior chamber antigen injection resulted in systemic protection from pulmonary damage [31].

All of these situations of natural immune deviation have certain common cellular processes: a) specialized antigen presenting cells; b) induction of T cells with regulatory activity; and c) deviation of cytokine production and/or suppression of effector cell activity.

3. Dendritic Cells as Initiators of Tolerance

Dendritic cells (DC) may be conceptualized in a very general sense as dual purpose cells: In conditions of homeostasis, DC reside in an immature state and promote tolerance, whereas when exposed to injury or damage signals they mature and induce T cell activation. This general paradigm can be observed in the four conditions of tolerogenesis that were previously discussed.

In pregnancy circulating factors such as TGF-β family members [32] and hCG [33], have been reported to inhibit DC maturation and function [34, 35]. DC with tolerogenic properties are found at the maternal-fetal interface and express high concentrations of the immune suppressive enzyme indolamine 2,3 deoxygenase (IDO). Through local tryptophan depletion, as well as production of immune suppressive metabolites, cells expressing IDO have been demonstrated to induce T cell apoptosis, and more recently to elicit generation of T regulatory (Treg) cells [36, 37]. The critical role of this enzyme in pregnancy can be seen in studies where IDO inhibition results in immunologically mediated spontaneous abortion [38].

Inhibition of DC maturation and/or reprogramming by the tumor microenvironment has been well documented in numerous clinical system and animal experiments. DC isolated from tumor draining lymph nodes in melanoma [39, 40], ovarian [41], breast [42], and lung cancer [43] have been characterized as having an immature/plasmacytoid phenotype, suppressing T cell activating ability and possessing elevated levels of IDO. Manipulation of DC by silencing the gene IDO using siRNA has been demonstrated by us to evoke productive T cell immunity towards melanoma [44]. Secretion of VEGF by tumor cells is one of several proposed mechanisms for increased immature DC in tumor patients [45]. Administration of the anti-VEGFR antibody bevacizumab in patients with a variety of tumors was demonstrated to increase DC maturation and restore T cell activating activity [46].

In the situation of oral tolerance, a population of T cell suppressive CD11c+,CD11b+ DCs and CD11c+,CD8α+ DCs has been reported in the Peyer's patches [47]. These cells have been described to express high levels of IDO and possess ability to activate Treg cells [48]. Interestingly, administration of flt-3L, which expands DC systemically has been demonstrated to augment effects of oral tolerance induction [49]. A more recent report described IL-10/IL-27 expressing CD11b- DC as inducers of oral tolerance in a

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transgenic system. The relationship between these cells and IDO expressing DC remains to be elucidated [50].

Unique antigen-presenting cells bearing the macrophage marker F4/80 reside in the anterior chamber of the eye, whose migration to the spleen and activation of regulatory cells of the NKT lineage is essential for ACAID to occur [51]. The importance of this antigen presenting cell in ACAID can be seen from studies in which similar concentrations of TGF-β as those found in the anterior chamber are added exogenously to naïve monocytes. The resulting cell population, which phenotypically resembles ocular macrophages have the potential to induce immune modulation in vivo through induction of Treg cells [52].

Thus it appears that the process of tolerogenesis is associated with a critical function of the DC/antigen presenting cell. Given this knowledge artificial manipulation of DC for induction of tolerance has been performed in several settings. For example, tolerogenic modifications of DC performed by our group have included exposure of the DC to small molecule immune suppressants [53-55], gene transfection with tolerogenic genes [56, 57] and gene silencing of immune activatory genes [58-61].

4. T Regulatory Cells as Effectors of Tolerance

The concept of T cells suppressing other T cells as a mechanism of tolerance was accepted for decades. Initial studies in the 1970s focused on "T suppressor" cells, which were CD8 positive cells with the ability to restrain autoimmunity, support transplant tolerance, and were elevated in cancer. The existence of these cells came into doubt when molecular studies demonstrated fundamental proteins ascribed to these cells could not be found [62]. In the 1990s the focus started to shift to cells expressing the CD4+, CD25+ phenotype. Hall et al were the first to describe a cell population with this phenotype capable of transferring tolerance in a rat model of transplantation [63, 64]. Subsequently, Sakaguchi's group, which are commonly given credit for identification of the Treg cell, confirmed the importance of the CD4+ CD25+ phenotype based on experiments demonstrating neonatal thymectomy causes loss of Treg, which results in systemic autoimmunity, which is prevented by transfer of the cell population [65]. Since those early days, the field of Treg has blossomed, with numerous molecular details of their function having been elucidated. Interestingly, observations made with the ill-defined T suppressor cells in the early 1980s, such as ability to suppress antigen presenting cell function [66], are now being rediscovered with Treg cells [67].

In the four conditions of natural tolerogenesis described above, the DC causes generation of regulatory cells capable of inhibiting effector T cells directly, or indirectly through inhibiting other DC from maturing [68]. In pregnancy Treg with the CD4+ CD25+ FoxP3+ phenotype have been found in mouse and human fetal-placental interface [69]. Suggesting a possible role in successful pregnancy.Immunologically mediated abortions have been noticed in patients having reduced number of FoxP3 positive cells [70, 71]. In animal models of recurrent immunologically mediated abortion, administration of CTLA4 has been shown to prevent pregnancy loss through augmenting activity and number of FoxP3 positive Treg [72]. Infiltration of tumors by Treg cells has been

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correlated with poor prognosis in numerous clinical tumors including gastric cancer [73], lung cancer [74], colon cancer [75], and breast cancer [76]. Conversely, reduction of Treg through antibody depletion has demonstrated derepression of immunity in animal models [77] and limited patient experiences [78]. In oral tolerance, conventional FoxP3 expressing Treg [48], as well as TGF-beta secreting "Th3" cells have been defined [79]. Although heterogeneity of effector function may be explained by different model systems used, at least one report suggests involvement of FoxP3 in Th3 cell function, indicating that suppressor mechanisms may not be mutually exclusive [80]. Mechanisms of suppression in ACAID involve a type of regulatory natural killer T (NKT) cell which upon activation secretes urokinase-type plasminogen activator locally. This causes activation of latent TGF-beta and suppressor of effector function [81].

Numerous mechanisms of Treg inhibition of immune effector function have been described. Originally, suppression of T cell activation by membrane-bound TGF-b was proposed [82]. Subsequent studies have demonstrated Treg inhibit DC maturation, thus providing an indirect mechanism of effector suppression. Treg-mediated suppression of NK [83] and macrophage function [84] has been reported. Perhaps one of the most intriguing mechanisms of suppression is direct lysis of effector cells through a granzyme B/perforin-dependent mechanism [85].

5. Tissue Injury as Enemy of Tolerogenesis

The balance between the host's need for induction of immunity versus tolerance in response to antigen is dictated by integration of several factors which are globally associated with the concept of "danger". Early experiments demonstrated that offspring of mice with transgenic T cell receptors towards an autoantigen crossed with mice expressing the antigen do not develop autoimmunity despite large numbers of circulating autoreactive cells. However, when a "danger signal" was administered, self-tolerance would be lost and autoimmunity ensued [86, 87]. Essentially, the concept was that despite existence of autoreactive T cells, the immature DC in the basal state led to generation of Treg cells, as well as anergy, due to lack of costimulation and expression of co-inhibitory receptors. The identification of toll like receptors (TLRs) and subsequently other pattern recognition receptors, provided a molecular basis for the concept of "danger" [88]. Essentially innate reactions, primarily mediated by the DC controlled whether the adaptive response would mature into a productive immunity or ignorance of the antigen.

Non-TLR sensors of "danger" include retinoid acid inducible gene (RIG)-I-like receptors (RLRs) such as retinoid acid inducible gene (RIG)-I, melanoma differentiation antigen (MDA)5, and DNA-dependent activator of IFN-regulatory factors (DAI), and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), which include NOD1, NOD2, NLRP3 and absent in melanoma (AIM)2 [89]. RIG-1 and MDA5 are intracellular receptors that recognize single-stranded RNA bearing 5'-triphosphates as found in some viruses [90], as well as free DNA [91]. DAI is activated by double stranded DNA, originally being identified as a cytosolic receptor capable of inducing interferon responses in cells lacking TLR9 [92]. Generally RLRs are associated with interferon induction in response to nucleic acids, whereas NLRs recognize a wider set of

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pathogen associated molecular patterns (PAMP) and damage associated molecular patterns (DAMP) [89]. NLRs are similar to RLRs in that they also are cytosolic, however one of their effector mechanisms is production of IL-1 through activation of the caspase-1/inflammasome pathway.

In the conditions of natural tolerogenesis described above, experimental data have demonstrated breaking of tolerance through injury or various inflammatory signals. In pregnancy it is known that various TLR activators are associated with complications and fetal loss [93]. Induction of anticancer immunity has been reported with DC activators such as TLR-9 agonists, which are currently in clinical trials [94, 95]. Blockade of oral tolerance and actual conversion to immunity has been demonstrated with agents that induce DC maturation [96, 97]. Breaking of ocular tolerance has been observed in experimental autoimmune uveoretinitis to be mediated by the TLR4 agonist HMGB1 [98]. Furthermore, experimental ocular injury has been shown to inhibit ACAID through suppression of TGF-beta, however molecular mediators remain unclear [99].

In conditions of autoimmunity, such as RA, there is a local inflammatory response occurring, which has extra-articular implications. For example, patients with RA exhibit classical symptoms of general inflammation such as elevated ESR, C-reactive protein, and inflammatory cytokines such as IL-1, IL-6, and TNF-alpha [100]. The effects of this systemic inflammation may be profound. A paradoxical T cell hyporesponsiveness has been observed in RA patients, which is believed to be mediated by oxidative stress released from inflamed sites [101]. This constant inflammatory damage potentially leads to self amplification of the disease. For example, it is known that inflamed synovium is associated with increased expression of TLR-4 and 2, and that in absence of TLR-4, CIA development is inhibited [102]. Several TLR agonists have been found to be constitutively expressed in the synovium of RA patients. HSP22 is a heat shock protein that was shown to activate DC in a TLR-4 dependent manner and correlated with disease [103]. Another heat shock protein, gp96 was found in synovial fluid of RA patients and induced macrophage activation through TLR-2 [104]. Free RNA released by injured cells in the synovium as also been related to induction of inflammation through a TLR-3 dependent pathway [105]. It is thus likely that by maintaining a persistent inflammatory environment with numerous endogenous TLR-ligands available , it is difficult to induce antigen-specific suppression of immunity.

Experimental evidence exists for the role of tissue injury blocking tolerogenesis. In addition to DC maturation, which has been demonstrated to occur in many situations by injury signal-generated TLR agonists, these signals also block Treg generation. For example, DC generated IL-6 make in response to TLR-4 activations renders T cells non-responsive to suppressive effects of Treg cells [106]. TNF-alpha, which is elevated in RA patients and is produced, in part by macrophage activation, has been demonstrated to directly inhibit Treg activity in vitro and in vivo [107]. Notably, RA patients treated with Remicade have been demonstrated to recover deficiencies in Treg activity.

The current notion is that tolerance requires antigen presenting cells to be in an unprimed, immature state, and that ongoing inflammatory conditions inherently stimulate

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maturation of DC. Therefore it would be logical to aim to first reduce inflammation and "danger" signals, before utilization of tolerance promoting strategies. The ability of the immune system to actively "self tolerize" when a foreign antigen is present in absence of danger can be seen in studies where allogeneic pancreatic islets are depleted of antigen presenting cell content by high concentrations of oxygen. This results in long-term survival and generation of cells with regulatory activity capable of transferring tolerance [108]. Thus we are proposing that administration of regenerative cells may on the one hand reduce "danger" but on the other hand may have direct tolerance-promoting effects.

6. Stromal Vascular Fraction: MSC, HSC, and Tregs

The stromal vascular fraction (SVF) is comprised of a mixed population of pericytes, EPCs, MSCs, hematopoietic stem cells [109], Treg [9], and alternatively activated monocytes. This mixture conceptually may be a useful source of cells with both immune modulatory and regenerative properties. The thesis of the current paper is that SVF may be a useful adjuvant for induction of tolerance. Accordingly we will describe some of the constituent cells of relevance.

6.1 MSC

SVF is believed to contain a higher population of MSC as compared to other sources, allowing for obtaining regenerative effects without need for ex vivo expansion. MSC are a population of immune modulatory adherent cells capable of differentiating into bone, cartilage and adipose tissue. These cells have been isolated from numerous tissues including adipose [110], heart [111] , Wharton's Jelly [112], dental pulp [113], peripheral blood [114], cord blood [115], and more recently menstrual blood [116-118]. In addition to their tissue regenerative/growth factor secreting activities, these cells possess anti-inflammatory activities which appear to be present regardless of tissue of origin [119, 120]. Mechanistically, MSC appear to suppress inflammation through secretion of anti-inflammatory mediators such as IL-10 [121], TGF-beta [122], LIF [123], soluble HLA-G [124] and IL-1 receptor antagonist [125]. Additionally, MSC express immune regulatory enzyme such as cycloxygenase [126] and indolamine 2,3 deoxygenase [127] which appear to synergize with ongoing tolerogenic processes. Suppression of the autoimmune-associated cytokine IL-17 has been reported by MSC [128]. Indirectly MSC appear to inhibit autoimmunity through ability to induce generation of T regulatory cells [129].

The in vivo anti-inflammatory effects of MSC may be witnessed by success in treating animal models of immune mediated/inflammatory pathologies such as multiple sclerosis [130], colitis [131], graft versus host disease [132], rheumatoid arthritis [133], and ischemia/reperfusion injury [134]. Clinically, MSC have demonstrated ability to inhibit conditions such graft versus host (GVHD) [135-140], systemic lupus erythematosis (SLE) [141], and end stage liver disease [142]. Based on their ability to induce regeneration of injured tissue, combined with anti-inflammatory effects, we believe the MSC population may be useful as an adjuvant to tolerogenic strategies in treatment of autoimmune conditions.

6.2 Hematopoietic Stem Cells

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Numerous studies have demonstrated CD34 hematopoietic stem cells (HSC) have therapeutic activity in animal models of diverse conditions such as stroke [143], myocardial infarction [144], and liver failure [145], with clinical trials are currently ongoing for these indications [146-148]. Mechanistically, the function of CD34 cells for non-hematopoietic conditions is the subject of discussion. Previous thoughts that CD34 cells have transdifferentiation ability to convert into damaged tissue have to some extent been challenged [149, 150], with the current prevailing concept being that trophic/paracrine activities may be more relevant. Indeed, basal and induced expression of growth factors such as VEGF, HGF, IGF-1, and FGF-2 have been described in conditioned media of isolated CD34+ cells [151]. Additionally, CD34+ cells are known to be angiogenic, as demonstrated by ability to induce functional collateralization in hindlimb ischemia models and patients with critical limb ischemia [152]. Since angiogenesis is a critical component of tissue healing, this has also been proposed as a mechanism of action [143].

In addition to regenerative activities, it has been shown that CD34+ cells possess direct immune suppressive/tolerance inducing ability. This was postulated based on studies demonstrating that during bone marrow transplantation, "megadose" CD34 cells would preferentially induce graft acceptance [153]. Investigation into the mechanisms of this effect led to studies in which in vitro mixed lymphocyte culture (MLR) assays were used to show CD34 cells are capable of inducing death in CD8 cells responding to alloantigen of the same origin as the CD34 cell. The effect does not occur against third-party cells and is believed to be associated with expression of MHC class I and class II antigens but not costimulatory molecules [154]. Additionally, a possible role for FasL in this "veto effect" has been proposed [155]. Indeed the association between high expression of HLA-DR and FasL suggests the possibility of antigen presentation and concurrent T cell deletion by the FasL [156]. Local production of TNF-α by CD34 cells has been described as another possible mechanism of depletion of reactive T cells [157]. TGF-β, one of the effector cytokines responsible for Treg suppression of T cells [158], neutrophils [159], macrophages [160], and dendritic cells, has been implicated as an autocrine factor released by CD34 cells which maintains a G0 state [161-164]. The possibility of CD34 cells acting through a TGF-β dependent mechanism is an area requiring future experimental investigation. In addition to direct suppression, an interesting paper by Kared et al, demonstrated that hematopoietic progenitors, by expression of Jagged, can induce generation of Treg cells, which are capable of inhibiting autoimmune diabetes [165]. The recent finding that circulating CD34 cells traffic through blood, lymph, and peripheral organs, suggests that in addition to hematopoietic functions, CD34+ cells may play an "immunosurveillance" role in that upon activation by TLR agonists they differentiate into DC, whose maturity is associated with presence of innate immune activation signals [166].

6.3 Treg Cells

Feuerer et al. [9] examined adipose tissue for content of Treg cells based on functionality and expression of the CD4+, CD25+, FoxP3+ phenotype. Increased numbers of these cells were observed in adipose compared to other peripheral tissues. The authors made a case for the role of Treg in controlling inflammation associated with obesity.

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Interestingly, the adipose Treg's appeared to have a "primed" phenotype, as witnessed by highly elevated IL-10 transcript and protein levels in adipose Treg.

The possibility of adipose-derived Treg cells having enhanced in vivo expansion and functional activity may be conceptually supported by studies showing that adipose derived cytokines such as leptin and TNF-α inhibit Treg proliferation and activity in vivo [107, 167]. The local effects of these cytokines would conceptually, be altered by liberating Treg from fat followed by systemic re-administration. Administration of a large number of Treg cells with augmented in vivo proliferative and functional potential may result in a reduction of the threshold needed to attain tolerance to an ongoing immune response. Indeed interventions inducing an antigen-nonspecific immune modulation have previously been demonstrated to cause antigen-specific Tregs, and tolerance [168].

The rationale for administration of autologous SVF as a source of immune modulation is also based on expression of high numbers of alternatively activated macrophages, which has been discussed by us in a previous report [1].

7. The Problem of Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disorder affecting approximately 0.5-1% of the global population [169], characterized by immune-mediated synovial inflammation and joint deterioration. In general, because of the critical role of inflammation in the pathology of RA, patients have in the past been started on NSAIDS, however more recent practice has been concurrent initiation of disease modifying antirheumatic drugs (DMARDs). These agents are slow acting but have been demonstrated to inhibit radiological progression of RA. Such agents typically include: 1) hydroxychloroquine, which acts in part as a toll like receptor (TLR) 7/9 antagonist, thus decreasing innate immune activation [170]; 2) Leflunomide, an antimetabolite that inhibits pyrimidine synthesis and protein tyrosine kinase activity [171], which results in suppression of T cell responses [172], and has been also demonstrated to inhibit dendritic cell (DC) activation [173]; 3) Injectable gold compounds such as auranofin which directly or through metabolites such as dicyanogold(i) have been demonstrated to inhibit T cell and antigen presenting cell activation [174, 175], as well as cause Th2 deviation [176]; 4) Sulfasalazine, was used since 1950, acts primarily through inhibition of cycloxygenase and lipoxygenase [177]; and 5) Methotrexate, an antifolate that inhibits T cell activation and proliferation, that has been one of the golden standards for RA [178]. Typically combinations of DMARDs with glucocorticoids are used in clinical practice [179].

The TNF-α-targeting agents, Remicade, Enbrel, and Humira, sometimes referred to as "biologic agents" are used primarily after response to conventional DMARDs has failed [180]. Although improvement in quality of life has occurred as a result of biological DMARDs, substantial progress remains to be made. For example, TNF-α blockers have been associated with reactivation of infectious disease, autoantibody formation and the possibility of increased lymphoma risk [181, 182]. Thus to date, one of the major

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limitations to RA therapy has been lack of ability to specifically inhibit autoreactive responses while allowing other immune components to remain intact.

7.1 Tolerance Induction in RA

The autoimmune nature of RA suggests the possibility of specifically inhibiting the pathological response through "reprogramming" of immune effectors. However, in order to evoke antigen-specific immune modulation, it is necessary to have knowledge of autoantigens that are present in a majority of the population and contribute to disease. Collagen II is an extracellular matrix component found primarily in the synovial tissue that is usually sequestered from immunological attack. Induction of a RA-like disease has been reported in inbred strains following immunization of collagen II in the presence of adjuvant [183]. Autoimmunity was not induced by collagen I or III, nor by denatured collagen II protein. Supporting a causative immunopathological effect of collagen I-I specific T cells were experiments undertaken in which the RA-like disease could be transferred to naïve recipients by administration of lymph node cells [184]. Subsequent work cloning T cell lines from synovial membranes of patients with RA demonstrated existence of collagen II-specific cells that persisted for a period of 3 years in vivo [185]. Subsequent PCR-studies of T cell receptor beta chains confirmed the oligoclonal expansion of collagen II-reactive cells in patients [186]. In 1993 Weiner's group reported a double blind, placebo-controlled trial of 60 patients with advanced RA treated by oral administration of chicken collagen II for a period of 3 months. Responses in terms of decreased number of swollen joints were observed in the treated population but not placebo controls. Of the treated patients four presented with complete remission of disease. No treatment-associated adverse effects were noted [187]. Unfortunately, Phase III trials using oral tolerance in RA have not met primary efficacy endpoints [188].

Given the general failure of oral tolerance in RA, more specific approaches have involved stimulation of tolerogenic responses using ex vivo manipulated DC. Dendritic cells (DC) under physiological conditions promote tolerance, and when exposed to injury/damage signals mature and induce T cell activation. By ex vivo manipulating antigen pulsed/donor specific DC, we have previously been able to induce antigen-specific suppression of immunity and generation of T regulatory (Treg) cells. Tolerogenic modifications of DC performed by our group have included exposure of the DC to small molecule immune suppressants [53-55], gene transfection with tolerogenic genes [56, 57] and gene silencing of immune activatory genes [58-61]. In our previous work, we have demonstrated ability to prevent CIA induction by pulsing DC with collagen II (CII) and suppressing DC maturation with chemical or genetic means. Limitations of these data, however, have been the lack of robust inhibition of inflammatory responses when administration of manipulated DC was performed at various time points subsequent to disease onset. The general failure of antigen specific approaches, both in oral tolerance, as well as DC-based approaches may be the result of underlying inflammatory reactions.

8. MSC as Tolerizers

We previously discussed the possibility of using SVF as a source of regenerative and immune modulatory cells. While having touched on the MSC component briefly, here we will discuss some unique aspects of this population relevant to tolerance-inducing

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regiments. Specifically, the possibility of using systemically-administered mesenchymal stem cells (MSC) as a cellular therapy for RA has several conceptual advantages that address the previously mentioned drawbacks of current approaches. One such advantage is that the MSC may be viewed as a "smart" immune modulator. In contrast to current therapies, which globally cause immune suppression, production of anti-inflammatory factors by MSC appears to be dependent on their environment, with upregulation of factors such as TGF-b, HLA-G, IL-10, and neuropilin-A ligands galectin-1 and Semaphorin-3A in response to immune/inflammatory stimuli but little in the basal state [122, 123, 189-191]. Additionally, systemically administered MSC possess ability to selectively home to injured/hypoxic areas by recognition of signals such as HMGB1 or CXCR1, respectively [192-195]. The ability to home to injury, combined with selective induction of immune modulation only in response to inflammatory/danger signals suggests the possibility that systemically administered MSC do not cause global immune suppression. This is supported by clinical studies using MSC for other inflammatory conditions, which to date, have not reported immune suppression associated adverse effects [196-198]. Another important aspect of MSC therapy is their ability to regenerate injured tissue through direct differentiation into articular tissue [199], as well as ability to secret growth factors capable of augmenting endogenous regenerative processes [200].

Physiologically, the role of MSC in RA is a matter of debate. Nakagawa et al used radiolabeling of bone marrow cells to demonstrate migration of bone marrow stromal cells into synovium of rats suffering from CIA. While inference was made to contribution of the MSC to synovial proliferation, a causal relationship was not demonstrated [201]. Subsequently, it was reported that MSC differentiate into nurse-like cells that promote adhesion of lymphocytes to the synovium [202]. Indeed, in patients with RA, but not healthy controls, bone marrow MSC-generating capacity is markedly reduced [203], whether this is due to systemic TNF-alpha suppression of bone marrow [204], or exhaustion of MSC precursors by heightened demand is not known. However, there are suggestions of the latter based on observations of shorter telomeres in MSC derived from RA patients [203]. The concept of MSC contributing to pathology was demonstrated in the CIA model by Djouad et al who reported administration of MSC resulted in upregulation of Th1 immunity and worsening of symptoms [205]. The investigators attributed this to their observations that TNF-alpha abrogates immune regulatory activities of MSC. This study however was contradicted by several more recent studies in which inhibition of arthritis progression, or even regression of disease was observed. Mao et al demonstrated administration of rat MSC intravenously into DBA mice with full-blow CIA resulted in regression of disease, which was correlated with decreased production of TNF-alpha and IL-17 [206]. Gonzalez et al administered ex vivo expanded human adipose-derived MSC into the same animal model. Inhibition of disease progression was observed, which correlated with increased Treg numbers that were specific for CII. This study supports the previous principle discussed that an antigen-nonspecific tolerizing event may contribute to development of antigen specific suppression [207]. In addition to immune modulation, it is possible that cartilage tissue generated de novo from MSC possesses a decreased level of immunogenicity [208]. The overall anti-inflammatory/immune modulatory effects of MSC have been demonstrated in a variety of settings including the mouse model of multiple sclerosis [209, 210],

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transplant rejection [129], diabetes [211], the mouse model of SLE [212], and autoimmune enteropathy [131].

9. Case Report

A 67 year old female with a history of severe pain and swelling in her fingers, stiffness in hands and wrists especially upon rising in the morning lasting approximately 10 to 15 minutes which began approximately in August 2007. The patient self-medicated with NSAIDS until seeking medical attention in April of 2008 as her symptoms continued to worsen. Her symptoms at this time included progressively worsening fatigue, excess sleeping, redness of both hands, and now pain and swelling in both ankles and knees, difficulty walking even short distances—limited by pain, and profound fatigue. No fever, rash, neurologic symptoms were described.
Other than a surgical history of 3 caesarean sections, partial colon resection due to ruptured diverticulum, and laparoscopic cholecystectom her past medical history was unremarkable.

Physical exam revealed swollen, inflamed MCP's & PIP's in both hands. Both wrists and 1st right MTP joint were also swollen and inflamed. Range of motion of shoulder, neck and knees were normal. No rheumatoid nodules, vasculitic lesions, ulnar deviation of the MCP joints or swan deformity were noted. The rest of the physical exam was unremarkable. Lab data revealed rheumatoid factor level of 75 IU/ml, (normal range 0-39) and anti-cyclic citrullated peptide antibody (CCP Ab) titer of >250, (normal range <25); erythrocyte sedimentation ration (ESR) –4), antistreptoysin O Ab – 6.3: parvovirus H19 IgG elevated to 5.0 (range<0.9). CBC LFTs renal function were all normal. Based on these findings a diagnosis of RA was made. Patient was given 40 mg of Kenalog IM for immediate pain management and was recommended to start treatment with plaquenil and methothrexate but refused and self-medicated with NSAIDS and Tylenol PRN for pain from April to August 2008.

The patient arrived at the ICM Clinic in Costa Rica on August 6, 2008 for stem cell therapy with autologous fat derived cells (stromal vascular fraction). A liposuction was performed and 500 cc of adipose tissue was obtained. The tissue was digested and the SVF was isolated, tested for sterility and endotoxin and frozen in liquid nitrogen [1]. Cells were prepared under the guidelines of Good Tissue Practices 21 CFR 1271 as relates to sample screening and processing. The patient was allowed to heal from the liposuction for one week. She then received a total of 53 million SVF cells in two successive day intravenously infusions. No side effects from the infusions were reported.

The patient reported considerable resolution of her joint pain and stiffness after the second infusion and began walking normally by the third day after treatment with no pain or symptoms. Physical exam at this time revealed no joints effusions in her hands, wrists or feet. Rest of the physical exam was normal.

The patient continues to do well after the 15 months of stem cell therapy, trains daily with a personal trainer without limitations. Her last lab data was from September, 2009

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and revealed a decrease of a rheumatoid factor from 75 to 51.8 IU per ml and CCP IgG from >250 to > 100.

Future Directions

Immunoregulatory circuits responsible for tolerance induction are complex and multi-cellular. Based on the conditions of "natural tolerance" in pregnancy, cancer, oral tolerance and ACAID, common themes appear such as need for antigen presentation in a "tolerogenic context", the generation of Treg cells, and the maintenance of tolerance by constant suppression of inflammation. Conceptually, a therapeutic approach for induction of tolerance in a clinical situation would need to mimic events occurring in one of the four conditions described. Clinically it is very difficult to implement multiple acting therapies simultaneously, especially when some of the components are novel. That said, in the development of a "tolerogenic protocol" it may be necessary to consider agents that have a history of clinical use.

Creation of a tolerogenic protocol would require several components: a) a source of antigen; b) a response to the antigen in the form of an antigen presentation event; c) manipulation of the response so as to endow creation of a regulatory cell population; and d) maintenance/amplification of the regulatory cell population. Using this framework, several possibilities emerge. Antigen load may be administered exogenously, in the form of peptides or proteins given intravenously [213, 214], selected for tolerogenic epitopes [215, 216], administered in the context of tolerogenic DC [217] or administered orally [218]. Alternatively, the antigenic source may be already existing in the host, but the host would have to be manipulated in a manner so as to promote tolerogenesis. In both situations the SVF population may be beneficial. Expansion of Treg cells has been shown to occur in response to tolerogenic peptides [219], during intravenous [220, 221], and oral tolerance [48]. According to our hypothesis, the concurrent administration SVF would provide a ready-source of Tregs that could be expanded in vivo by the tolerogenic regime. In the situation of tolerance to endogenous antigens, the MSC component of the SVF may induce a localized anti-inflammatory environment which would be pro-tolerogenic. Manipulation of the antigen presenting event may be performed using agents clinically available such as short course of rapamycin [222], or DMARDs that inhibit DC maturation such as hydroxychloroquine, which inhibits DC maturation by suppressing TLR 8/9 activation [170], or lefluonamide [173]. Hypothetically, the MSC and Treg content of SVF may also be capable of inhibiting DC maturation, since both of these cell types have been reported to possess this property [223-225]. The generation of Treg cells could hypothetically be amplified by agents such as anti-CD3 monoclonal antibody, which has been used with some success in autoimmune diabetes [226]. Other agents could include TNF-alpha blockers that have previously been shown to restore Treg functional deficiencies in RA patients through induction of FoxP3 expression [107]. Administration of SVF may conceptually allow for amplification of Tregs that would recognize the autoantigen being presented. Maintenance of the tolerogenic feedback loop could be accomplished by providing regenerative cells, such as MSC in the SVF, which would hypothetically result in suppression of "danger signals" by reduction of inflammation.

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In conclusion, we propose that SVF cells represent a novel, easy to implement cell therapy that warrants investigation as a monotherapy or adjuvant to tolerance induction protocols. The fact that autotransplantation of adipose tissue is part of standard cosmetic surgery practice without adverse events [227, 228], as well as our pilot clinical data with SVF in multiple sclerosis [1], and RA, supports the notion of feasibility. Of the components of SVF, the MSC fraction may provide direct immune regulatory activities, as well as stimulation of tissue regeneration, thus decreasing "danger signals" which inhibit tolerogenesis. CD34 cells found in SVF have the potential to immune regulate, although further work in this area is necessary. The recent finding of enhanced Treg numbers and activity in adipose tissue suggests SVF may be a previously unrecognized source of regulatory cells capable of in vivo expansion subsequent to administration [9]. In a previous study we reported treatment of 3 patients with multiple sclerosis with autologous SVF, which underwent a profound clinical response [1]. The cases presented here serve to expand on the "clinical signal" that an anti-inflammatory/disease modifying effect may be achieved using the simple process of autologous SVF administration. While future studies are obviously needed to confirm these preliminary observations, the establishment of feasibility and administration protocols serves as a basis for future studies. An interesting question presented by these studies is whether the adipose resident Treg cells may also have deregulated function as found in the periphery of patients with RA [229]. This is currently being investigated.

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