Chapter 13

Stem Cell-Based Cellular Therapy in Rheumatoid Arthritis

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1. Introduction

1.1. Potential mechanisms of pathogenesis of RA

Rheumatoid arthritis (RA) is a chronically systemic inflammatory disorder that ultimately leads to the destruction of joint architecture. RA affects more than two million people in America and about 1% of the world's population, women are three times more prone to RA than men (1, 2). About 58 million people suffer from RA all over the world. The disease has a variable course, from a mild, occasionally symptomatic illness requiring only symptomatic therapy to a fully onset requiring aggressive immunosuppressive therapy, surgery or both. The hallmarks of RA is the systemic loss of regulation of immune system characterized by either acute or chronic inflammation, in which the immune system primarily attacks the joints of the body leading to tissue pathology and clinical disease. The etiological factor causes the immune reaction is still unknown. The pathological insult starts with inflammation of the synovium (sinovita), which then takes the form of proliferative nature (pannus) with the damage of cartilage and bones. Plasmatic cells of joint produce antibody that form aggregates of IgG. In turn, IgG aggregates have been recognized by the immune system as foreign antigens and plasmatic cells (B cells, dendritic cells, T cells, and macrophages formed lymphoid follicle-like structures within the synovial membrane) begin to produce autoantibodies against those structures known as rheumatoid factors (RF). Autoantibodies directed against the Fc fragment of IgG. (3, 4). The most important type of RF is the IgM class, which appears in 70-80% of patients with RA. In the course of the disease, the normal, relatively avascular synovium then becomes heavily infiltrated by a wide variety of cells, including B cells, macrophages, fibroblasts, neutrophil granulocytes, dendritic cells, and many other cells (5). Because of the autoimmune inflammatory process, which leads to formation of pannus - granulation tissue that occurs from in the inflamed synovium, which is consists of actively proliferating fibroblasts, lymphocytes, macrophages

and rich with vessels. The synovial lining increases to a thickness of up to 30 cell layers, presumably through influx of macrophages, and by expansion of synovial fibroblasts. Pannus grows intensively, percolates from the synovial tissue to the cartilage and destroys it by the influence of enzymes, which are induced by the production of high amount of proinflammatory cytokines mainly tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6 within the pannus. Furthermore, many other cytokines such as IL-17, IL-18, IL-15, chemokines, and angiogenic molecules are present in the inflamed synovial membrane and drive the development of the disease. Subsequently, these proinflammatory cytokines activate signal transduction pathways and transcription factors, which, in turn, control the production of cytokines (6). Gradually, intrasynovial cartilage disappears, it is replaced by granulation tissue and ankylosis develops. Chronic inflammation of periarthric tissues, joint capsule, ligaments and tendons lead to deformation of joints, semiluxations, and contractures. Currently, there is a large body of literature which suggests that autoimmune processes play a central role in the early stages of RA, and in later stages non-immune mechanisms (*i.e.*, the ability of pannus for growth, invasion and destruction of joint cartilage) are more important. However, the crucial triggers for the onset of diseases that lead to pathogenic events in RA are still not fully understood. It is generally believed that RA is the result of interactions amongst genetic, environmental, biomechanical factors, neuroimmunological interactions and altered articular microvascular function (7). Several genetic loci have been proposed based on the genome wide association studies to have an association with the susceptibility and severity of RA (8-12). A disease association with HLA-DR4 alleles (which contain the shared epitope) is well established (13). However, apart from the major histocompatibility complex, genetic factors associated with RA severity have not yet been convincingly proved. However, other loci, including PTPN22 (protein tyrosine phosphatase, non-receptor type 22), PADI4 (peptidyl arginine deiminase, type IV), CTLA4 (cytotoxic T-lymphocyte antigen 4), Fc◎Rs (Fc receptors for IgG), CD40 and CDK6 were identified that involved in susceptibility and higher rate of joint destruction (14-17). Various cytokines and cytokine-receptor loci, such as those encoding TNF, IL-1, IL-10, and IL-18, have been implicated in disease association to various degrees in distinct populations (12). The area of identification of susceptible gene loci in RA is currently under intense investigation. The frequencies of bone marrow and circulating $CD34^+$ HPC (hematopoietic progenitor cells) in RA patient are reduced (18-20), the median apoptotic index of early bone marrow myeloid precursors is significantly higher than in controls (21, 22) and their proliferative activity is impaired (18, 19). Reduced colony formation by highly purified RA $CD34^+$ cells may be linked to an intrinsic defect in the clonogenic potential of RA progenitors. However, the molecular pathways responsible for this abnormality remain undetermined. HPC isolated from RA patients are hyporesponsive to hematopoietic growth factors and fail to expand appropriately. This defect appears specifically connected to the extracellular signal-regulated protein kinase (ERK) signaling pathway, a signaling cascade critically involved in cell cycle entry and progression of progenitor cells (23). The molecular defect causing RA has not been fully characterized although it is reported that many cell population have aberrant function, including monocytes, macrophages, B cells, T cells, endothelial cells, and fibroblasts, participate in the ongoing inflammatory process. Multiple

inflammatory pathways contribute to the persistent chronic inflammation in RA (24). Recent advances in our understanding of the key cells (*i.e.*, T cells, B cells) and inflammatory cytokines (TNF and IL-6) have provided therapeutic opportunities, which are now directly targeted by biologic agents approved by the US Food and Drug Administration (FDA) for the treatment of RA.

2. Current biological therapies for RA

Biologic therapies have brought improved efficacy in the treatment of RA. However, these therapies sometimes fail or produce only partial responses, because of the lack of reliable predictive biomarkers of prognosis, therapeutic response, and toxicity data. Sustained remission is rarely achieved and requires ongoing pharmacological intervention. Although RA often responds to immunosuppressive medication including corticosteroids, methotrexate, azathioprine and cyclophosphamide, or non-steroidal anti-inflammatory drugs, none of these drugs showed a curative effect (25). Synthetic disease-modifying antirheumatic drugs (DMARDs), such as methotrexate, leflunomide, and sulfasalazine, have evidently improved clinical symptoms and reduced joint damage in RA patients. However, despite the effectiveness of synthetic DMARDs, many patients who have been taking those drugs continue to have clinical symptoms of inflammation and progressive joint destruction. Recently, FDA approved many TNF-α inhibitors including infliximab, etanercept, adalimumab, golimumab, and certolizumab pegol in the RA treatment. In randomized clinical trials, all of these agents have been shown effective in reducing clinical symptom of inflammation in RA patients who have failed synthetic DMARDs (26). Multiple studies have demonstrated significant benefits of early treatment with TNF-α inhibitors combined with methotrexate (27-29). Other FDA-approved biologic agents for treating moderate-to- severe RA include abatacept, rituximab, and tocilizumab (30-33). However, all biologic agents carry an increased risk of infections.

3. Current concept of stem cell therapy in RA

The natural healing process for RA requires a combination of stem cells, growth factors, and matrix to optimize tissue repair and regeneration. The use of these bioactive cells to supplement and hasten the natural healing process is considered by many to be a new era of clinical treatment. This ideal cell population for the treatment of RA should have a series of properties, namely a high osteogenic and chondrogenic potential, and at the same time, it should be easily expanded, *i.e.*, capable of self replicating and can be maintained in cultures for a long period of time. Due to their natural and intrinsic properties, stem cells are one of the best available cell types. There are mainly four types of stem cells undergoing current study. They are embryonic stem cells (ESCs), allogeneic (donor) stem cells (SCs), induced pluripotential adult stem cells (iPSCs), and last but not least autologous stem cells. Of these four, only two, donor SCs and autologous SCs have been used to treat arthritis in human at present. The multipotent autologous stem cells also know as mesenchyme stem cell (MSC) are sufficient to be used to treat disorders involving connective tissue, such as blood,

tendon, ligament, cartilage, bone, nerve, muscle, and liver. During the last 1-2 decades, the scientific community witnessed and reported the appearance of several sources of stem cells with both osteogenic and chondrogenic potential. There are many different sources of adult stem cells (*e.g.*, bone marrow, periosteum, adipose tissue, skeletal muscle and umbilical cord) for bone and cartilage regenerative medicine, namely those focusing on the differentiation potential of the latter, as well as *in vivo* proof of concept of their applicability.

4. MSC based stem cell therapy in RA

MSCs are the non-hematopoietic progenitor cells found in various adult tissues. MSCs have been characterized by the ease of isolation and rapid growth *in vitro* while maintaining their differentiation potential, allowing for extensive culture expansion to obtain large quantities suitable for therapeutic use. MSCs are characterized by the capacity to adhere to plastic surface, the phenotype ($CD90^+$, $CD73^+$, $CD105^+$ and absence of hematopoietic markers including $CD45^-$, CD34, $CD11b^-$, $CD14^-$ CD79α or CD19 and HLA-DR^-), the ability to differentiate into at least three distinct lineages including adipocytes, chondrocytes, osteoblasts, and the capacity for self-renewal (34, 35). MSCs are hypoimmunogenic and are able to evade the host immune surveillance. MSCs express low (fetal) to intermediate (adult) level of major histocompatibility complex (MHC) class I molecules and do not express MHC class II molecules on the cell surface, although an intracellular pool of MHC class II molecules can be stimulated to express on the cell surface by interferon-gamma (IFN-γ) (36). However, because MSC do not express any costimulatory molecules, including B7-1 (CD80), B7-2 (CD86), or CD40, MSCs do not activate alloreative T cells (37). After differentiation into adipocytes, osteoblasts, and chondrocytes, MSCs continue to express MHC class I but not class II molecules on the cell surface, even under stimulation, and continue to be non-immunogenic (36, 38). However, the immune privilege of MSCs seem to be limited. A few studies in mouse system have reported that allogeneic mismatched MSCs were rejected by the host and could not form ectopic bone *in vivo*, while syngeneic recipient allowed ectopic bone formation despite the fact that, the MSCs showed immunosuppressive activity *in vitro* (39, 40). Recently, MSCs have been shown to possess several immunomodulatory properties. These include suppression of T cell proliferation, influencing dendritic cell maturation and function, suppression of B cell proliferation and terminal differentiation, and immune modulation of other immune cells such as NK cells and macrophages (41-44). Meanwhile, MSCs also express the Toll-like receptors (TLR)-2 -8. The addition of polyI:C (TLR3 ligand), Pam3Cys (TLR2 ligand) or LPS (TLR4 ligand) inhibited the differentiation potential of MSCs, but spared their immunosuppressive function (45). MSCs also inhibit the maturation and decrease the expression of antigen presentation molecules and costimulatory molecules on the surface of antigen-presenting cells (APCs) (42). Furthermore, MSCs not only have a direct inhibitory effect on T cells but also affect the first critical step of immune response in which they can inhibit the differentiation and maturation of the APCs and cause the dendritic cells to switch cytokine secretion profile to decrease the secretion of proinflammatory cytokines such as TNF-α, IFN-γ, and IL-12, and importantly, increase the production of IL-10, which is a suppressive, tolerogenic and potent inducer of regulatory T cells (Tregs) (42, 46, 47). An additional study,

however, reported that TLR3 or -4 ligation resulted in a strong down-regulation of the Notch ligand Jagged-1, leading to the down-regulation of suppressive capacities (48). Thus, in the context of an inflammatory process, MSCs may lose the immunosuppressive function. MSCs have been shown in recent studies to have significant effects on a variety of conditions including both RA and osteoarthritis (49). MSCs are shown to have significant effects on a variety of autoimmune type of problems such as rheumatoid arthritis, osteoarthritis, lupus, colitis, mixed connective tissue disease, and scleroderma. The mechanism of the immunomodulatory effects of MSCs is not completely understood, although both direct and indirect actions have been suggested through either cell-cell interaction or soluble factors that create a local immunosuppressive environment. MSCs also alter the cytokine secretion profiles of dendritic cells, naive and effector T cells, and NK cells to induce a more profound anti-inflammatory or tolerant phenotype. Secretion of the proinflammatory cytokines, TNF-α and IFN-γ, is decreased whereas that of the suppressive IL-4 and IL-10 is strongly stimulated (46). Other factors involved in the immunosuppression have been shown to include hepatocyte growth factor, TGF-β1, IL-10, IL-6, prostaglandin E_2, nitric oxide, and possibly indoleamine 2,3-dioxygnease. Although the precise mechanism has yet to be clarified (38, 50), a large body of evidences suggests that MSCs are immunosuppressive and anti-inflammatory and can be transplanted between MHC-incompatible individuals. Single injection of primary murine MSCs could prevent the occurrence of severe arthritis, associated with a decreased pro-inflammatory cytokines in serum (51). Recently, the systemic injection of MSCs engineered to overexpress IL-10 significantly reduces the arthritic symptoms contrary to the injection of naïve MSCs (52). Several other studies have indicated the limitation of MSC based therapy due to its limited regeneration potentials, unidentified homing mechanism as well as long-term culture may leads to unstable culture and the possibility of malignancy. The first study on the use of MSCs in CIA (collagen induced arthritis) showed that the allogeneic C3H10T1/2 cells were unable to decrease the clinical signs of arthritis and even worsen the disease in a murine model (53). One of the groups suggested that the systemic injection of allogeneic, as well as autologous BM-derived primary MSCs could prevent the disease, but did not have any curative effects (54). Overall, the effect of MSCs on the immune cells is to tip off the immune response toward a tolerant and anti-inflammatory phenotype. These immunomodulatory effects appear not to be limited to MSCs themselves but also are shared by other mesenchymal cells. MSC- differentiated cells as well as various stromal cells from different tissues, including chondrocytes and fibroblasts, have also been shown to have immunosuppressive effects under certain conditions (36, 55). MSCs have been shown to decrease the severity of CIA, but so far very few clinical trials is going on despite the convincing evidence that primary MSCs could inhibit human T-cell proliferation by *in vitro* (56). Similar results have been recently obtained with human adipose-derived stem cells (ADSCs), which share similar properties with MSCs. The authors concluded that ADSCs suppressed T-cell response through the generation/activation of antigen-specific Tregs (57). The immunomodulatory role of MSCs is dose dependent, and two injections of bone marrow (BM)-MSCs on days 18 and 24 significantly improved arthritic symptoms (58). The mechanisms involved are suggested as to be through $CD4^+CD25^+Foxp3^+$ Treg cell induction as well as T-cell anergy or Treg activation (59, 60). MSCs treatment in immunized mice induced the proliferation of antigen-specific

clones of Tregs with a CD4+CD25+CD27+Foxp3+ phenotype, suggesting that the immunosuppressive activity of MSCs could be prolonged by the action of Treg clones that is activated by an antigen-specific stimulus (51). A recent result showed that ADSCs both prevented and treated CIA by significantly reducing the incidence and severity of experimental arthritis (61). In this study, treatment with ADSCs inhibited the production of various inflammatory mediators, decreased the expansion of antigen-specific Th1/Th17 cell, and induced the production of anti-inflammatory cytokine interleukin-10. Moreover, ADSCs could induce the generation of antigen-specific Tregs with the capacity to suppress collagen-specific T cell responses. Currently, a clinical trial based on MSC transplantation has been initiated to treat RA; clinicians are trying to asses the safety and efficacy of umbilical cord-derived MSCs for RA at phase I/II levels (NCT01547091, www.Clinical.Trials.gov).

5. HSC (hematopoietic stem cell)-based therapy in RA

Out of several advantages of HSC-based therapy, one potential of using HSC lines for transplantation in patients with autoimmune diseases is that these cells can be recovered from unaffected individuals. Thus, genetic makeup is defined and will not be affected by genetic influence whereas ESCs will be affected by genetic influences (62). In addition, by using genetically selected or engineered cell types may further limit the possibility of disease progression or re-emergence. There are several studies in the past that used cell-based approaches to treat active, destructive, refractory and inflammatory arthritis that involved HSC transplantation (HSCT) (62-65) and juvenile idiopathic arthritis (66, 67). Although this treatment was curative for some patients, prolonged immunosuppression after HSCT was associated with a significant risk of infection, which limited the potential of this therapy (68, 69). One other potential problem of using non-self HSCs is the possible immune rejection of the transplanted cells. Under these circumstances, ESC-derived HSCs or other blood cells may offer distinct advantages over cord blood and BM-derived HSC lines in avoiding rejection of the transplant. In future, there is a hope for the development of deposit banks of ESCs expressing various combinations of the three most critical MHC molecules that can be utilized to allow close matching to the recipient's MHC composition. However, data from these trials implicated that Tregs, as a critical component of the regulatory state, will be functioning in the newly reconstituted immune system after transplant. In patients with juvenile idiopathic arthritis treated with HSCT, post-transplant reconstitution of $CD4^{+}CD25^{high}$ Treg was more rapid than that of $CD4^{+}CD25^{-}$ cells. This preferential recovery of Tregs could result from the relative resistance of Tregs to apoptosis after genotoxic stress (70). These results imply that there are other approaches to shift the balance of the immune system away from inflammation and toward regulation, *e.g.*, those that selectively bolster Treg numbers or function, may prove to be successful and less risky than HSCT.

6. Regulatory T cell-based therapy in RA

Tregs play an important role in the prevention of autoimmunity, and have ability to modulate the severity of CIA (71, 72). Deficiencies in Treg function have been identified in a

wide variety of human autoimmune disorders, including RA (73-76). The majority of the reports suggested that biological or stem cells therapy will induce a potent population of Tregs in patients with RA (57, 59, 60), but the natural Treg defect will persist in responding patients after anti-TNF treatment (77). A few studies suggested that Tregs isolated from patients with active RA were competent at suppressing conventional T cell proliferation but not cytokine production (73). A recent report suggested that reduced expression and functional abnormalities in Tregs associated with cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) could account for the Treg defect in patients with RA (78).

Tregs comprise about 5-10% of the mature CD4+ T helper cell subpopulation in mice and about 1-2% of CD4+ T cells in human. Tregs are composed of thymus-derived, naturally occurring $CD4^+CD25^+Foxp3^+$ Tregs (nTregs) as well as adaptive (also called 'induced') Tregs (iTregs) that are generated from $CD4^+CD25^-Foxp3^-$ naive T cells in the periphery. Unlike other cell-surface markers used to identify Tregs, Foxp3 is not up-regulated upon activation, and discriminates Tregs from activated effectors T cells. On the other hand, Foxp3 is also expressed in T cells without conferring any regulatory functions. nTregs are characterized by the expression of CD25, CD45RB, CD62L, CD103, CD95 (Fas), MHC class II, CD127, neuropiln-1 lymphocyt activation gene-3 (LAG-3), CTLA-4, glucocorticoid-induced TNFR family related gene (GITR), and Foxp3. iTreg cells are also characterized as expressing the same markers as nTreg cells but arise from $CD4^+D25^+FoxP3^-$ precursor cells. iTreg cells are further classified into two subgroups on the basis of TGF-β or IL-10 production. Recently, it was reported that human $CD4^+FoxP3^+$ T cells have three distinct populations with precise phenotypes and fates which includes, $CD25^{++}$ $CD45RA^+$ ($Foxp3^{lo}$) resting Tregs (rTreg cells), $CD25^{+++}$ $CD45RA^-$ ($Foxp3^{hi}$) activated Tregs (aTregs), and $CD25^{++}$ $CD45RA^-$ ($Foxp3^{hi}$) cytokines-secreting T cells which lack suppressive activity (79). The first two groups of phenotypes represent different stages of Treg cell differentiation and are both playing suppressive function *in vitro*. However, the new and exclusive cell surface marker of Tregs is still an area of intense research.

One critical factor in Treg cell-based therapy is the survival of Tregs. Tregs are highly susceptible to apoptosis in the absence of common gamma chain (γc) cytokines (*i.e.*, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) because Tregs do not produce these cytokines and depend on other cells (80, 81). Tregs are also susceptible to apoptosis due to the low expression of the anti-apoptotic bcl-2 family members (82). Bcl-xL is an anti-apoptotic factor that is able to prolong the life span of apoptosis-prone lymphocytes. ß-catenin enhances the survival of $CD4^+CD25^+$ Tregs *in vitro* without altering their anergic state or suppressive function by modulating the antiapoptotic bcl-xl gene expression in Tregs (83). Recent reports (84) suggest that bcl-xL is involved in the development and function of Tregs by inducing the expression of Foxp3, CTLA-4, TGF-β, and repressing the programmed death receptor-1 (PD-I) expression. Additionally, Foxp3 and bcl-xL could cooperatively promote Treg cell survival and prevention of arthritis development due to the increased life span of Tregs (85). These approaches promote Treg cell differentiation and long-term survival thus results in the long-lasting Treg cell persistence.

Several studies have shown improvement of inflammation, *e.g.*, that seen in murine antigen-induced arthritis, by transfusion of functional Tregs (86-89). It has ignited some debates

regarding the potential use in cellular therapy for RA. One approach to boost number of Tregs without myeloablative conditioning is to isolate Tregs from patients, expand them in culture, and then re-infuse the cells (90). Expansion of pre-existing Treg cell subsets *ex vivo* and transfuse back into patients in carefully controlled conditions to ensure the preservation of the regulatory capacity may be helpful, but could require large numbers of Tregs to be effective (91, 92). *Ex vivo* Treg cell expansion and re-infusion has successfully prevented or reversed a number of autoimmune diseases in mouse models, including models of inflammatory arthritis (93-97). There are several limitations and pitfalls in the treatment of patients with Tregs. Incomplete lineage commitment enables some human Tregs to express nuclear factor ROR-γt and to develop into IL-17-producing effector cells (98-100). It is also possible that cells become unstable after transfusion, particularly in an inflammatory environment, pathogenic responses might be exacerbated. Contamination of therapeutic Tregs with these unstable Tregs, or even with true autoreactive effector T cells, could lead to deterioration rather than attenuation of the autoimmune process. Tregs may be less effective in suppressing effector T cells in an autoimmune setting, and may even augment IL-17 production *in vitro (100-102)*. The ability to generate iTreg cells *in vitro* by culturing $CD4^+$ T cells in the presence of TGF-β provides a mechanism by which large numbers of functional iTreg cells could be generated for adoptive transfer into patients with RA, however, TGF-β-induced Tregs are potentially unstable and would pose a risk of converting into pathogenic responder T cells (103, 104). There are also several other disadvantages of the *in vitro* expansion or generation of Tregs for transfer back to patients, which include the risk of contamination from pathogenic responder T cells that could exacerbate the disease, and the generation of polyclonal Tregs that could initiate a generalized immunosuppression. In spite of these potential concerns, however, clinical trials are underway to test the therapeutic potential of Tregs in graft-versus-host diseases. Ectopic expression of the regulatory transcription factor, Foxp3, has been used to convert conventional $CD4^+$ T cells into Tregs with regulatory function (87, 105). A powerful approach to generating large numbers of antigen-specific Tregs has been demontrated by the introduction of T cell receptor (TCR) gene into purified CD4+CD25+ T cells as well as co-transfer of Foxp3 and TCR genes to convert conventional $CD4^+$ T cells into antigen-specific Tregs (88). However, important safety concerns remained for the application of this approach. TCR-transduced Tregs as well as Foxp3+TCR-transduced $CD4^+$ T cells selectively accumulated in the draining lymph nodes of the antigen-challenged knee, indicating that both were able to respond to the antigen stimulation in *vivo* compared to PBS-treated control mice. There was no increase in Th17 cells in the T cell-treated mice, indicating that in the *in vivo* environment of inflammatory arthritis neither the engineered Tregs nor the engineered $CD4^+$ T cells converted into Th17 cells. In a series of studies, both populations displayed characteristic markers of nTregs (CD25, CTLA-4, and GITR) and demonstrated suppressive activity. These studies open the possibility to target Tregs to tissue-specific antigens for the treatment of autoimmune tissue damage. Recent strategies have used the foot-and-mouth disease virus 2A or 2A-like elements to create multicistronic vectors capable of generating multiple proteins from the same transcript. A single 2A peptide-linked retroviral vector has been used successfully to generate reliable and versatile gene therapy vectors that can be used in

biomedical research (106). In addition, co-expression of Foxp3 and bcl-xL linked by a 2A sequence in $CD4^+$ cells is critical for augmenting the differentiation and persistence of Tregs. Most significantly, the co-introduction of these molecules into $CD4^+$ T cells resulted in their ability to significantly block the development of arthritis in a murine model (87). These data provide new insights toward the generation of highly reactive Tregs for adoptive immunotherapy of autoimmune disease. However, adoptive cell transfer did not cure established arthritis, which could be explained by the absence of specificity that directs the movement of Tregs to the inflamed paw. Thus, generation of antigen-specific highly reactive Tregs may be a promising approach for the treatment of established autoimmune diseases.

7. Concept of stem cell-derived Treg-based therapy in RA

Because of the intrinsic resistance of Tregs to exogenous expansion and a high number of Tregs are required to perform the Treg-based immunotherapy, it becomes imminent to isolate a larger Treg cell subset for exogenous expansion and adoptive transfer, as discussed above, there are number problem and limitations to generate a large number of Tregs regardless of an increasing number of protocols for isolating subsets of Tregs, no approach to date has been confirmed the capacity to isolate the entire Treg cell population with 100 percent specificity. ESCs or iPSCs have the remarkable potential to develop into many different cell types in the body during early life and growth. ES or iPSC can give rise to all the blood cell types including myeloide (*e.g.*, monocytes and macrophages, neutrophiles, basophiles, eosinophiles, erythrocytes, megakaryocytes/platelets, dendritic cells) and lymphiode lineage (*e.g.*, T cells, B cells, NK cells). Whether ESCs or iPSCs will provide advantages over cord blood or adult BM-derived HSC remains to be determined. However, HSCs or MSCs, have limited potentials for self-renewal and the differentiation decline in response to differentiation signals with with each cycle compared to pluripotent ESCs or iPSCs (107). HSCs are present in differentiated cultures from human ESCs and from human fetal-derived embryonic germ stem cells (108-112). In contrast, iPSCs could be generated from mouse and human somatic cells by introducing Oct3/4 and Sox2 with either Klf4 and c-Myc or Nanog and Lin28 by using retroviruses or lentiviruses-mediated transduction (113-116). In addition, iPSCs are similar to natural pluripotent stem cells (*e.g.*, ESCs) in many respects, such as the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, and potency and differentiability, but the full extent of the relations to natural pluripotent stem cells are still being assessed (117, 118). The use of iPSCs can bypass ethical and feasibility issues related to the use of ESCs and HSCs. The iPS technology continues to progress rapidly, and clinically applicable iPSCs can be generated from patients with noninvasive medical procedures. Collectively, iPSCs have a greater potential to be used in adoptive cell transfer (ACT)-based immunotherapy for autoimmune diseases compared to ESCs and HSCs.

Previously, pluripotent stem cells (*e.g.*, ESCs, HSCs) have been shown to differentiate into T cells *in vitro*, and recently iPSCs have been demonstrated to have the ability to differentiate into T cells (123) and antigen-specific CD8+ cytotoxic T lymphocytes (CTLs) (124). In these studies, murine iPSCs were genetically modified with ovalbumin (OVA)-specific MHC I-

restricted TCR (OT-I) by retrovirus-mediated transduction. These iPSCs differentiated into functional antigen-specific CTLs *in vivo* and significantly protected the hosts from a tumor challenge (124). Thus, a new approach to generate a high number of functional Tregs from ESCs or iPSCs may be used for the treatment of autoimmune diseases (Figures 1-3). ESCs and iPSCs can be induced to differentiate into antigen specific or naïve Tregs *in vitro* through a Notch-mediated signaling. Genetic modification of iPSCs or ESCs with the Foxp3 gene and antigen specific TCR as well as stimulation with an *in vitro* Notch ligand will direct iPSC differentiation into Tregs, which are able to produce suppressive cytokines and inhibit

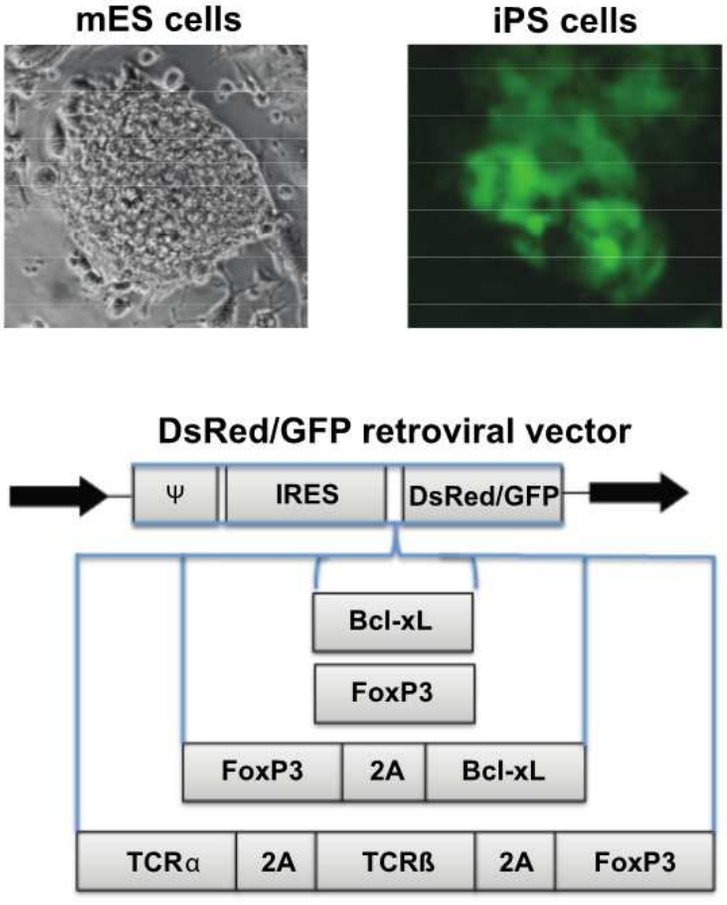

Figure 1. Generation of muticistronic construct for antigen-specific Tregs. (A) Mouse ES cells & iPS cells were maintained on feeder layers of irradiated SNL76/7 cells in 6-well culture plates and were passaged every 3 days. In brief, iPSCs were maintained in DMEM culture medium supplemented with 15% fetal calf serum (FCS), 0.1 mmol/L nonessential amino acids, 1 mmol/L l-glutamine, and 0.1 mmol/L β-mercaptoethanol. (B) Schematic representation of the retrovirus construct based on MiDR vector contain bcl-xL, or Foxp3, or Foxp3 + bcl-xL or TCRα + TCRβ + FoxP3, ψ, packaging signal; 2A, picornavirus self-cleaving 2A sequence; LTR, long terminal repeats; DsRed and IRES bidirectional promoter.

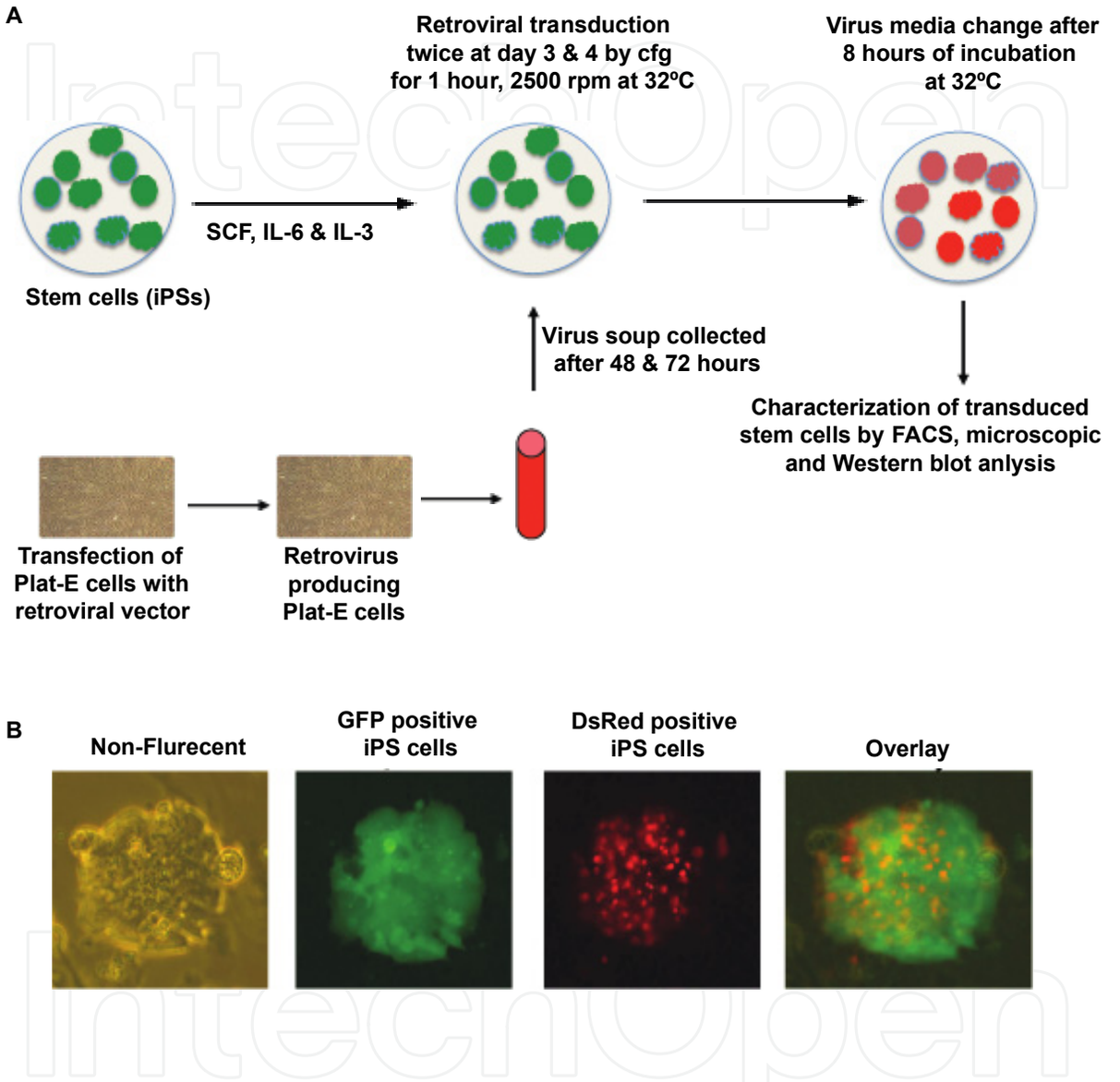

Figure 2. Retroviral transduction of stem cells. (A) Schematic representation of retroviral transduction. Retroviral transduction was performed as described before (124). 5×10^4 iPSCs were cultured in the presence of stem cell factor (SCF), IL-6 and IL-3 for 48 hours in 24 well plate coated with 1% gelatin. At the same time virus producing packaging cells line were transfected with retroviral construct containing gene of interests. After 2 days, the supernatant was replaced with 1 ml viral supernatant containing 10 μg/ml Polybrene, and the cells were spun for 1 h at 32°C and incubated at 32°C for 8 h. This was repeated the following day. Viral supernatant was removed and replaced with fresh medium, and iPSCs were re-cultured on fresh feeder layer. Expression of DsRed was determined by flow cytometry by gating on double positive (GFP + DsRed) iPSCs. DsRed-expressing iPSCs can be enriched and purified by cell sorting using a high-speed cell sorter and can be further use for differentiation and characterization. (B) Gene-transduced iPSCs were visualized by fluorescence microscopy.

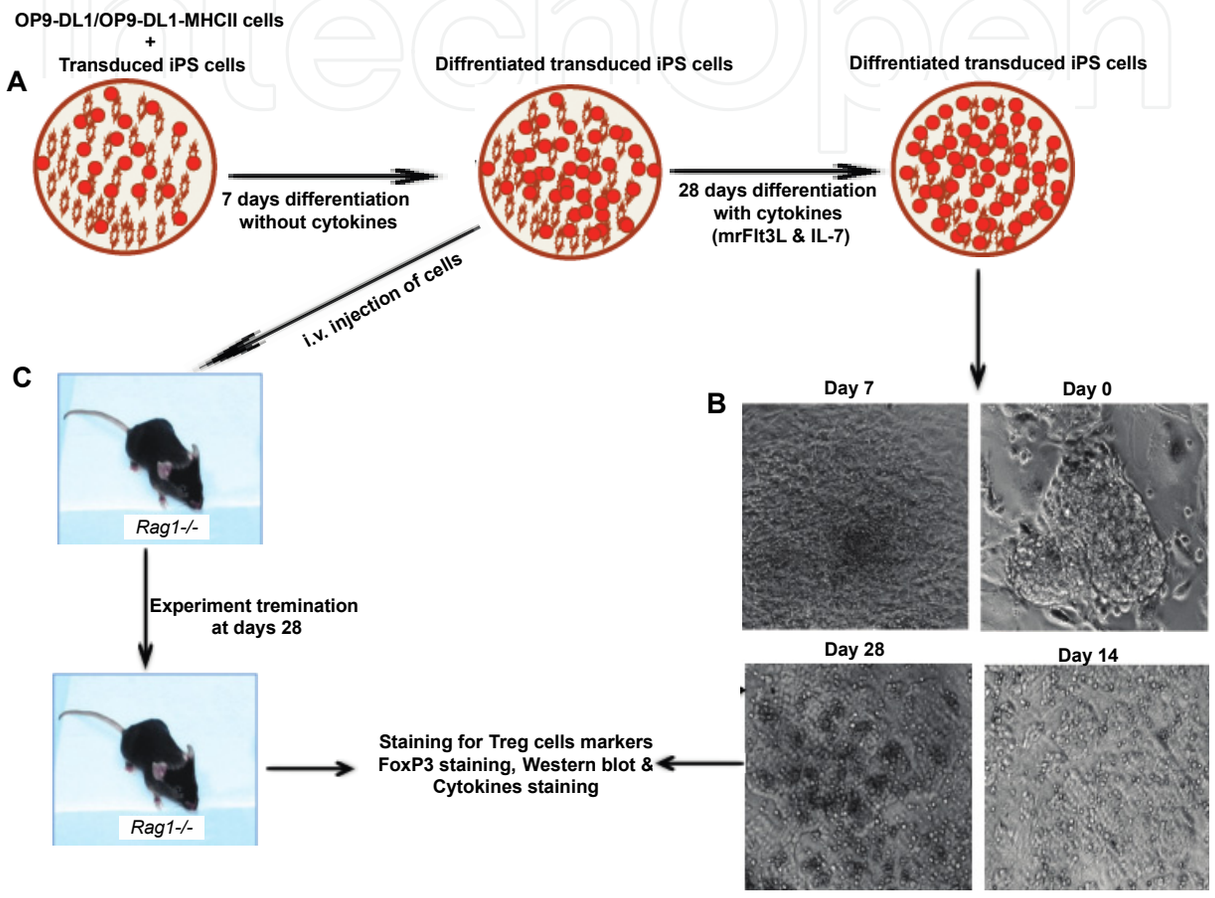

Figure 3. *In vivo* & *in vitro* differentiations of transduced iPSCs. (A) Schematic representation of *in vitro* stem cells differentiation. Monolayers of OP9-DL1/OP9DL-1-MHC-II cells were cultured in α-MEM medium supplemented with 20% FCS and 2.2 g/L sodium bicarbonate. Gene-transduced, sorted and purified iPSCs were washed once in OP9-DL1 medium before plating onto subconfluent OP9-DL1 monolayers for Treg lineage differentiation in the presence of murine recombinant 5 ng/ml Flt-3 ligand and 1 ng/ml murine rIL-7 after day 7. (B). Morphology of Treg cell differentiation on days 0, 7, 14, and 28. (C) Foxp3-transduced iPSCs were co-cultured on OP9-DL1 cells for 7 days, and adoptively transferred into *Rag1-/-* mice that had no mature T cells or B cells. After 6 weeks, antigen-specific Treg cell development can be determined (*e.g.*, we observed CD4+ CD25+ Foxp3+ Tregs in the lymph nodes (LN) and spleen and the cells had the ability to produce TGF-β and IL-10). Thus, Foxp3-tranduced iPSCs are capable of differentiating into functional Tregs both *in vitro* and *in vivo*.

other immune cell activities. In addition, gene transduction of antigen-specific TCR will result in iPSC or ESC-derived Tregs exhibiting a uniform expression of antigen-specific TCR following *in vitro* or *in vivo* development. This approach will help generate large numbers of functional antigen-specific Tregs. Therefore, gene transduction of iPSC or ESCs followed by T lineage differentiation through an *in vitro* Notch signaling or an *in vivo* approach will generate large numbers of monoclonal antigen-specific Tregs, which can be used for Treg-based immunotherapy. These cells could be able to persist *in vivo*, unlike cells generated via the current therapeutic regimen, and will likely contribute toward the treatment of autoimmune diseases (such as type I diabetes, RA, and system lupus erythematosus). The use of iPSCs as a mean to develop disease-specific immune cells for immunotherapy has a great potential in the prevention of many diseases. Collectively, iPSCs have a greater potential to be used in Treg-based immunotherapy for autoimmune diseases compared to ESCs and HSCs.

8. Conclusion

Biologic agents have revolutionized the treatment of RA by reducing the signs and symptoms and improving physical function and quality of life in affected patients, and also showed promising data to treat many diseases. However, question remains for their side effects. Improved knowledge of the pathophysiology will lead to the recognition of more reliable and practical predictors of the disease course and treatment response. Stem cell-based therapies offer many exciting opportunities for the development of novel treatment and cure for autoimmune diseases. The multipotent autologous stem cells have potential to treat disorders involving connective tissue because of their differentiation potential. In addition, the multipotent autologous stem cells have significant effects on a variety of autoimmune diseases because of their immunomodulatory immune response. Despite of the great potential and convincing evidence that primary MSCs inhibit human T-cell proliferation *in vitro* and decrease the severity of CIA in mouse models, there are very few clinical trials are going on worldwide and recent trials have produced mixed results. The results of clinical trails question the efficacy of MSCs in disease conditions in part due to incomplete understanding of the fate of MSC after infusion despite of the positive safety aspects. Because of the plasticity and potentially unlimited capacity for self-renewal, iPSC-derived Tregs may be applied for Treg cell adoptive immunotherapy, such as cancer, autoimmune disorders, and infectious or aging-related diseases. A challenging research effort remains to fully examine this potential and to address several remaining questions, which include how to direct the differentiation of specific cell types, generate large number of differentiated cells in the best possible ways and determine which particular type of stem cell will be optimal for each therapeutic approach. However, stem cells or their progeny may provide one of several better roads for future delivery of immune cell-based therapies. The approaches for generation of antigen-specific Tregs from iPSCs provide an important foundation for the ultimate generation of patient- and/or disease-specific Treg-based therapies. However, to explore the potential to alleviate these devastating chronic diseases with the use of stem cell-based technologies is still enormous.

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