Chapter

Biological Modulation of the Treg: Teff Ratio: From Immunosuppression to Immunoactivation

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Abstract

T cell-mediated immunomodulation can be, in simple terms, defined as altering the normal Treg:Teff ratio. Immunosuppression skews the net Treg:Teff ratio toward the 'tolerogenic' Treg component, while immunoactivation skews the response toward the 'proinflammatory' Teff component. In the treatment of autoimmune diseases, achieving an immunosuppressive state is a desirable goal in order to prevent ongoing injury by activated Teff cells. In contrast, an innate, or induced, immunosuppressive state can be deleterious and prevent pathogen-induced disease while allow for the progression of cancer. Indeed, a current goal of cancer therapy is attenuating an existing endogenous immunosuppressive state that prevents effective T cell-mediated immunorecognition of cancer cells. Thus, the biological modulation of the Treg:Teff ratio provides a unique approach for treating both autoimmune diseases and cancers. Using a biomanufacturing system, miRNA-enriched immunotherapeutic has been generated that either induce (TA1) or overcome (IA1) an immunosuppressive state. As will be shown, these therapeutics show efficacy both in vitro and in vivo in the prevention of autoimmune Type 1 diabetes and in enhancing the ability of resting immune cells to recognize and inhibit cancer cell growth. The successful development of these cost-effective, and easily biomanufactured, secretome-based therapeutics may prove useful in treating both autoimmune diseases and cancer.

Keywords: T lymphocyte, immunosuppression, immunoactivation, Treg, Teff, proinflammatory, autoimmunity, cancer, biomanufacturing, miRNA

1. Introduction

The core function of the immune system is preserving *'self'* and rejecting 'non-self'. Biologically, 'non-self' is most often seen as exogenous biologicals (e.g., viruses and bacteria), abnormal autologous cells (e.g., cancers) and, more recently, 'man-made diseases' arising from the purposeful introduction of *'non-self'* (e.g., enzyme-replacement therapy, transfusion and transplantation medicine). Immunological *'self'* of most tissues is imparted by the major histocompatibility complex (MHC) which encodes a variety of proteins that provide a means for identifying, targeting, and eliminating foreign invaders and diseased cells while preserving

normal *'self'* tissue [1–3]. The MHC proteins themselves consist of three classes. MHC Class I molecules are expressed on virtually all nucleated cells while Class II molecules are expressed exclusively on antigen presenting cells (APC; e.g., monocytes, macrophages, dendritic cell, B lymphocytes, and endothelial cells) and activated T lymphocytes. MHC Class III genes encode components of the complement system. The human MHC is referred to as the Human Leukocyte Antigen (HLA) complex while the murine equivalent is referred to as the Histocompatibility-2 (H2) complex. In the context of MHC-mediated immune recognition, the T lymphocyte (T cell) is of particular importance and plays a (the) central role in transfusion-associated graft versus host disease, transplant rejection, autoimmune diseases and cancer therapy. In terms of human diseases, autoimmune disorders and cancer are of most significance clinically.

The activation status of T cells plays a critical role in normal immunological homeostasis, the response to cancers, rejection of tissue/organ grafts, and the ontogeny and pathophysiology of autoimmune diseases. T cells encompass multiple subpopulations that can exert either a protolerogenic effects (regulatory T cells; Tregs) or a proinflammatory responses (effector T cells; Teff). Hence, in examining the immune status, the relative abundance of Tregs and Teff, i.e., the Treg:Teff ratio, is critical. Indeed, skewing the immune response towards either end of the continuum leads to significant medical consequences. As shown in **Figure 1**, an immunosuppressive state (increased Treg and/or decreased Teff) may facilitate the growth and spread of abnormal (i.e., cancer) cells, or in the context of transplantation medicine enhance engraftment, while a proinflammatory state (decreased Tregs and/or increased Teff) that may give rise to an autoimmune disease, graft rejection or, in the case of cancer, enhance tumor cell elimination. Indeed, modern clinical approaches attempt to pharmacologically modulate the Treg:Teff ratio in the treatment of autoimmune disease, tissue transplantation and cancer therapy.

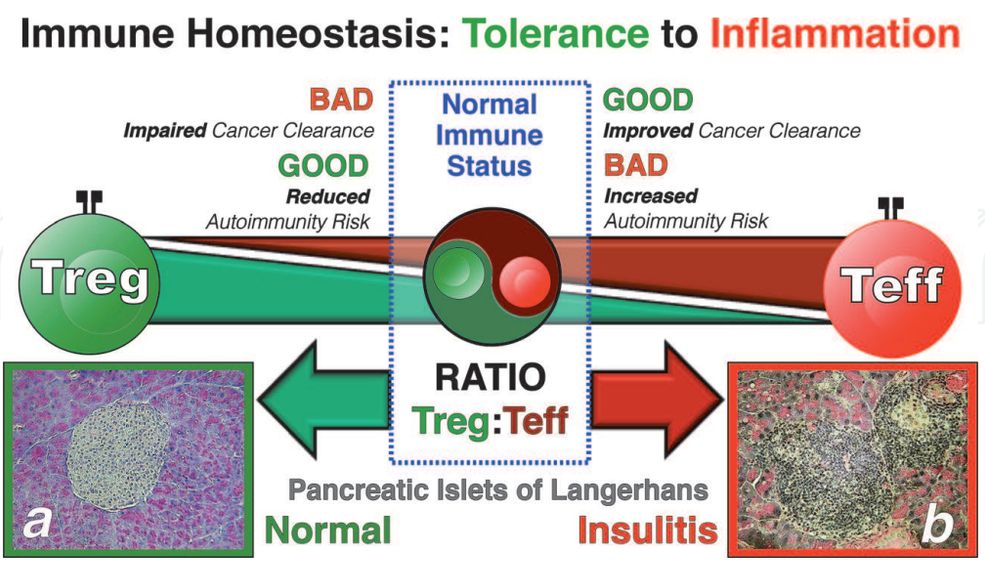

Figure 1.
The yin and yang of the cellular immune response. A key aspect of immune regulation is the dualism of the tolerogenic (Treg; e.g., Foxp3$^+$, IL-10$^+$, TGF-β$^+$ and IL-4$^+$) and effector (Teff; e.g., Th17$^+$, IL-2$^+$, INF-γ$^+$ IL-12$^+$, and TNF-β$^+$) CD4$^+$ T cells. Effector T cells also include cytotoxic CD8$^+$ T cells (CTL). These seemingly disparate cellular subpopulations are actually complementary, interconnected, and interdependent in regulating the immunological response. As such, the immune response is a continuum that may be best reflected by the Treg:Teff ratio. Indeed, the skewing of the Treg:Teff ratio towards either the left or right influences the immunological risks/benefits of an animal. As shown, a skewed response towards the Treg cells may prevent T1D or could be used to prevent rejection of transplanted islets. In contrast, skewing towards the Teff populations increases the risk of autoimmune diseases such as type 1 diabetes (T1D) consequent to the development of insulitis of the islet cells.

2. Autoimmune diseases: increasing the Treg:Teff ratio

Autoimmune diseases affect virtually all tissues and organs and encompass such diverse diseases as Type 1 Diabetes (T1D), Crohn's disease (CD), Multiple Sclerosis (MS), Rheumatoid Arthritis (RA) and immune thrombocytopenia (ITP). Despite the diversity of tissues affected, extensive research has demonstrated the central role for T cells with Treg being downregulated and Teff upregulated leading to a reduced Treg:Teff ratio and a chronic pro-inflammatory state (**Figure 1**). Current clinical approaches to regulating the Treg:Teff ratio are almost entirely focused on reducing the Teff component. Most commonly, treatments for chronic autoimmune diseases include administration of systemic steroids (e.g., dexamethasone), cytotoxic anti-proliferative/activation agents (e.g., cyclosporine), and interruption of proinflammatory cytokine signaling cascades (e.g., Enbrel) resulting in the induction of a general immunosuppressive state in the individual (**Figure 2**). While these pharmacological approaches are often highly effective in controlling the autoimmune disease, they also pose significant risks to the individual including increased risks of opportunistic infections, cancer and organ injury. Perhaps surprisingly, very few clinical tools exist to increase the Treg component of the Treg:Teff ratio. Importantly, an increase in the functional Treg component would be very effective at reducing the damage induced by the Teff subsets in autoimmune diseases and decreasing the risk of Host versus graft disease in tissue/organ transplantation.

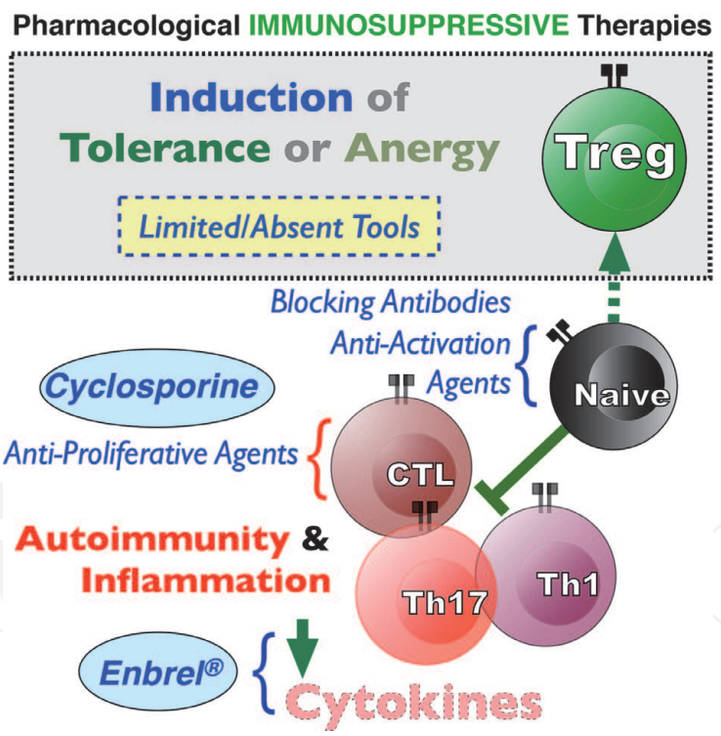

Figure 2.
Current pharmacologic therapies almost exclusively targets T cell activation and the Teff subpopulations. The proliferation of pro-inflammatory T cells (e.g., CTL, Th17, Th1 populations) and decrease in regulatory T cells (Treg) are commonly observed in both autoimmune and allorecognition immune responses. The majority of current therapeutic agents are primarily cytotoxic agents preventing T cell activation (e.g., cyclosporine and rapamycin) or T cell proliferation (e.g., methotrexate, corticosteroids and azathioprine). Additionally, some blocking antibodies have been investigated. In contrast, very limited, if any, pharmaceutical approaches are effective at increasing the Treg populations.

3. Cancer immunotherapy: decreasing the Treg:Teff ratio

In contrast to autoimmune diseases, immunosuppressive states (i.e., increased Treg:Teff ratio) exist resulting in a failure to appropriately respond to abnormal

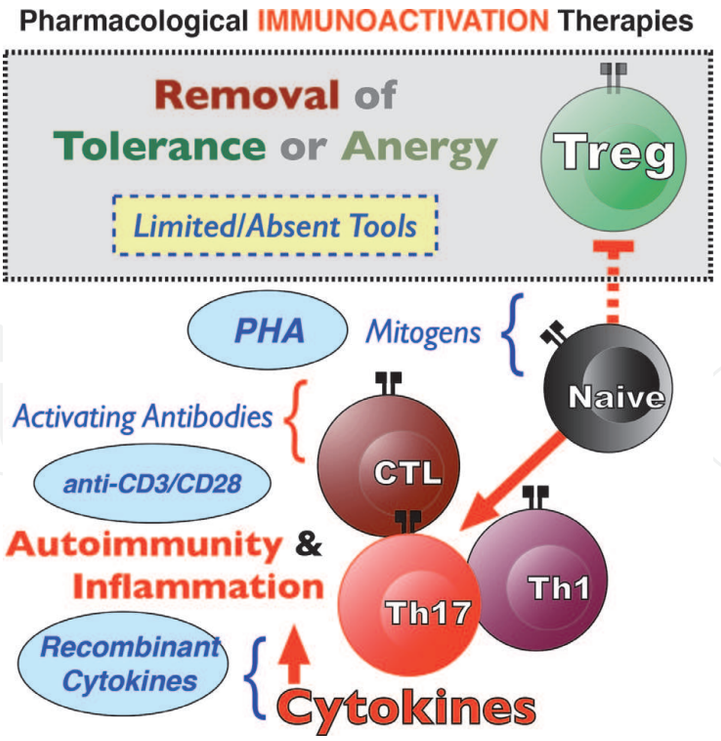

Figure 3.
Pharmacologic immunoactivation approaches have proven problematic due to their induction of poorly controlled inflammatory responses. A common cause of toxicity to these approaches has been the induction of the cytokine release syndrome (i.e., cytokine storm) [4–6].

cells (e.g., cancer) or infective agents (e.g., viruses and bacteria). This immunosuppressive state is most commonly exemplified by the progression and metastases of cancers arising from a poor or impaired cellular immune response to abnormal cells. Indeed, cancer progression is most often characterized by an impaired Teff response; either due to failure in recognizing abnormal cells (i.e., *'non-self'*) or via an existing immunosuppressive state arising from pharmacological agents or an inherent skewing of the Treg:Teff response towards the Treg cells. Unlike immunosuppressive diseases, the focus of clinical therapy has historically focused on cytotoxic chemicals that exert an enhanced lethality to cancer cells; though it is crucial to note that these agents also exhibit toxicity to normal cells, especially populations characterized by high proliferation rates (e.g., bone marrow; intestinal epithelial cells). Only more recently has cancer therapy began to focus on immunomodulation; in essence actively modulating the Treg:Teff ratio. Cancer immunotherapy has most commonly utilized cytokine therapy or direct activation of T cells via mitogens or monoclonal antibody therapy (**Figure 3**). However, both of these approaches are beset by systemic toxicity limiting their practical use. More recently, adoptive cellular therapeutic (ACT) approaches, using either allogenic T cells and/or CAR-T cells, have been used. However, while clearly an increasingly important cancer immunotherapy, these cellular approaches are expensive and, to date, continue to pose a risk of uncontrolled immune activation and bystander cell injury [4–6].

4. Biological modulation of the immune response

While pharmacologic agents remain the mainstay of modern medicine in treating both autoimmune diseases and cancers, a more direct ability to biologically modulate the Treg:Teff ratio could, potentially, be a safer and more effective tool in

treating disease. It is worth noting that the biological modulation of the immune response is not a new concept. Indeed, the theory and practice of proinflammatory (*i.e.,* decreasing the Treg:Teff ratio) immunotherapy originated with William Coley's treatment in 1891 of cancer patients with bacteria (and other toxins) in order to induce an immune response that would exert a bystander effect on the tumor mass [7–10]. This pioneering clinical research has led to the recognition of Coley as the *"Father of Immunotherapy"*. Indeed, *Coley's Toxins* were a mainstay of cancer therapy for much of the early twentieth century and were marketed up to ~1962. However, these biologics were poorly defined, subject to diverse manufacturing standards (or lack thereof), and highly variable in their efficacy. By the mid twentieth century, criticisms from within the medical community led to less usage of *Coley's Toxins* as they were supplanted by the newer, and *'safer'*, developments of radiation therapy and chemotherapy; ironically both of which are now recognized to pose very significant short- and long-term risks to the patient. Indeed, it is these risks that are today driving forces in rediscovering immunotherapy.

Today, ~130 years after *Coley's Toxins* made their initial debut, modern immunotherapy has begun to revisit Coley's core principles of modulating (i.e., inducing) the endogenous immune response. Several approaches have been pursued to enhance the patient's own immune response. Ironically, similarly to Coley's use of *Streptococcus pyogenes* and *Serratia marcescens*, genetically modified strains of *Salmonella sp.* as well as recombinant polioviruses have been used in tumor therapy to induce an inflammatory microenvironment at the tumor site [11–14]. Tumor-specific immunotherapy has also been explored in which cancer cells from patients have been isolated, irradiated and modified for the re-infusion into the patient in an attempt to enhance anti-tumor immune activation and improve tumor killing [15–19]. But perhaps the favored approach has been the application of adoptive cell transfer (ACT) immunotherapy and, especially, chimeric antigen receptor (CAR)-T cell therapy [20–22]. While CAR-T cells will prove to be a crucial tool in cancer immunotherapy, they are beset by significant issues including cost, manufacturing time (weeks-months) and the risk of inducing cytokine release syndrome [4–6].

However, of significant clinical importance, few studies/approaches to date have elucidated effective biological immunotherapeutic approaches for modulating the Treg:Teff ratio (**Figures 2** and **3**). The ability to biologically manipulate the Treg:Teff ratio in a controllable manner would be of significant benefit in the treatment of cancer as well as the treatment of autoimmune diseases and the prevention of graft rejection.

5. A new approach to the biomodulation of the Treg:Teff ratio

As described in the preceding sections, pharmacologic agents, the current mainstay of clinical medicine, are, relatively speaking, non-specific agents beset with often significant adverse side effects. Hence, over the last decade increasing research has been done on biologically modifying the innate Treg:Teff immune response. In this chapter we will discuss a novel biomodulatory approach that more effectively, and directly, target the Treg:Teff ratio by increasing or decreasing Treg cells while simultaneously, and inversely, decreasing of increasing Teff subsets (**Figure 4**). This approach, derived from our work on the polymer-based bioengineering of allogeneic T cells and their use directly, or via the production of acellular microRNA (miRNA), to induce a tolerogenic or proinflammatory state characterized by significant changes in the Treg:Teff ratio [23–35].

Biomanufacturing of these immunomodulatory therapeutics was accomplished using a rapid and inexpensive leukocyte allorecognition-based system (**Figure 4**) [34, 35]. The core component of the biomanufacturing system is, in essence, a two-way mixed lymphocyte reaction (MLR) in which MHC-disparate leukocyte populations (either human PBMC or murine splenocytes) are co-incubated. Previous work from our laboratory demonstrated that the covalent grafting (PEGylation) of methoxy(polyethylene glycol) [mPEG] to one leukocyte population resulted in abrogation of the MHC-mediated proliferation of Teff cells [23–42]. Moreover, these studies demonstrated that, consequent to impaired cell:cell communication (**Figure 4C**), the weak allostimulation induced a tolerogenic/anergic state both in vitro and in vivo (**Figure 4A**) [29–34, 41]. The PEGylated cells themselves, or the resultant purified Treg cells, can be adoptively transferred to induce systemic tolerance in the recipient. Importantly, our studies demonstrated that the secretome of PEGylated-MLR also exerted a tolerogenic effect in vitro and in vivo [25–27, 29–34]. In parallel to the PEGylated cells, the control MLR (**Figure 4B**) was used to

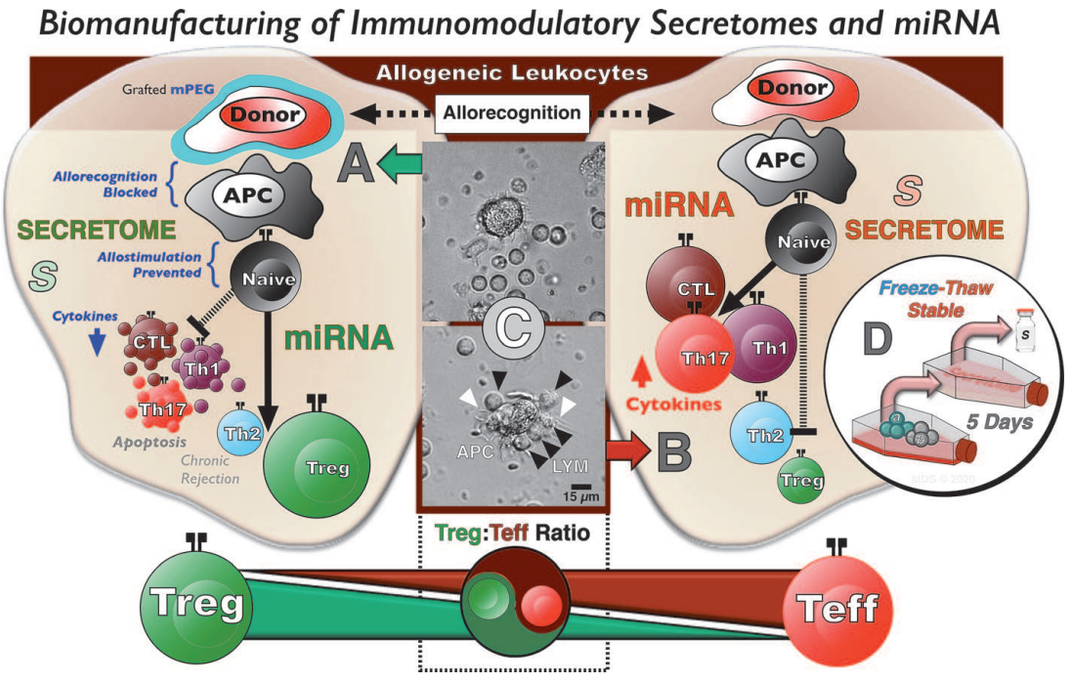

Figure 4.
Biomanufacturing immunomodulatory secretomes and purified miRNA. Panel A: Immunocamouflage of donor cells by the covalent grafting of methoxy(polyethylene) glycol (mPEG) to one donor population in a mixed lymphocyte reaction (MLR) results in the disruption of the essential cell–cell interactions (blue test) decreasing T cell proliferation and altered subset differentiation patterns. As shown, Treg cells are vastly increased while Teff subsets (CTL, Th1 and Th17 shown) are decreased resulting in an increase in the Treg:Teff ratio. Importantly, the secretome from the mPEG-MLR exerts a tolerogenic response when used either in vitro or in vivo. The key component of the secretome are miRNA. Panel B: Current pharmacologic therapy almost exclusively targets T cell activation and proliferation consequent to allorecognition. Response to non-self is in large part mediated by cell–cell interactions between antigen presenting cells (APC; e.g., dendritic cells) and naive T cells. This cell–cell interaction is characterized by essential adhesion, allorecognition and co-stimulation events. Consequent to allorecognition, a proliferation of proinflammatory T cells (e.g., cytotoxic T lymphocyte, CTL; Th17, IL-17$^+$; Th1, IFN-γ$^+$; and IL-2$^+$ populations) and decrease in regulatory T cells (Treg, Foxp3$^+$ and CD25$^+$) is observed. Panel C: As shown in photomicrographs, in a control MLR, significant and persistent interactions (black arrows) occur between allogeneic lymphocytes (LYM) and dendritic cells (APC). The lymphocyte adhesion and antigen presentation interactions typically occur at pseudopodal extensions from the APC (white arrows). PEGylation of either allogeneic PBMC population decreases the stability and duration of initial cell:cell interactions between lymphocytes due to the global charge and steric camouflage of membrane proteins. Panel D: Importantly, the secretomes/miRNA bioproduction is both simple and rapid. As shown, allogeneic leukocytes (a, b) are incubated for 5 days and the secretome is collected. The secretome itself can be used or the miRNA component of the secretome can be further isolated for use. Both the secretome and miRNA can be stored frozen as they are stable under freeze–thaw conditions. The key component of the secretome are soluble (free and exosome) miRNA. Size of the T cell populations denotes increase or decrease in number. Apoptosis is indicated by blebbing. Data derived from Refs. [23–35]. Figure modified from Scott et al. [35].

generate a proinflammatory secretome, or with further purification, miRNA preparation that could induce a controlled inflammatory response in unactivated T cells [25–27, 29–34]. Most importantly, the process is rapid (5 days), inexpensive and can be accomplished using stand tissue culture facilities—though also suitable for larger scale bioreactor systems (**Figure 4D**).

The two biomanufactured miRNA-enrich therapeutics, denoted as TA1 for the tolerogenic preparation and IA1 for the proinflammatory preparation, exert potent immunomodulatory effects on T cells differentiation (**Figure 5**). TA1 drives the differentiation of $CD3^+CD4^-CD8^-$ T cells towards Treg cells ($CD4^+Foxp3^+CD25^+$) while IA1 drives T cell proliferation towards both $CD4^+$ Th17 and Th1 cells and also towards $CD3^+CD8^+$ cytotoxic T lymphocytes (CTL). Thus, stable and storable (freeze–thaw stable) tolerogenic and proinflammatory biologics can be rapidly (5 days) and reproducibly biomanufactured.

Importantly, the active component of the TA1 and IA1 therapeutics are miRNAs—not cytokines or other potential immunomodulatory effectors [31, 34, 35]. The role of miRNA can be seen by the loss of immunomodulatory activity of TA1 and IA1 conditioned murine plasma upon treatment with RNase (**Figure 6**). As shown, naïve control mice (N) have high levels of Treg cells relative to Th17 cell. However, when challenged with a transfusion of allogeneic cells (AC), by day 5 Treg cells have decreased significantly with a concomitant increase in Th17. However, if naïve mice are pretreated with TA1 or IA1, the immune response to the allogeneic cells is dramatically altered. TA1 pre-treatment resulted in a maintenance, and slight elevation, of normal murine Treg levels and prevention of the Th17 upregulation upon allogeneic challenge. In contrast, IA1 pre-treatment enhanced the inflammatory response to the allogenic cells; i.e., significantly decreased Treg and increased Th17 cells relative to both naïve mice and control AC challenged mice. Importantly, RNase treatment of the TA1 or IA1 samples to degrade the miRNA component resulted in the attenuation of their respective immunomodulatory activity resulting in a T cell response virtually identical to the AC treated control mice.

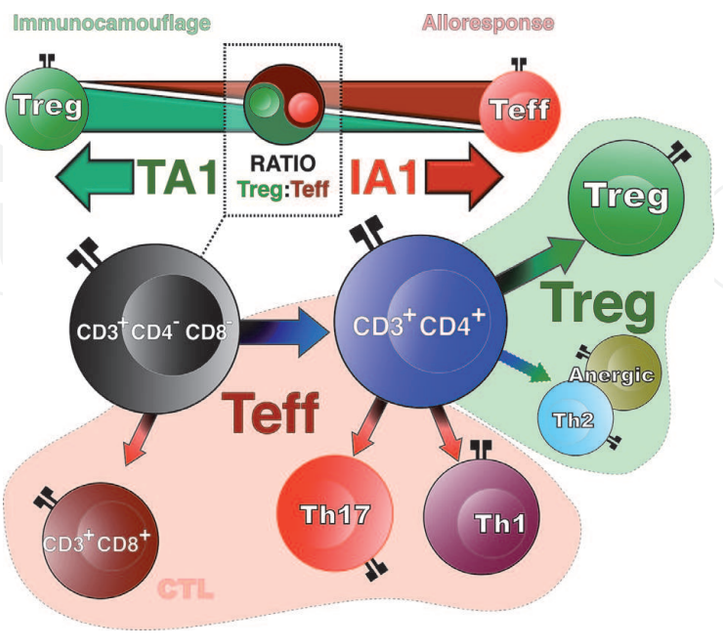

Figure 5.
In vitro and in vivo flow cytometric and functional analyses of T cells demonstrates that the TA1 and IA1 therapeutics differentially skew the differentiation pattern of naive $CD3^+CD4^-CD8^-$ T cells. As shown diagrammatically, TA1 favors tolerogenic/anergic T cell subsets while significantly inhibiting proinflammatory Teff populations. Conversely, as shown by the skewing of the Treg:Teff ratio, IA1 induces differentiation and proliferation of both $CD4^+$ and $CD8^+$ Teff subsets while reducing Treg populations.

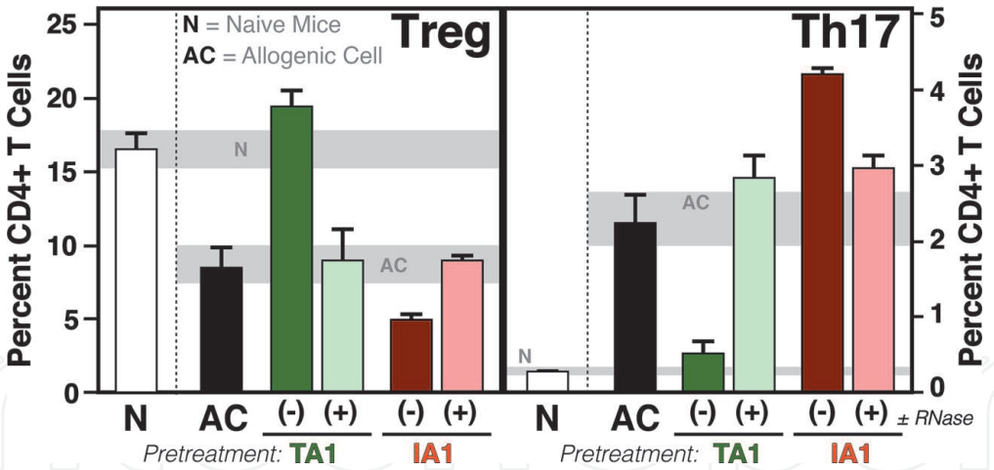

Figure 6.
The active component of TA1 and IA1 are miRNA as evidenced loss of immunomodulatory activity consequent to RNase treatment. A microRNA (miRNA) specific preparation made from mice previously treated (5 days prior) with mPEG-allogeneic leukocytes yielded a systemic immunomodulation (increased Tregs, decreased Th17 T cells) very similar to the mPEG-cellular product within the spleen of mice 5 days post treatment with the miRNA preparation. As shown, the immunomodulatory effect is lost by treatment with RNase enzymes. N = naïve mice; AC = allogeneic cells; miRNA alloplasma fraction ± RNase; and mPEG-alloplasma ± RNase.

6. Treatment of autoimmune diseases via Treg:Teff modulation

Autoimmunity arises consequent to an animal/individual's immune system recognizing their own tissues as *'non-self'*. The Non-Obese Diabetic (NOD) mouse is an inbred strain that exhibits the spontaneous development of a variety of autoimmune diseases including insulin dependent T1D. The murine autoimmune diabetes develops spontaneously around 16–20 weeks of age though studies indicate that the autoimmune process begins by weeks 3–4 [43–51]. Of note, the NOD mouse has been extensively used to study the mechanisms underlying autoimmune-mediated diabetes as well as to evaluate therapeutic interventions on disease pathogenesis. To investigate the ability of TA1 to attenuate disease progression and incidence, 7-week-old NOD mice were treated with TA1—no other interventions were done.

As demonstrated in **Figure 7A**, the onset and incidence of diabetes was assessed and correlated with the Treg:Teff ratio of the mice [31–35]. As shown, 75% of the untreated NOD mice, but only 40% of the TA1 treated mice, developed T1D. The onset of T1D was correlated with the Treg:Teff ratios of the individual mouse (Panel A). As shown, the TA1 treated mice exhibited significantly increased Treg:Teff ratio which correlated with significantly delayed onset of the disease in the mice that became diabetic. Mice with very high Treg:Teff ratios (average > 250) in either the control or TA1 treated mice remained normoglycemic. Moreover, TA-treatment was associated with improved islet histology (**Figure 7B**) as reflected by the lower incidence of overt insulitis and peri-insulitis. Indeed, no normal islets were observed in the control diabetic NOD mice. In contrast, in TA1 treated mice that became diabetic, almost 20% of their islets exhibited normal morphology—more than that observed in normoglycemic NOD mice at 30 weeks. In the normoglycemic TA1 treated mice > 40% of the islets exhibited normal histology.

Mechanistically, the changes noted in the Treg:Teff ratio (using $Foxp3^+$ and $Th17^+$ lymphocytes as surrogates for Treg and Teff, respectively) correlating with the changes noted in multiple T cell subsets [31]. As shown in **Figure 8**, analysis of the pancreatic lymph node demonstrated that TA1 induced multiple tolerogenic T cell subpopulations (e.g., $Foxp3^+$, IL-10^+, TGF-β^+ and IL-4^+ $CD4^+$ T cells) and down regulated multiple Teff subgroups ($Th17^+$, IL-2^+, INF-γ^+ and TNF-β^+ $CD4^+$ cells).

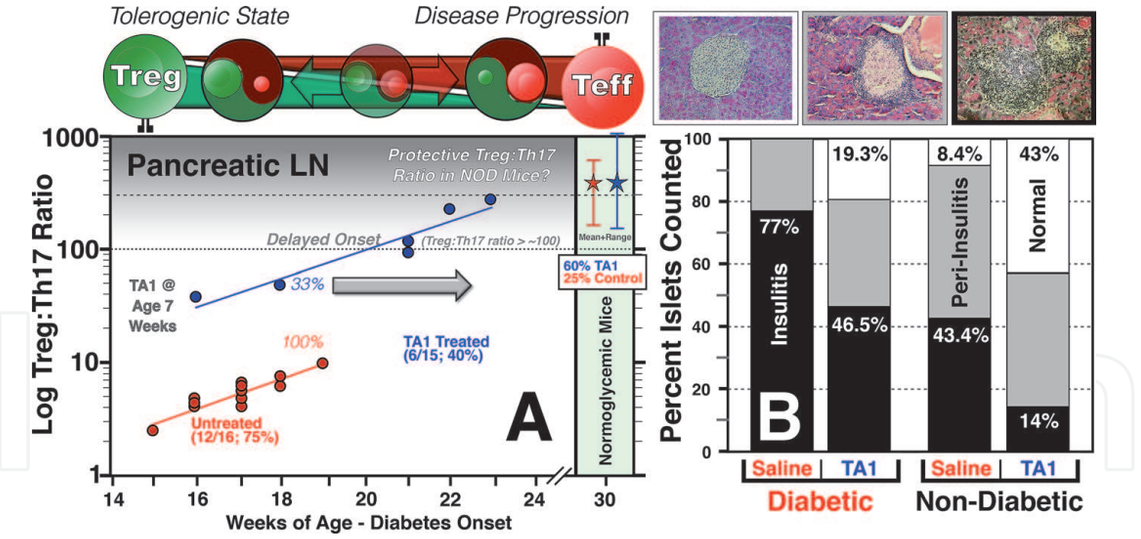

Figure 7.
Inhibition of T1D in the NOD mouse via induction of immunosuppression by the administration of immunomodulatory miRNA. Panel A: Age of onset for T1D versus the Treg:Teff ratio in the control and TA1-treated NOD mouse. Note that TA1 therapy dramatically increased the Treg:Teff ratio and delayed both onset and incidence of T1D. In contrast, in control NOD mice the Treg:Teff ratio shifted left towards the expansion of Teff cells and disease progression. Panel B: Shown are the percentages of pancreatic islets exhibiting normal morphology or evidence of insulitis or peri-insulitis. Also shown are photomicrographs of islets exhibiting (left to right) normal morphology, peri-insulitis and insulitis. Data from Wang et al. [31].

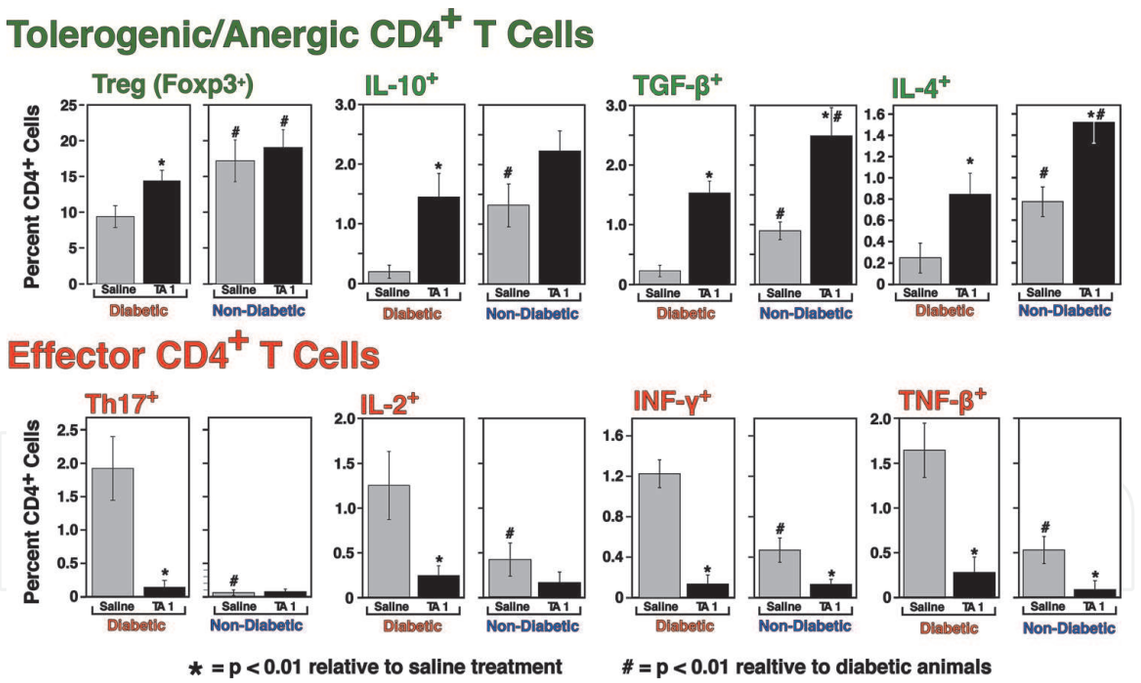

Figure 8.
Effect of TA1 therapy on T CD4⁺ T cell populations. As shown, TA1 therapy significantly increased multiple Treg populations in comparison to the control NOD mice. Concurrent with the increase in Treg subsets, TA1 very dramatically reduced the Teff subpopulations. The net consequence of TA1 therapy was a significant shift in the Treg:Teff ratio towards a tolerogenic state. Data from Wang et al. [31].

Hence, TA1 effectively skewed the Treg:Teff ratio towards a tolerogenic-immunosuppressive environment within the pancreas that consequently inhibited the effector T cell dependent autoimmune disease process. Importantly, the TA1 induced immunomodulation was not limited to the pancreas as T cell subtyping of multiple lymphoid tissues, as well and the blood, demonstrated that the induced tolerogenic environment was systemic in nature [31]. These systemic

findings suggest that TA1 could be used to treat a broad range of T cell mediated autoimmune diseases.

7. Enhancing the anti-cancer response via Treg:Teff modulation

In contrast to autoimmune diseases, systemic immunosuppressive states can be highly problematic in the context of infectious agents (e.g., bacteria and viruses) and cancer. Indeed, this lack of immune response to cancer was the problem that Coley attempted to address with his immunomodulatory preparations. By injecting a toxic mixture, a broad immune response would be induced that, it was HOPED, would exert a non-specific bystander effect on cancer cells. This was, in fact, a relatively viable clinical approach as cancer cells tend to be more sensitive to metabolic (e.g., high fever, energy starvation) and immunological (e.g., T cell and complement activation) extremes. Indeed, *Coley's Toxins* were a mainstay of advanced cancer therapy for much of the early/mid twentieth century until their use was supplanted by *'safer'* radiation and chemo- therapy approaches. But *'safe'* is not always *'safe'*. The long-term toxicity effects of both radiation and chemotherapy are now becoming appreciated—especially when used in young patients. Hence, significant clinical efforts are now being directed towards increasingly expensive and labor/time intensive, immunotherapies that can either enhance the endogenous immune response to cancers or be engineered to attack specific cancers.

In contrast to these expensive and time-extensive cellular therapies, the bioproduction of IA1 (as well as TA1) is rapid and inexpensive. Moreover, minimal time (24 hours) is required to skew the Treg:Teff ratio of resting PBMC towards an inflammatory response arising from the simultaneous decrease of Treg and increase in Teff [34]. Hence IA1 could be used to enhance the immune response of autologous leukocytes thus obviating the risks associated with the adoptive transfer of allogenic T cells. Moreover, the strength of the inflammatory response is substantially less than that observed with other activation strategies (e.g., mitogens, anti-CD3, of allogeneic stimulation) reducing the risk of cytokine release syndrome [34]. Also, of potential value, the strength of the IA1 stimulation can, if necessary, be titrated using TA1.

To evaluate the potential anti-cancer efficacy of IA1 activated leukocytes, in vitro studies were conducted using HeLa and SH-4 melanoma cell lines (**Figure 9**) [34]. The direct toxicity and anti-proliferative effects of control and the SYN (prepared from resting cells) or IA1 treated PBMC against the HeLa (epithelial) and SH-4 (melanoma) human cancer cell lines were assessed using an ACEA iCELLigence (ACEA Biosciences, Inc., San Diego, CA). The iCELLigence provides a continuous, real-time, measurement of cell proliferation using changes in the electrical impedance within tissue culture wells. The change in impedance is induced by the increase in adherent cells and is unaffected by cells (e.g., PBMC) that remain non-adherent. All studies were done with an initial seeding density of 5000 HeLa, or 20,000 SH-4, cells per well. To assess the ability of SYN- or IA1-activation to enhance the anti-cancer efficacy of naïve lymphocytes, donor PBMC were pretreated with SYN or IA1 for 24 hours and then overlaid on seeded cancer cells at a ratio of 50 PBMC per cancer cell.

As shown, direct addition of IA1 to HeLa cells demonstrated that the IA1 therapeutic itself exhibited no direct effects on cancer cell proliferation (**Figure 9A**). However, when HeLa cells were overlaid with unactivated or SYN-activated allogenic donor PBMC, the T cells eventually recognized the allogenic HeLa cells and, after ~90 hours, inhibited cell proliferation and, ultimately, killed the HeLa cells as

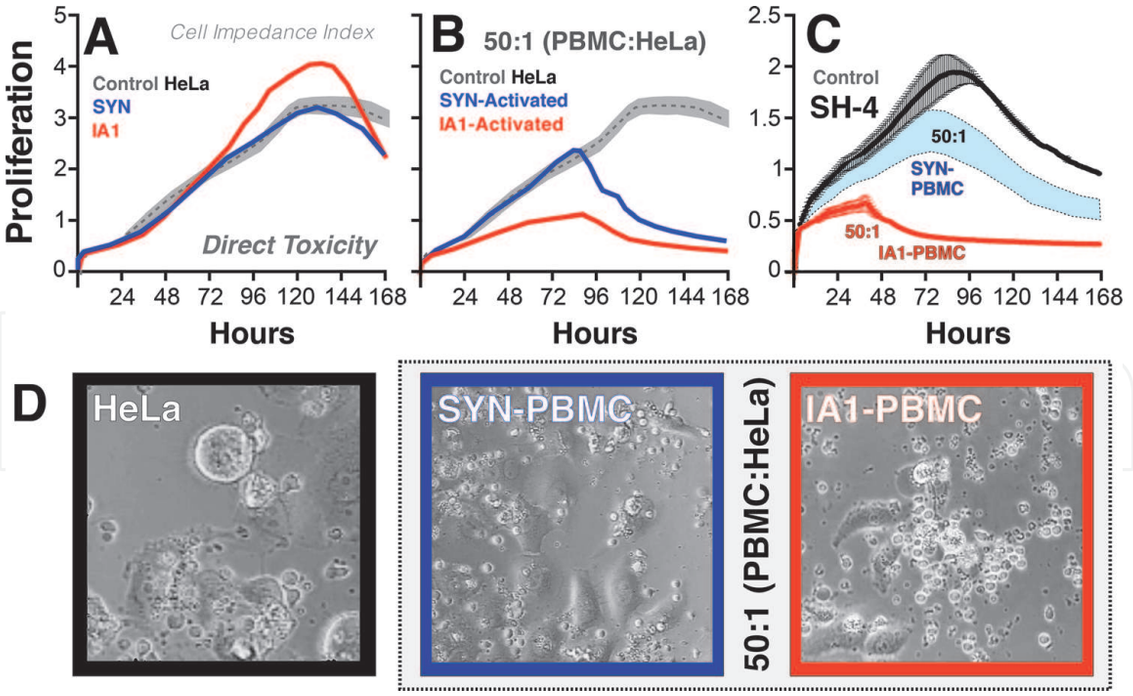

Figure 9.
IA1 enhances the anti-cancer efficacy of resting PBMC. Panel A: IA1 exhibits no direct toxicity to HeLa cells (shown) or PBMC (not shown). Panel B: IA-1 pre-treatment, but not SYN-pre-treatment, exhibited a greatly enhanced anti-cancer effect on HeLa cells. Panel C: Similarly to HeLa cells, IA1, but not SYN, pre-treated PBMC exhibited significant anti-SH-4 (melanoma) activity. Panel D: The enhanced efficacy of treated PBMC is supported by photomicrographs of allogenic PBMC responding to HeLa cells. As shown, after 72 hours incubation, resting unactivated PBMC show limited interaction when overlaid on HeLa cells. In contrast, the same PBMC, when treated for 24 hours with IA1, show a robust enhanced interaction with the HeLa cell monolayer. Cell proliferation was measured by changes in electrical impedance. SYN (derived from the secretome of resting PBMC) or IA1-pretreated utilized PBMC from the same donor. Modified from Yang et al. [34] and Scott et al. [35].

reflected by the decrease in the impedance index. In contrast, when IA1-activated PBMC were overlaid, the inhibition of HeLa cell proliferation was noted within the first 8–12 hours (versus ~90 hours) dramatically reducing the overall proliferation of the HeLa cells (**Figure 9B**). The anti-cancer efficacy of IA-activated PBMC was not limited to HeLa cells. Further studies using SH-4 melanoma cells also demonstrated that IA1-activation of naïve PBMC induced a potent anti-cancer effect (**Figure 9C**). As noted, control SH-4 melanoma cells showed rapid proliferation over 96 hours. However, when untreated SYN-pretreated PBMC (50 PBMC per SH-4 cell) were overlaid onto the seeded SH-4 cells at 0 hours, a significant, but modest, inhibition of SH-4 growth occurred. However, when IA1-pretreated (24 hours) PBMC from the same donor are overlaid on the SH-4 cells, a greatly enhanced anti-cancer effect was noted relative to untreated PBMC. The enhanced efficacy of treated PBMC was supported by photomicrographs of allogenic PBMC responding to HeLa cells (**Figure 9D**). As shown, after 72 hours incubation, SYN-activated PBMC exhibited limited interaction with the HeLa cells. In contrast, the same PBMC, when pre-treated for 24 hours with IA1, demonstrated a significantly enhanced interaction with the HeLa cell monolayer. Hence, in vitro, IA1 is capable of significantly enhancing the anti-cancer efficacy of resting PBMC. As such the secretome generated IA1 proved to be a potent adjuvant therapy for the activation of autologous lymphocytes in cancer patients. This approach could be done either by collection of PBMC with ex vivo activation for 24 hours, or as shown in **Figures 6–8**, direct systemic administration of the IA1-therapeutic to the patient. Moreover, this methodology could be used in conjunction with other ACT approaches.

8. Regulating the Treg:Teff ratio: toxicity and ping-pong immunology

Importantly, treatment of mice or cells with mPEG-splenocytes or the TA1 and IA1 (see **Figure 9A**) secretome products exerted no evidence of direct acute toxicity [27, 31, 34, 35]. Indeed, the safety of allogenic mPEG-splenocytes was demonstrated in a murine model of transfusion associated graft versus host disease in which it was shown that transfusion of mPEG-splenocytes were incapable of inducing graft versus host disease in immunocompromise (irradiated) mice [25]. This is not to say that these approaches may not be prone to chronic side effects. Immunosuppressive therapy, i.e., tolerization, is known to increase the risk of cancer. Thus, the long-term persistence of the effects of mPEG-leukocytes or TA1 [35] could pose a similar risk. Indeed, our previous studies have demonstrated that the immunomodulatory effects of both the PEGylated allogenic splenocytes and the TA1 and IA1 secretome products extend well beyond the circulation time of donor lymphocytes and exhibit functional activity both in vitro and in vivo [27, 31, 33–35]. For example, in mice treated with allogenic mPEG-splenocytes, the Tregs remained significantly elevated at 30 days post treatment and, when challenged with a secondary transfusion of unmodified allogenic splenocytes, prevented the expected (decreased Treg and increased Teff) proinflammatory effects of the allogenic splenocyte transfusion.

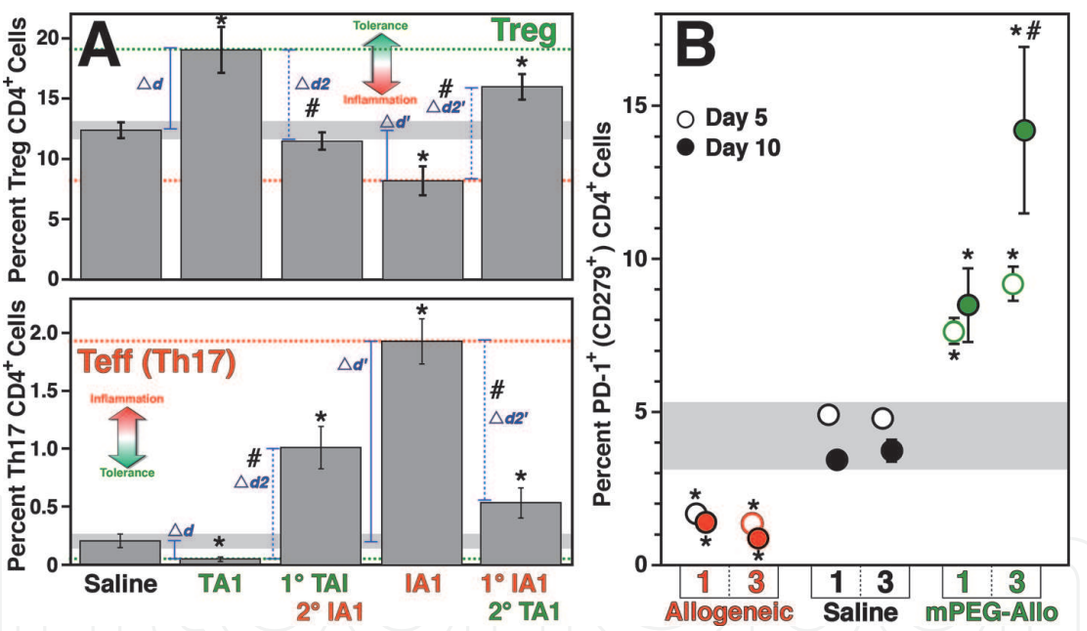

Figure 10.

*Ping-pong immunology of TA1 and IA1. Panel A: Shown are the Treg (Foxp3⁺) and Teff (Th17) CD4⁺ cells in the spleen of mice treated with a primary (1°) infusion of either TA1 or IA1. A subset of mice were subsequently received a secondary (2°) infusion with the opposing therapeutic (IA1 or TA1) at day 9. Lymphoid organs (spleen shown) were harvested at day 40. As noted by the absolute percentage of CD4⁺ T cells and the delta ( ) d/d' from naïve mice, primary (1°) treatment with TA1 and IA1 alone gave the expected Treg and Teff response. The 2° treatment with the opposite miRNA preparation was able to significantly counterbalance the effect of the 1° treatment. This is reflected by the d2 and d2' bars and the regression of the Treg and Th17 values towards the mean of naïve mice. As expected based on the magnitude of the 1° treatment, the d2 and d2' bars were greater than the initial d/d' values. This is most obvious with the Th17 cells where both the magnitude and actual decrease in the 2° d2 (~1.5%) for TA1 was significantly greater than 1° d (~0.15%). Panel B: PD-1⁺ (CD279⁺) CD4⁺ T cells are important in downregulating the immune response and promoting self-tolerance via suppression of Teff cell populations. As shown, transfusion of allogeneic splenocytes downregulated, while mPEG-allogenic splenocytes upregulated, PD-1⁺ cells relative to naïve, saline treated, mice. Shown are the PD-1⁺ cells in the spleen of mice treated with a primary (1°) infusion of either allogenic or mPEG-allogenic at day 0 (denoted as 1) or a total of 3 injections given at days 0, 2 and 4 (denoted as 3). Spleens were harvested at either day 5 or 10 for determination of T cell subpopulations. N ≥ 5 for all samples shown. Significance: * $p < 0.01$ from naïve mice; # $p < 0.01$ from primary TA1 or IA1 (panel a) or; panel B from 1 or 3 doses.*

Indeed, the Treg remained high and no Th17 cells were induced [27]. Long-term studies of mice treated once with TA1 also demonstrated a persistent, and significant, elevation in their Treg cells for ≥270 days [35]. Hence, the potent immunomodulatory effects of this approach could be of concern.

As noted above, the persistence of the Treg response, even upon allogenic challenge, while beneficial in the treatment of autoimmune diseases could pose immunological risks. However, TA1 and IA1 target the same miRNA-based bioregulatory pathway governing lymphocyte differentiation and proliferation. Because of this, TA1 and IA1 are capable of counter-acting the activity of the other. This *'ping-pong immunology'* in mice is demonstrated in **Figure 10A**. As noted, treatment with TA1 or IA1 inversely affects the Treg and Teff populations (Δd, Δd'). However, subsequent administration of the IA1 to TA1 treated mice, or vise-versa, resulted in the Treg and Teff cell populations reverting towards the homeostatic level noted in naïve, untreated, mice (Δd2, Δd2'). The immunomodulatory *'ping-pong'* activity of TA1 and IA1 can be further fine-tuned via dosing, as both TA1 and IA1 show dose dependent activity [31–35]. Also of note, the immunomodulatory activity of this technology is correlated with other markers of tolerance or inflammation. Transfusion of mPEG-splenocytes triggers a significant upregulation of PD-1$^+$ (CD279) CD4$^+$ lymphocytes (**Figure 10B**). These PD-1$^+$ cells are important in downregulating the immune response and promoting self-tolerance via suppression of Teff cell populations. The expression of PD-1$^+$ T cells may underlie the production of IL-10$^+$ T cells as noted in **Figure 8**. In contrast to TA1 treatment, both unmodified allogenic cells (shown) and IA1 treatment (not shown) decrease PD-1 expression relative to naïve mice. A dose effect on PD-1 expression can be seen with both allogenic and, especially, mPEG-allogenic cells in mice receiving 1 or 3 injections (at days 0, 2 and 4) of cells when assessed at days 5 and 10 post treatment.

9. Conclusions

Immunosuppression and immunoactivation represent the divergent ends of the Treg:Teff ratio continuum (**Figure 1**). While pharmacologic agents have historically been the primary tools for modulating the Teff response, few options have existed for modulating (especially upregulating) the Treg response. However, the direct immunomodulation of the endogenous immune system may have significant clinical benefit in treating a broad range of clinical conditions ranging from autoimmune diseases, tissue/organ engraftment, and cancer. Extensive in vitro and in vivo studies in our laboratory have demonstrated that PEGylated lymphocytes as well as the biomanufactured TA1 and IA1 exhibited significant immunomodulatory activity [23–42, 52]. Indeed, these agents directly altered the Treg:Teff ratio by simultaneously modulating both regulatory and effector T cell subsets. Consequent to their immunomodulatory activity, the immunosuppressive TA1 therapeutic significantly delayed the onset and overall incidence of autoimmune diabetes in the NOD mouse [31]. Conversely, the proinflammatory IA1 therapeutic directly activated T cells overcoming their inherent immunological inertia resulting in enhanced recognition and killing of cancer cells [34]. The immunomodulatory effects of these agents were highly persistent [35]. The TA1 and IA1 agents showed dose dependency and could be used to counteract the effect of on another [31–35]. The successful development of these immunomodulatory therapeutics may prove useful in facilitating organ engraftment, treating autoimmune disease and enhancing the endogenous anti-cancer response.

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Conflict of interest

Canadian Blood Services is pursuing patents related to the production and utilization of the described acellular immunomodulatory agents. Canadian Blood Services, a not-for-profit organization responsible for collecting, manufacturing and distributing blood and blood products to all Canadians (except Quebec), is the assignee for relevant patents. MDS is an inventor on these patents. XY has no conflicts of interests.

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