Chapter

Modulating the T Lymphocyte Immune Response via Secretome Produced miRNA: From Tolerance Induction to the Enhancement of the Anticancer Response

Mark D. Scott, Duncheng Wang, Wendy M. Toyofuku and Xining Yang

Abstract

T cells are key mediators of graft tolerance/rejection, development of autoimmunity, and the anticancer response. Consequently, differentially modifying the T cell response is a major therapeutic target. Most immunomodulatory approaches have focused on cytotoxic agents, cytokine modulation, monoclonal antibodies, mitogen activation, adoptive cell therapies (including CAR-T cells). However, these approaches do not persistently reorient the systemic immune response thus necessitating continual therapy. Previous murine studies from our laboratory demonstrated that the adoptive transfer of polymer-grafted (PEGylated) allogeneic leukocytes resulted in the induction of a persistent and systemic tolerogenic state. Further analyses demonstrated that miRNA isolated from the secretome of polymer-modified or control allogeneic responses effectively induced either a tolerogenic (TA1 miRNA) or proinflammatory (IA1 miRNA) response both *in vitro* and *in vivo* that was both systemic and persistent. In a murine Type 1 diabetes autoimmune model, the tolerogenic TA1 therapeutic effectively attenuated the disease process via the systemic upregulation of regulatory T cells while simultaneously downregulating T effector cells. In contrast, the proinflammatory IA1 therapeutic enhanced the anticancer efficacy of naïve PBMC by increasing inflammatory T cells and decreasing regulatory T cells. The successful development of this secretome miRNA approach may prove useful treating both autoimmune diseases and cancer.

Keywords: T lymphocyte, miRNA, polymer, secretome, tolerance, Treg, proinflammatory, Teff, autoimmunity, cancer, adoptive cell transfer

1. Introduction

Biologically, and clinically, the concept of "self" is of crucial importance in protection against foreign biologicals (e.g., viruses and bacteria), abnormal autologous cells (e.g., cancers) and more recently developed "diseases" (i.e., the purposeful introduction of "nonself") such as enzyme-replacement therapy and transfusion

and transplantation medicine. The immune system is tasked with preserving "self" and rejecting "nonself" and has multiple components-any of which will be of variable importance depending on the context of the immunological assault. 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. 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. T cells themselves consist of a diverse array of subsets that fall into two general categories: 1) Regulatory T cells (Treg) which modulate the strength of an immune response and maintain "self"; and effector T

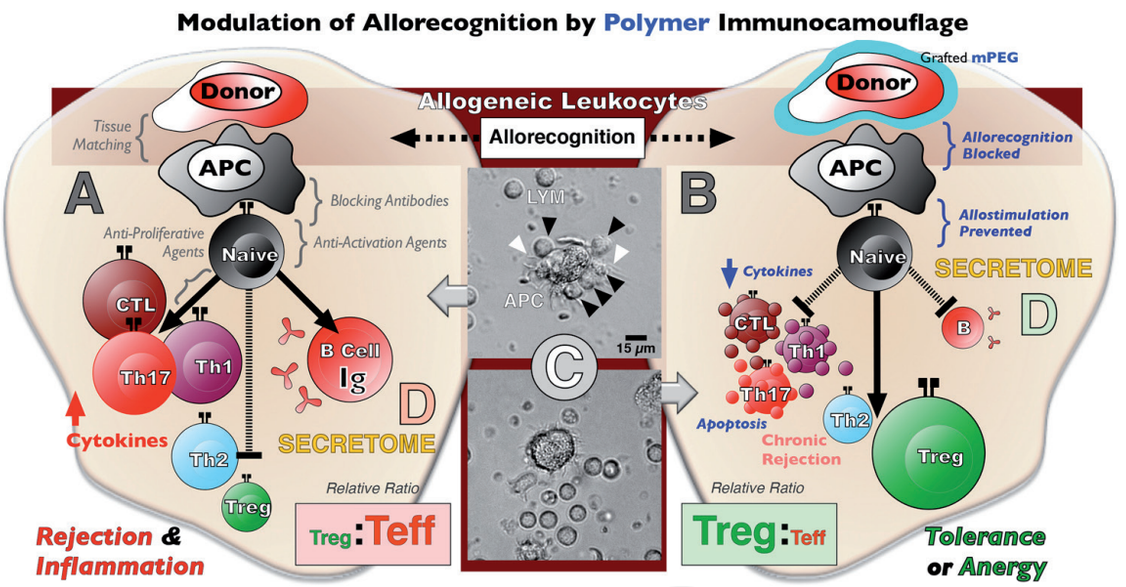

Figure 1.
Immune modulation via pharmacologic and immunocamouflage therapy. (A) Current pharmacologic therapy almost exclusively targets T cell activation and proliferation consequent to allorecognition. Response to nonself is in large part mediated by cell-cell interactions between antigen presenting 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. 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, blocking antibodies have been investigated. Gray text indicates current techniques to prevent/limit alloimmune responses. (B) In contrast, immunocamouflage of donor cells by methoxy(polyethylene) glycol (mPEG) results in the disruption of the essential cell-cell interactions decreasing T cell proliferation and altering differentiation patterns (decreased Th17 and increased Treg). In aggregate, the polymer induced changes induces a tolerogenic/anergic state both in vitro *and* in vivo. *Size of T cell population denotes increase or decrease in number. Size of B cell indicates antibody response. Blue text represents the consequences of polymer-mediated immunocamouflage of the alloresponse. (C) As shown in photomicrographs, in a control mixed lymphocyte reaction (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. (D) Importantly, the secretomes derived from the MLR and mPEG-MLR exert potent effects on a secondary MLR encompassing fresh PBMC from the same or different donors. The key component of the secretome are soluble (free and exosome) miRNA. Data derived from Refs [32–43].*

cells (Teff) that mediate the inflammatory response and consists, in part, of Th1, Th17 and Th2 subsets. Hence, the functional ratio of Treg to Teff (Treg:Teff) cells is critical and an imbalance of this ratio from the norm can induce either an autoimmune (excess Teff or decreased Treg) state or impaired response to "nonself" (e.g., cancer) consequent to biologically ill-advised tolerance (too many Treg or weak Teff response). Indeed, the T cell response plays a (the) central role in autoimmune diseases, transplant rejection, graft versus host disease (GVHD), graft versus leukemia (GVL), cancer and, more recently, cancer therapy. Hence, consequent to the central role of T cells as a key cellular component in the development of autoimmune diseases, graft tolerance or rejection, and the anticancer response, the T cell response has been a major focus in the development of clinical therapies (**Figure 1A**) [1].

2. Immunomodulation of the T cell response in autoimmunity and cancer

Autoimmune diseases arise when the immune system recognizes the individual's own tissues or organs as "foreign" and targets them for destruction. Autoimmune diseases can affect virtually all tissues and organ systems and encompass such diverse diseases as Type 1 Diabetes (T1D; pancreas), Idiopathic Thrombocytopenic Purpura (ITP; platelet destruction), Crohn's disease (CD; bowel), Multiple Sclerosis (MS; brain) and Rheumatoid Arthritis (RA; joints). Despite the diversity of tissues affected, extensive research has demonstrated that Treg are downregulated while Teff are upregulated (i.e., leading to a reduced Treg:Teff ratio) leading to a chronic proinflammatory state. Current therapeutic approaches to managing autoimmune diseases are typically focused on symptom relief and the use of immunosuppressive agents capable of inhibiting the proinflammatory response arising from "self-recognition." Most commonly, treatment for chronic autoimmune disease is via administration of systemic steroids (e.g., dexamethasone), cytotoxic anti-proliferative/activation agents (e.g., cyclosporine) that induce a general immunosuppression, and/or IVIG (pooled, polyvalent, IgG purified from the plasma of >1000 blood donors) [2–6]. Other experimental approaches to the treatment of autoimmune diseases include blocking monoclonal antibodies directed against the TCR, CD4, costimulatory ligands and receptors, adhesion molecules, and cytokine receptors [7–9]. A more recent approach has been to interrupt the cytokine signals necessary for the activation and proliferation of autoreactive T cells. The current gold standard for this approach is Enbrel® (etanercept), a solubilized TNF-α receptor fragment that intercepts and sequesters the TNF-α cytokine thereby inhibiting the proliferation of proinflammatory T cells [10–15]. However, Enbrel® has been given a USA FDA "Black Box" warning due to significantly increased risks of serious infections that may lead to hospitalization or death [16–22]. Common to all of these approaches is an attempt to increase the Treg:Teff ratio by either directly increasing Treg or selectively decreasing Teff populations. However, despite their importance in clinical medicine, many of these agents have been plagued by both significant toxicity/adverse events and an inability to adequately eliminate or inhibit reactive T cells [8].

In contrast to autoimmune diseases, an insufficient/inefficient immune response may underlie the proliferation and dissemination of abnormal cells (i.e., cancer cells). While this may occur for a number of reasons, immunosuppression is a known risk factor. Indeed, acquired or inherited T cell defects as well as long-term therapy with immunosuppressive drugs are clearly associated with an increased risk of neoplasia. The impaired immune response to cancer cells can arise, at least in part, from an increase in the Treg:Teff ratio (too many Treg and/or insufficient Teff cell production). To address this imbalance in the Treg:Teff ratio, experimental

therapies are currently focused on the *ex vivo* expansion and subsequent transfusion of autologous Teff capable of killing the cancer cells [23–31]. However, these current immune enhancing methods, while promising, are expensive, complicated to accomplish (e.g., insertion of specific target cancer genes in APC) and requires weeks of tissue culture expansion to meet the threshold for cell infusion.

Perhaps most importantly, current tolerogenic or proinflammatory therapeutic approaches fail to persistently reorient the systemic T cell immune response thus necessitating continual therapy. Moreover, despite the importance of the Treg:Teff ratio, in both autoimmune diseases and cancer, there are a paucity of pharmacologic tools that can directly, and in tandem, target the regulation of both the Treg and Teff subsets. Hence, to diminish or overcome the need for chronic administration of immunotherapeutic agents, new approaches capable of persistently reorienting the endogenous immune (Treg:Teff) response would be of value.

3. Immunomodulation via immunocamouflage and differential miRNA production

Previous studies from our laboratory demonstrated that a persistent and systemic reorientation of the animal (murine; or *in vitro* human) immune response towards a tolerogenic response could be induced via the adoptive transfer of immunocamouflaged allogeneic leukocytes to a recipient animal [32–43]. Immunocamouflage of cells is mediated by the covalent grafting of methoxypoly(ethylene glycol) (mPEG) to the leukocyte membrane surface. Consequent to mPEG-grafting (PEGylation), MHC-mediated T cell alloproliferation is dramatically inhibited due to consequent to impaired cell:cell interaction and weak allostimulation (**Figure 1A** and **B**). These studies demonstrated that the PEGylated allogeneic leukocytes diminished intracellular communication preventing a Teff response while simultaneously inducing the generation of Treg cells skewing the Treg:Teff ratio towards a tolerogenic state (**Figure 1B** and **C**) [36, 38–43]. Further *in vitro* and *in vivo* studies demonstrated that, using MLR-based secretome biomanufacturing systems, distinct acellular microRNA (miRNA) based therapeutics could be manufactured from control and PEGylated allorecognition reactions that systemically and persistently reorient the immune response to either a proinflammatory (IA1) or tolerogenic (TA1) state (**Figure 1**) [40, 43]. In this chapter, we will demonstrate how these miRNA-based therapeutics can inhibit the progression of T cell mediated autoimmune diseases (TA1) or conversely enhance the proinflammatory anticancer T cell response (IA1).

4. Production of miRNA therapeutics via the alloresponse pathway

Since their discovery in 1996, the role of circulating (cell-free) miRNA in disease processes has become an active research area and recent findings suggest that they may be biomarkers, or possibly mediators, of cancers as well as autoimmune diseases such as T1D [44–46]. To understand mechanistically how the TA1 and IA1 miRNA biologics function, an appreciation of the biological role and regulatory complexity of miRNA is needed. Recent studies have demonstrated that miRNA are key epigenetic regulators of cellular processes including immune responses, inflammation, proliferation, survival, and cellular differentiation [47, 48]. miRNA are short (~22 nucleotides) single-stranded RNA molecules found in all eukaryotes and it is estimated that ~60% of mammalian genes are targeted by one or more miRNA [49, 50]. Moreover, because of their evolutionary importance in gene regulation, miRNA and their

sequence and processing are highly conserved between mammalian species (e.g., mouse and human) [49]. While miRNA are most commonly found intracellularly, significant amounts of stable miRNA are also found in the serum of mammals suggesting an important messenger/regulatory role. While the nomenclature of miRNAs is relatively straightforward it is important to note that similarities in miRNA number designation is not indicative of similarity in functionality (**Figure 2**). Moreover, the literature is replete with conflicting claims for the specific actions of a single miRNA.

Indeed, there is a significant lack of clarity regarding the function of a single miRNA. This lack of functional clarity likely arises consequent to the complexity and low fidelity of the miRNA bioregulatory process. Of note, a single miRNA can potentially affect tens to hundreds of genes and individual genes can be regulated by multiple miRNA [50]. Hence, the effect of modifying the expression of a single miRNA on protein regulation and bioregulatory networks is unpredictable. Because of this regulatory complexity, most studies have focused on miRNA as disease biomarkers, not as therapeutic agents as there is a low probability that altered expression of a single, or even a few, miRNA would exert a potent and definitive biological response [51–54]. From a bioregulatory approach, it is more probable that multiple miRNA control protein expression, proliferation and differentiation and it is this "pattern of miRNA expression" (encompassing increased, decreased and static levels) that must be mimicked to achieve pharmacologically effective miRNA-based therapeutics. To achieve this goal our laboratory approach has been purposefully chosen to biologically manufacture relatively complex miRNA preparations mimicking normal biology in order to achieve maximal biological functionality.

Using a Mixed Lymphocyte Reaction (MLR) production model the T cell centric proinflammatory IA1 and tolerogenic TA1 therapeutics can be reproducibly manufactured using the control-MLR and mPEG-MLR (respectively; **Figure 1**). As demonstrated, the allogeneic PBMC populations within the control- and mPEG-MLR express significantly different patterns of miRNA expression relative to resting

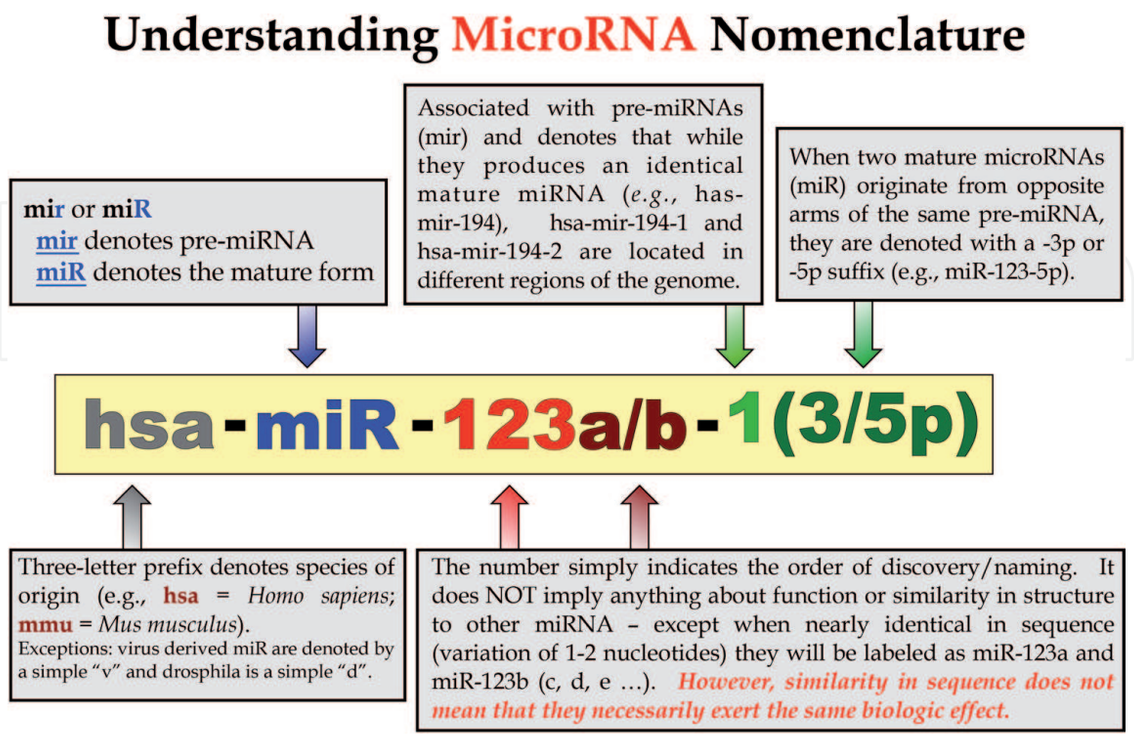

Figure 2.
miRNA nomenclature explained. An important concept to understand is that the miRNA number (e.g., 123 as shown) has no relationship to function or structure. For example, "hsa-miR-123" has no implied structural or functional similarity to "hsa-miR-128." However, because of the highly conserved nature of miRNA, human "hsa-miR-123" has very similar structure and function to murine "mmu-miR-123."

PBMC as evidenced via clustergram (**Figure 3A**), volcano plot (**Figure 3B**) and Log2 Fold (**Figure 3C**) miRNA expression analyses. Importantly, as shown in **Figure 3C**, the control- and mPEG-MLRs show unique patterns of expression. While there are some similarities in the pattern of expression there are significant disparity in miRNAs expressed as well (not shown are the miRNA unchanged from resting cells).

Importantly, the differences in miRNA expression between the Control- and mPEG-MLR leukocyte yield secretomes that exert dramatically different effects when used to treat resting human PBMC or murine splenocytes. Collection of the secretome produced (**Figure 4A**) during the control and polymer modified allorecognition-based MLR yields a reproducible, acellular, miRNA-rich, material that is stable and can be frozen and thawed with minimal decrement to its activity. As schematically presented (**Figure 4B**), TA1 upregulates regulatory T cell populations (e.g., Treg) while simultaneously downregulating Teff (e.g., Th17 and Th1) cells. In contrast, the proinflammatory IA1 increases Teff while decreasing Treg cells. Of note, the secretome from resting cells (SYN) has minimal to no effect on human or mouse immune cells. Moreover, due to the conserved nature of mammalian miRNA, cross species efficacy is observed with both TA1 and IA1. As shown in **Figure 4C**, murine splenocyte produced TA1 and IA1 exerted dose-dependent effects on a human MLR with murine-sourced TA1 reducing $CD3^+CD4^+$ T cell proliferation and the murine IA1 enhancing $CD3^+CD4^+$ T cell proliferation. Hence, a polymer-based, alloresponse manufacturing system may provide a unique avenue for more effectively, and safely, modulating the Treg:Teff cell ratio via the production of therapeutically effective TA1 and IA1 miRNA-based therapeutics [32–43]. Importantly, the effects of TA1 and IA1 immunotherapy was persistent. In murine studies, a single dosing of TA1 to mice resulted in significant increase in Treg cells within the spleen of normal mice that persisted to ≥270 days post treatment (**Figure 4D**).

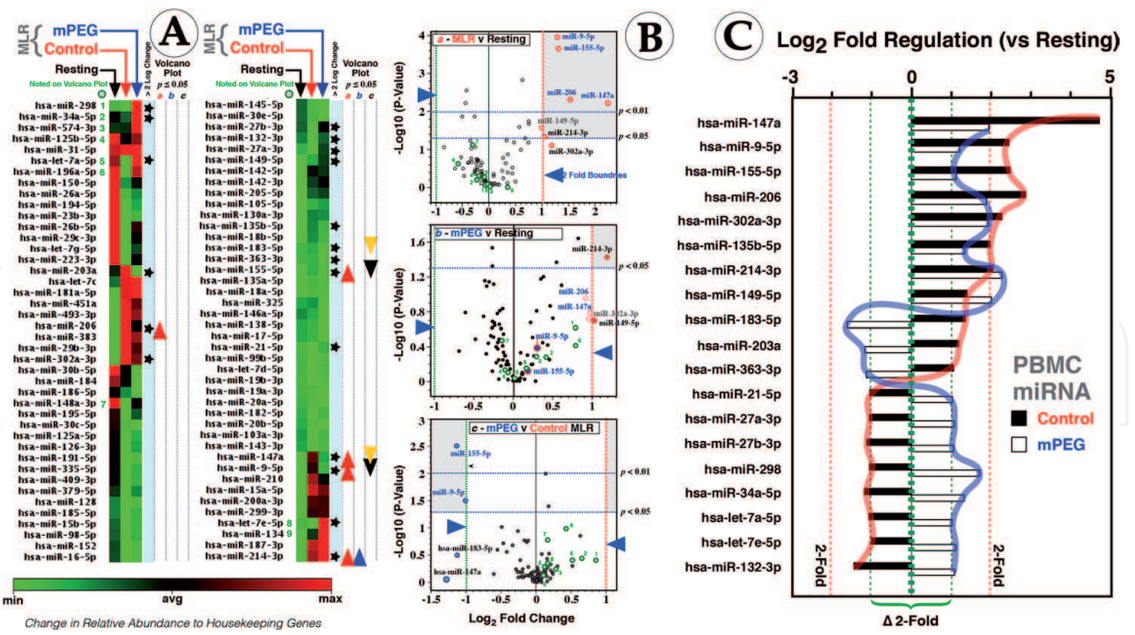

Figure 3.
Partial qPCR characterization of the miRNA expression in the Control- and mPEG-MLR. (A–C) Clustergram (A), Volcano Plot (B) and Log2 Fold (C) analyses of the miRNA expression in the mPEG-MLR and Control-MLR relative to resting cells. (C) Because of the complexity of miRNA regulation of genes, we have consciously chosen to produce a relatively complex miRNA preparations mimicking normal biology in order to achieve maximal biological functionality. Multiple miRNA changes are noted in the hTA1 miRNA compared to either resting cells (green dashed line = 0) or the proinflammatory hIA1 miRNA preparation. Using miRNA expressing a net ΔLog2 Fold change, significantly different "patterns of expression" are noted between the hTA1 and hIA1 miRNA. This pattern of expression, comprising both INCREASED and DECREASED miRNA species is essential for effective immunomodulation of recipient animals. Values derived from a minimum of 3 independent biological replicates. Unpublished data.

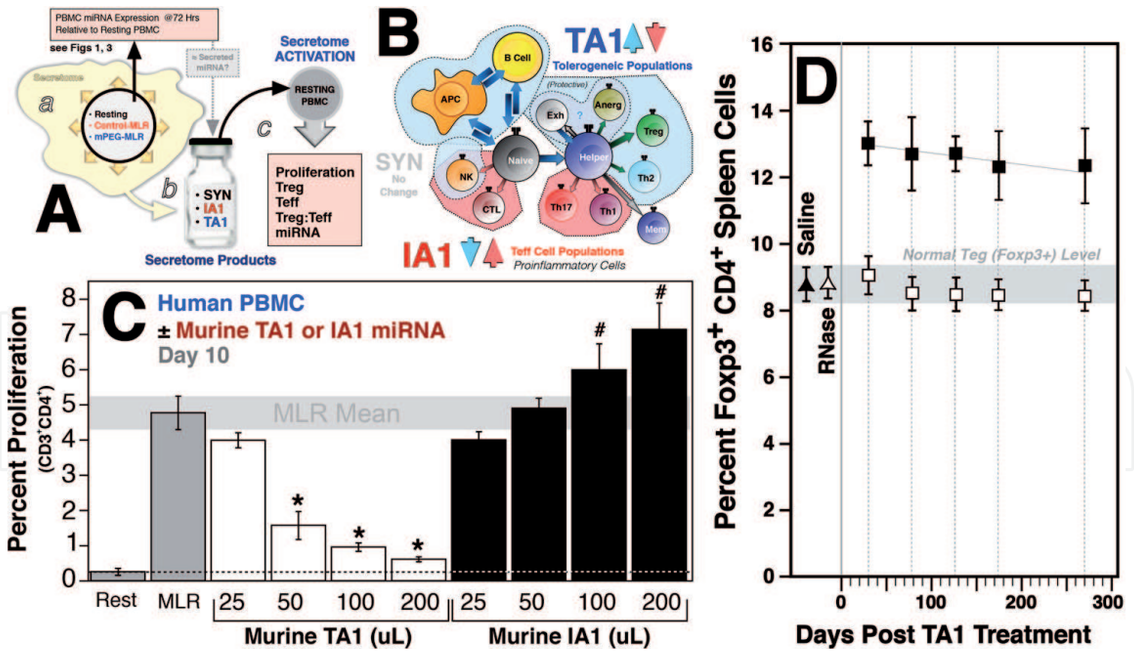

Figure 4.
Differential effects of TA1 and IA1 on the immune system. (A and B): Secretome production (A) of SYN (resting), IA1 (MLR) and TA1 (mPEG-MLR) gave rise to unique immunomodulatory activity (B). While IA1 enhanced proinflammatory subsets and reduced Treg cells, TA1 enhanced Treg while reducing Teff subpopulations. (C) Attesting to the conserved nature of miRNA, murine TA1 and IA1 exerted significant, dose-dependent, immunomodulatory effects on resting human PBMC. The SYN secretome product had no substantive effects on T cell proliferation and differentiation. Data derived from Refs: [32–43] TA1 administration induces a persistent tolerogenic state in immunocompetent mice. (D) As shown, $CD4^+Foxp3^+$ Treg cells remain elevated for ≥270 days following a SINGLE TA1 administration at age 7–8 weeks. In contrast, RNase-treated TA1 (to degrade the miRNA) had no immunomodulatory effect. Results shown are from a minimum of 8 animals per group Unpublished data.

5. Tolerogenic TA1: immunomodulation of autoimmune disease

Autoimmune destruction of pancreatic islets gives rise to T1D and occurs via T cell dependent pathways [55–57]. Elucidation of the role of T cells in T1D has been most effectively examined in the nonobese diabetic (NOD) mouse model. In the NOD mouse, evidence suggests that a deficit in Treg control over diabetogenic Teff cells leads to the development of insulitis and disease [56–66]. Indeed, changes in the Treg:Teff ratio (i.e., balance) can be observed as early as 3–4 weeks of age and becomes more pronounced with disease progression (**Figure 5**) [56]. Human studies have similarly demonstrated that T1D Treg exhibit an impaired ability to suppress Teff [67]. Thus, the emergence of an aggressive diabetogenic lymphocyte response in NOD mice, and likely humans, is dependent upon a change in the Treg:Teff ratio.

As demonstrated in **Figure 5**, the Treg:Teff ratio (defined as the ratio of $Foxp3^+$ to $Th17^+$ T cells) in control (saline treated) NOD mice decreased with disease progression from 103 in nondiabetic 7 week old mice to only 4.7 in diabetic mice at time of sacrifice (15–30 week). Moreover, control NOD mice exhibited a rapid onset of diabetes with 75% (12 of 16) of the mice becoming diabetic by week 19. Subsequent to week 19, no additional mice became diabetic. In contrast, a single dosing (3 injections at 2 days intervals) of the TA1 therapeutic at 7 weeks of age dramatically altered both the incidence and rate of progression of the T1D in the NOD mouse. By week 19 only 13% (2 of 15) of the TA1 treated mice became diabetic with an additional 4 mice becoming diabetic between weeks 21 and 23 (total diabetic 6/15; 40%). Mechanistically, these findings were associated with

a systemic alteration of the immune system as noted in **Figure 5**. In control NOD mice, the progression to diabetes was characterized by significantly elevated levels of most proinflammatory Teff (e.g., INF-γ^+, Th17$^+$, and IL-2$^+$) lymphocytes and a corresponding decrease in regulatory subsets. In contrast, TA1 therapy dramatically and significantly blunted the expansion of Teff cells (as exemplified by INF-γ^+, Th17$^+$, and IL-2$^+$ lymphocytes; **Figure 5A**) relative to diabetic or nondiabetic control NOD mice coupled with a simultaneous increase in a broad range of tolerogenic/anergic regulatory T cell subsets (e.g., foxp3$^+$, IL-10$^+$, TGF-β^+; **Figure 5B**) in the pancreatic lymph node. These studies also demonstrated that TA1 treated NOD mice had significant numbers of histologically normal pancreatic islets while no normal islets were identified in the untreated mice [40]. It is worth noting that all diabetic mice (control and TA1-treated) exhibited significantly lower levels of these tolerogenic cells than did the 30 week old nondiabetic (control or TA1) mice. Moreover, the effects of TA1-miRNA therapy were not localized to the pancreatic lymph node microenvironment. Analyses of the T cell subsets present in the spleen and brachial lymph node of control and TA1 treated NOD mice (diabetic and nondiabetic) similarly demonstrated dramatic changes in the

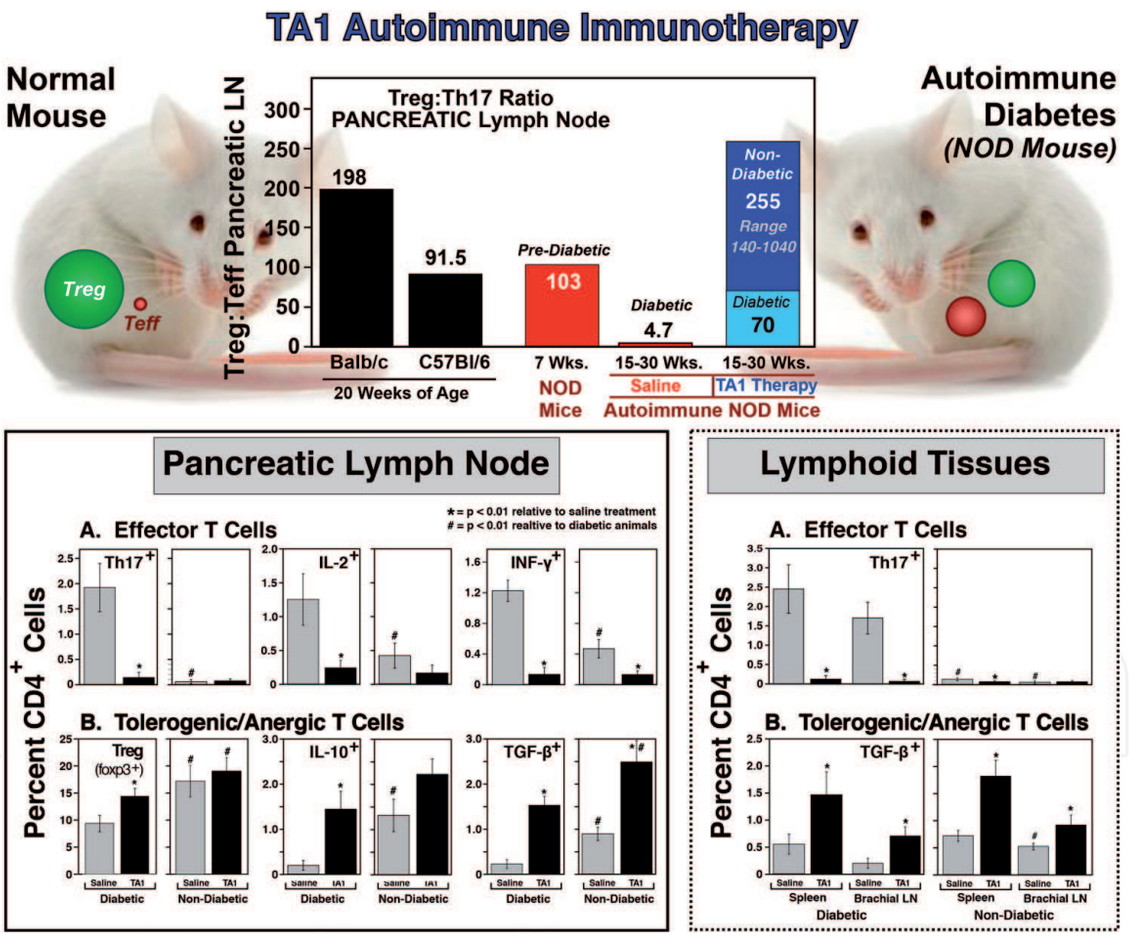

Figure 5.
The autoimmune disease of T1D process is mediated by a decrease in the Treg:Teff ratio and can be prevented by TA1 administration (top). Treatment with the TA1 miRNA product prevents the decrease and, in fact, significantly increases the Treg:Teff ratio. The increased Treg:Teff ratio is protective as evidenced by the finding that the majority of TA1 treated animals remained normoglycemic. Shown in the blue bars are the Treg:Teff ratio for TA1 treated mice who were diabetic (ratio of 70) and nondiabetic (ratio of 255). Mechanistically, TA1 immunotherapeutic significantly altered the expression of multiple proinflammatory (A) and tolerogenic/anergic (B) T cell subsets. These changes were systemic in nature as shown by changes in not only the pancreatic lymph node but in other immune tissues (spleen and brachial lymph node). Diabetic tissues were harvested at time of conversion, nondiabetic tissues were harvested at week 30. Diabetic values are the mean ± SD of 12 saline and 6 TA1 treated NOD mice. Nondiabetic results are the mean ± SD of 4 saline and 9 TA1 treated NOD mice. Derived from Ref. [40].

Teff cell populations (**Figure 5A**, right) and tolerogenic T cells (**Figure 5B**, right). These findings demonstrate that miRNA-based TA1 therapeutic, directly targets the Treg:Teff ratio yielding a systemic protolerogenic state both *in vivo* (mouse) and *in vitro* (human and mouse) suggesting that this approach would be of utility in a broad range of autoimmune diseases. Furthermore, due to the persistence of the immunomodulatory activity in mice (**Figure 4**), TA1-like drugs could, potentially, dramatically reduce the need for chronic administration of drugs.

6. Proinflammatory IA1: enhancing the immune response to cancer

T cells plays a critical role in the anticancer inflammatory responses. An effective anticancer proinflammatory T cell response is dependent upon the activation of Teff cells. Normally, T cells are activated upon ligation of their antigen receptors with specific cognate antigens [68]. However, because of the low frequency of cancer antigen-specific lymphocytes, the immune response to cancers can be initially, and all too often remains, weak. While previous studies have attempted to enhance the anticancer T cell response using pan T cell mitogens (e.g., phytohemagglutinin; PHA), cytokines (e.g., IL-2), or monoclonal antibodies (e.g., anti-CD3 and anti-CD28) the overly robust T cell response arising from these approaches often induced significant systemic toxicity leading to the suspension or abrogation of multiple clinical trials [69–74]. In contrast, in an allorecognition response only 1–10% of T cells are alloreactive [75]. Hence, the IA1 therapeutic, derived from a bioreactor allorecognition response (MLR), is expected to activate endogenous T cells in a more controlled manner, with less toxicity.

To assess IA1's ability to enhance the anticancer activity of resting PBMC, cells were treated for 24 hours with IA1 and overlaid on HeLa and SH-4 cancers cells. Cancer cell proliferation was then followed for 168 hours. Importantly, IA1 exerted no toxicity to resting PBMC but, as shown in **Figure 4**, induced significant activation (e.g., proliferation) of resting $CD3^+$ ($CD4^+$ and $CD8^+$) skewed towards proinflammatory subsets thus decreasing the Teff:Treg ratio. However, as predicted by the biology of the alloresponse, IA1-mediated T cell proliferation was much more restrained than that induced by the anti-CD3/anti-CD28 or PHA stimulation [43]. This finding suggests that the systemic toxicity, relative to pan T cell activators, should be greatly reduced. Crucially, IA1-activated PBMC demonstrated a potent inhibition of cancer cell (HeLa and SH-4 melanoma) proliferation relative to the resting PBMC (**Figure 6**). The anti-proliferation effect of IA1-activated PBMC was noted within ~12 hours vs. 4–5 days for resting cells. These findings demonstrate that miRNA-enriched therapeutics can be biomanufactured from the secretome and can induce a potent proinflammatory, anticancer, effect on resting lymphocytes.

The potential utility and use of IA1 in Adoptive Cell Therapy (ACT) is diagrammatically shown in **Figure 6**. The bioproduction of IA1 is both inexpensive and rapid (5 days) and the IA1 can be stored for long periods (several months frozen in the laboratory; data not shown). Moreover, neither IA1 or TA1 production actually requires donor specific tissues (PBMC) making these secretome-based therapeutics an "off-the-shelf" immune adjuvant. Most importantly for patient care, *ex vivo* activation of lymphocytes is rapid (24 hours). The rapidity of this approach is in stark contrast to the weeks to months necessary for production and expansion of CAR-T cells. Hence, IA1 activation of autologous PBMC could be employed as a first line therapy or, potentially, be used as an immunotherapeutic bridge while CAR-T cells are produced. Due to the simplicity and low cost of the approach, multiple rounds could be used as necessary with large numbers of autologous PBMC employed. Indeed, due to the ability to infuse large numbers of IA1 treated autologous cells, enhanced recognition

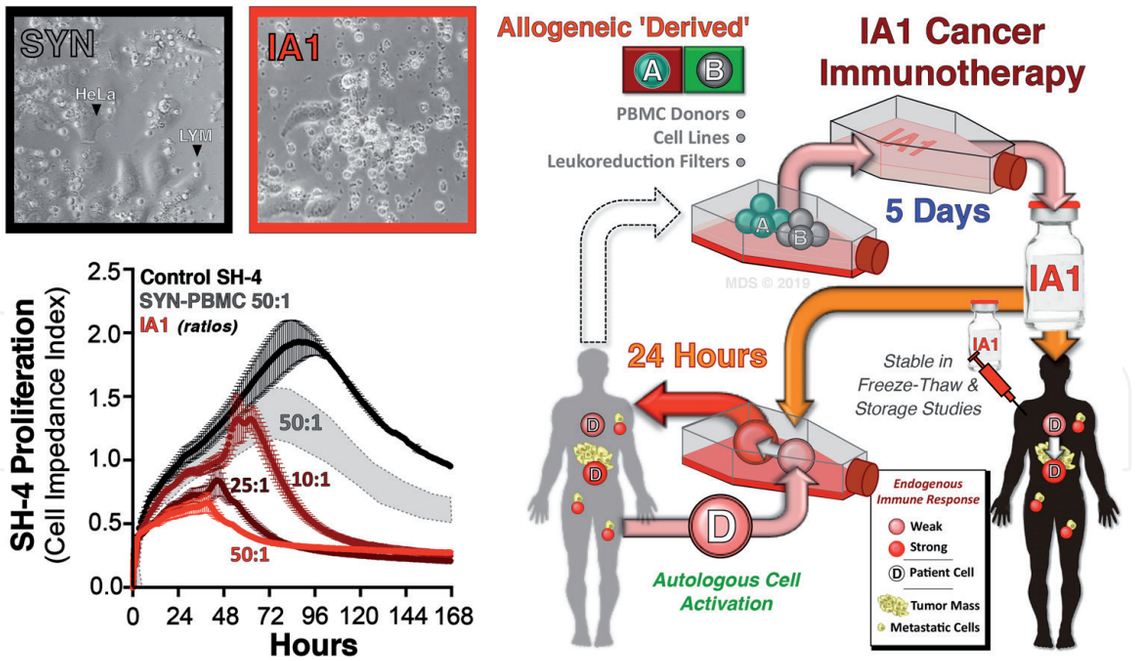

Figure 6.
*Schematic presentation of use and efficacy of the IA1 secretome therapeutic. Left panels: the enhanced efficacy of treated PBMC is supported by photomicrographs of allogenic PBMC responding to HeLa cells. As shown, after 72 hours incubation, resting (weak responders; left) PBMC show limited interaction when overlaid on HeLa cells. In contrast, the same PBMC, when treated for 24 hours with IA1, show a robust enhanced interaction (right) with the HeLa cell monolayer. Moreover, when IA1-treated PBMC are overlaid on SH-4 melanoma cells a greatly enhanced anti-cancer effect is noted relative to untreated PBMC. Shown are the growth profiles (as measured by electrical impedance) of SH-4 treated with either the SYN (derived from the secretome of resting PBMC) or IA1therapeutics. PBMC:SH-4 ratios included 50:1, 25:1 and 10:1. Right panels: bioreactor production of IA1 secretome is readily accomplished using an allogeneic MLR. Potential source materials include PBMC donors (A and B), autologous cells (dotted arrow), lymphocytic cell lines, or leukoreduction filters from blood collection bags. The secretome is collected at day 5 for processing into IA1 (**Figure 4**). IA1 is stable for months when aliquoted and frozen. Weak to absent immune response to both the primary tumor and metastatic sites allows for cancer progression. PBMC (D) from the patient can be treated* ex vivo *for 24 hours with IA1 and then reinfused into the individual where they show enhanced recognition and killing of the primary tumor and, potentially, improved immune surveillance at metastatic sites. Derived from Ref. [43].*

of not only the primary tumor but metastatic sites as well could be achieved thus improving long-term survival. Of note, similarly to our use of the tolerogenic TA1 in NOD mice (**Figures 4** and 5), IA1 could be directly injected into the recipient yielding a systemic proinflammatory reset of the immune system [40].

7. Conclusions

The immunomodulation of the endogenous immune system has become a major focus in treating a broad range of clinical conditions ranging from tissue/organ engraftment, autoimmune disease and cancer therapy. While significant clinical advancements have been made in immunotherapy, substantial challenges remain. One target of interest is the biologic/clinical desire to induce a persistent systemic immunological reset that could reduce both the need for chronic therapy and reduce the potential toxicities associated with current immunomodulatory approaches. Recent studies have demonstrated that miRNA are key regulators of cellular processes involved in both tolerogenic and proinflammatory immune responses and mediate immune cell proliferation and differentiation. Using an alloresponse bioreactor secretome system we have demonstrated that miRNA-based therapeutics can be reproducibly manufactured that can systemically reorient the immune system to either a tolerogenic or proinflammatory state by simultaneously

modulating both regulatory and effector T cell subsets thus skewing the Treg:Teff cell ratio to favor tolerance or inflammation. The tolerogenic TA1 therapeutic is derived from polymer-mediated immunocamouflage of the alloresponse reaction while the inflammatory IA1 preparation is derived from the alloresponse itself. The secretomes from these reactions are processed to maintain the miRNA within the secretome. In contrast to most miRNA therapeutic tactics, our approach has been to mimic the "complex pattern of miRNA expression" seen in protolerogenic or proinflammatory states. This "complex" approach was predicated by the inherent nature of miRNA bioregulation in that there is a low probability that altered expression of a single, or even a few, miRNA would exert a potent and definitive biological response. As shown, this approach successfully results in significant and, in mice, systemic and persistent changes to the immune system. The tolerogenic TA1 proved useful in reducing the onset and incidence of autoimmune diabetes in the NOD mouse while the proinflammatory IA1 therapeutic greatly enhanced the efficacy of human T cells to recognize and kill cancer cells without inducing the systemic inflammatory response seen with mitogens or monoclonal antibody (e.g., anti-CD3/CD28) therapies. Moreover, this approach can simultaneously modulate both regulatory and effect T cell subtype. The successful development of this miRNA-immunomodulatory approach may prove useful in facilitating organ engraftment, treating autoimmune disease and enhancing the endogenous anticancer 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, DW and WMT are inventors on these patents. XY has no conflicts of interest beyond being paid by Canadian Blood Services.

Author details

Mark D. Scott[1,2,3*], Duncheng Wang[4], Wendy M. Toyofuku[1,2] and Xining Yang[1,2,3]

1 Centre for Innovation, Canadian Blood Services, University of British Columbia, Vancouver, BC, Canada

2 Centre for Blood Research, University of British Columbia, Vancouver, BC, Canada

3 Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada

4 MD Anderson Cancer Center, Houston, Texas, USA

*Address all correspondence to: mdscott@mail.ubc.ca

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