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

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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 ΔLog_2 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 insulinitis 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., $\text{INF-}\gamma^+$, 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 $\text{INF-}\gamma^+$, 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^+ , $\text{TGF-}\beta^+$; **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 $\pm$ SD of 12 saline and 6 TA1 treated NOD mice. Nondiabetic results are the mean $\pm$ 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 play 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 cancer 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

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.

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References

- [1] Kaufmann SHE. Immunology's coming of age. *Frontiers in Immunology*. 2019;**10**:684. DOI: 10.3389/fimmu.2019.00684
- [2] Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, et al. In vitro generation of interleukin 10-producing regulatory CD4⁽⁺⁾ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *The Journal of Experimental Medicine*. 2002;**195**:603-616. DOI: 10.1084/jem.20011629
- [3] Crow AR, Lazarus AH. The mechanisms of action of intravenous immunoglobulin and polyclonal anti-D immunoglobulin in the amelioration of immune thrombocytopenic purpura: What do we really know? *Transfusion Medicine Reviews*. 2008;**22**:103-116. DOI: 10.1016/j.tmr.2007.12.001
- [4] Imbach P, Lazarus AH, Kuhne T. Intravenous immunoglobulins induce potentially synergistic immunomodulations in autoimmune disorders. *Vox Sanguinis*. 2010;**98**:385-394. DOI: 10.1111/j.1423-0410.2009.01264.x
- [5] Lazarus AH. Adoptive-transfer effects of intravenous immunoglobulin in autoimmunity. *Journal of Clinical Immunology*. 2010;**30**(Suppl 1):S20-S23. DOI: 10.1007/s10875-010-9410-9
- [6] Anthony RM, Kobayashi T, Wermeling F, Ravetch JV. Intravenous gammaglobulin suppresses inflammation through a novel T(H)2 pathway. *Nature*. 2011;**475**:110-113. DOI: 10.1038/nature10134
- [7] Blazar BR, Jenkins MK, Taylor PA, White J, Panoskaltsis-Mortari A, Korngold R, et al. Anti-CD3 epsilon F(ab')₂ fragments inhibit T cell expansion in vivo during graft-versus-host disease or the primary immune response to nominal antigen. *The Journal of Immunology*. 1997;**159**:5821-5833
- [8] Delmonico FL, Cosimi AB. Monoclonal antibody treatment of human allograft recipients. *Surgery, Gynecology & Obstetrics*. 1988;**166**:89-98
- [9] Blazar BR, Korngold R, Vallera DA. Recent advances in graft-versus-host disease (GVHD) prevention. *Immunological Reviews*. 1997;**157**:79-109. DOI: 10.1111/j.1600-065x.1997.tb00976.x
- [10] Toussiot E, Wendling D. The use of TNF-alpha blocking agents in rheumatoid arthritis: An overview. *Expert Opinion on Pharmacotherapy*. 2004;**5**:581-594. DOI: 10.1517/eoph.5.3.581.27357
- [11] Nam JL, Ramiro S, Gaujoux-Viala C, Takase K, Leon-Garcia M, Emery P, et al. Efficacy of biological disease-modifying antirheumatic drugs: A systematic literature review informing the 2013 update of the EULAR recommendations for the management of rheumatoid arthritis. *Annals of the Rheumatic Diseases*. 2014;**73**:516-528. DOI: 10.1136/annrheumdis-2013-204575
- [12] Ramiro S, Gaujoux-Viala C, Nam JL, Smolen JS, Buch M, Gossec L, et al. Safety of synthetic and biological DMARDs: A systematic literature review informing the 2013 update of the EULAR recommendations for management of rheumatoid arthritis. *Annals of the Rheumatic Diseases*. 2014;**73**:529-535. DOI: 10.1136/annrheumdis-2013-204575
- [13] Arora A, Mahajan A, Spurden D, Boyd H, Porter D. Long-term drug survival of TNF inhibitor therapy in RA patients: A systematic

review of European National Drug Registers. *International Journal of Rheumatology*. 2013;**2013**:764518. DOI: 10.1155/2013/764518

[14] Berard RA, Laxer RM. Etanercept (Enbrel) in the treatment of juvenile idiopathic arthritis. *Expert Opinion on Biological Therapy*. 2013;**13**:1623-1630. DOI: 10.1517/14712598.2013.840580

[15] Morgan CL, Emery P, Porter D, Reynolds A, Young A, Boyd H, et al. Treatment of rheumatoid arthritis with etanercept with reference to disease-modifying anti-rheumatic drugs: Long-term safety and survival using prospective, observational data. *Rheumatology (Oxford)*. 2014;**53**:186-194. DOI: 10.1093/rheumatology/ket333

[16] Blumenauer B, Judd M, Cranney A, Burls A, Coyle D, Hochberg M, et al. Etanercept for the treatment of rheumatoid arthritis. *Cochrane Database of Systematic Reviews*. 2003;CD004525. DOI: 10.1002/14651858.cd004525.pub2

[17] Chong BF, Wong HK. Immunobiologics in the treatment of psoriasis. *Clinical Immunology*. 2007;**123**:129-138. DOI: 10.1016/j.clim.2007.01.006

[18] Langley RG, Strober BE, Gu Y, Rozzo SJ, Okun MM. Benefit-risk assessment of tumour necrosis factor antagonists in the treatment of psoriasis. *The British Journal of Dermatology*. 2010;**162**:1349-1358. DOI: 10.1111/j.1365-2133.2010.09707.x

[19] Romero-Mate A, Garcia-Donoso C, Cordoba-Guijarro S. Efficacy and safety of etanercept in psoriasis/psoriatic arthritis: An updated review. *American Journal of Clinical Dermatology*. 2007;**8**:143-155. DOI: 10.2165/00128071-200708030-00002

[20] Sanchez Carazo JL, Mahiques Santos L, Oliver Martinez V. Safety of

etanercept in psoriasis: A critical review. *Drug Safety*. 2006;**29**:675-685. DOI: 10.2165/00002018-200629080-00004

[21] Inoue Y, Kaifu T, Sugahara-Tobinai A, Nakamura A, Miyazaki J, Takai T. Activating Fc gamma receptors participate in the development of autoimmune diabetes in NOD mice. *Journal of Immunology*. 2007;**179**:764-774. DOI: 10.4049/jimmunol.179.2.764

[22] Shoda LK, Young DL, Ramanujan S, Whiting CC, Atkinson MA, Bluestone JA, et al. A comprehensive review of interventions in the NOD mouse and implications for translation. *Immunity*. 2005;**23**:115-126. DOI: 10.1016/j.immuni.2005.08.002

[23] Bachanova V, Miller JS. NK cells in therapy of cancer. *Critical Reviews in Oncogenesis*. 2014;**19**:133-141. DOI: 10.1615/critrevoncog.2014011091

[24] Forget MA, Malu S, Liu H, Toth C, Maiti S, Kale C, et al. Activation and propagation of tumor-infiltrating lymphocytes on clinical-grade designer artificial antigen-presenting cells for adoptive immunotherapy of melanoma. *Journal of Immunotherapy*. 2014;**37**:448-460. DOI: 10.1097/cji.0000000000000056

[25] Liu S, Lizée G, Lou Y, Liu C, Overwijk WW, Wang G, et al. IL-21 synergizes with IL-7 to augment expansion and anti-tumor function of cytotoxic T cells. *International Immunology*. 2007;**19**:1213-1221. DOI: 10.1093/intimm/dxm093

[26] Miller JS. Therapeutic applications: Natural killer cells in the clinic. *Hematology. American Society of Hematology. Education Program*. 2013;**2013**:247-253. DOI: 10.1182/asheducation-2013.1.247

[27] Peng BG, He Q, Liang LI, Xie BH, Hua YP, Chen ZB, et al. Induction of cytotoxic T-lymphocyte responses

- using dendritic cells transfected with hepatocellular carcinoma mRNA. *British Journal of Biomedical Science*. 2006;**63**:123-128. DOI: 10.1080/09674845.2006.11732731
- [28] Sangiolo D. Cytokine induced killer cells as promising immunotherapy for solid tumors. *Journal of Cancer*. 2011;**2**:363-368. DOI: 10.7150/jca.2.363
- [29] Savage P, Millrain M, Dimakou S, Stebbing J, Dyson J. Expansion of CD8⁺ cytotoxic T cells *in vitro* and *in vivo* using MHC class I tetramers. *Tumour Biology*. 2007;**28**:70-76. DOI: 10.1159/000099152
- [30] Symes JC, Siatskas C, Fowler DH, Medin JA. Retrovirally transduced murine T lymphocytes expressing FasL mediate effective killing of prostate cancer cells. *Cancer Gene Therapy*. 2009;**16**:439-452. DOI: 10.1038/cgt.2008.96
- [31] Wu JY, Ernstoff MS, Hill JM, Cole B, Meehan KR. Ex vivo expansion of non-MHC-restricted cytotoxic effector cells as adoptive immunotherapy for myeloma. *Cytotherapy*. 2006;**8**:141-148. DOI: 10.1080/14653240600620218
- [32] Murad KL, Gosselin EJ, Eaton JW, Scott MD. Stealth cells: Prevention of major histocompatibility complex class II-mediated T-cell activation by cell surface modification. *Blood*. 1999;**94**:2135-2141
- [33] Chen AM, Scott MD. Current and future applications of immunological attenuation via pegylation of cells and tissue. *BioDrugs*. 2001;**15**:833-847. DOI: 10.2165/00063030-200115120-00005
- [34] Chen AM, Scott MD. Immunocamouflage: Prevention of transfusion-induced graft-versus-host disease via polymer grafting of donor cells. *Journal of Biomedical Materials Research. Part A*. 2003;**67**:626-636. DOI: 10.1002/jbm.a.10146
- [35] Chen AM, Scott MD. Comparative analysis of polymer and linker chemistries on the efficacy of immunocamouflage of murine leukocytes. *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology*. 2006;**34**:305-322. DOI: 10.1080/10731190600683845
- [36] Wang D, Toyofuku WM, Chen AM, Scott MD. Induction of immunotolerance via mPEG grafting to allogeneic leukocytes. *Biomaterials*. 2011;**32**:9494-9503. DOI: 10.1016/j.biomaterials.2011.08.061
- [37] Wang D, Toyofuku WM, Scott MD. The potential utility of methoxypoly(ethylene Glycol)-mediated prevention of rhesus blood group antigen RhD recognition in transfusion medicine. *Biomaterials*. 2012;**33**:3002-3012. DOI: 10.1016/j.biomaterials.2011.12.041
- [38] Wang D, Toyofuku WM, Kylaik DL, Scott MD. Use of flow cytometry in the in vitro and in vivo analysis of tolerance/anergy induction by immunocamouflage. In: Schmid I, editor. *Flow Cytometry-Recent Perspectives*. Croatia: InTech; 2012. pp. 133-150. DOI: 10.5772/37797
- [39] Kylaik-Price DL, Li L, Scott MD. Comparative efficacy of blood cell immunocamouflage by membrane grafting of methoxypoly(ethylene glycol) and polyethyloxazoline. *Biomaterials*. 2014;**35**:412-422. DOI: 10.1016/j.biomaterials.2013.09.016
- [40] Wang D, Shanina I, Toyofuku WM, Horwitz MS, Scott MD. Inhibition of autoimmune diabetes in NOD mice by miRNA therapy. *PLoS ONE*. 2015;**10**:e0145179. DOI: 10.1371/journal.pone.0145179
- [41] Kylaik-Price DL, Scott MD. Effects of methoxypoly (ethylene glycol) mediated immunocamouflage on leukocyte surface marker detection,

cell conjugation, activation and alloproliferation. *Biomaterials*. 2016;**74**:167-177. DOI: 10.1016/j.biomaterials.2015.09.047

[42] Kang N, Toyofuku WM, Yang X, Scott MD. Inhibition of allogeneic cytotoxic T cell (CD8⁺) proliferation via polymer-induced Treg (CD4⁺) cells. *Acta Biomaterialia*. 2017;**57**:146-155. DOI: doi.org/10.1016/j.actbio.2017.04.025

[43] Yang X, Kang N, Toyofuku WM, Scott MD. Enhancing the pro-inflammatory anti-cancer T cell response via biomanufactured, secretome-based, immunotherapeutics. *Immunobiology*. 2019;**224**:270-284. DOI: 10.1016/j.imbio.2018.12.003

[44] Wei B, Pei G. MicroRNAs: Critical regulators in Th17 cells and players in diseases. *Cellular & Molecular Immunology*. 2010;**7**:175-181. DOI: 10.1038/cmi.2010.19

[45] Guay C, Roggli E, Nesca V, Jacovetti C, Regazzi R. Diabetes mellitus, a microRNA-related disease? *Translational Research*. 2011;**157**: 253-264. DOI: 10.1016/j.trsl.2011.01.009

[46] Nielsen LB, Wang C, Sorensen K, Bang-Berthelsen CH, Hansen L, Andersen ML, et al. Circulating levels of microRNA from children with newly diagnosed type 1 diabetes and healthy controls: Evidence that miR-25 associates to residual beta-cell function and glycaemic control during disease progression. *Experimental Diabetes Research*. 2012;**2012**:896362. DOI: 10.1155/2012/896362

[47] Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005;**120**:15-20. DOI: 10.1016/j.cell.2004.12.035

[48] Chen K, Rajewsky N. The evolution of gene regulation by transcription

factors and microRNAs. *Nature Reviews. Genetics*. 2007;**8**:93-103. DOI: 10.1038/nrg1990

[49] Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research*. 2009;**19**:92-105. DOI: 10.1101/gr.082701.108

[50] Bartel DP. MicroRNAs: Target recognition and regulatory functions. *Cell*. 2009;**136**:215-233. DOI: 10.1016/j.cell.2009.01.002

[51] Bhardwaj A, Singh S, Singh AP. MicroRNA-based cancer therapeutics: Big hope from small RNAs. *Molecular and Cellular Pharmacology*. 2010;**2**:213-219. DOI: 10.4255/mcpharmacol.10.27

[52] Braicu C, Calin GA, Berindan-Neagoe I. MicroRNAs and cancer therapy-from bystanders to major players. *Current Medicinal Chemistry*. 2013;**20**:3561-3573. DOI: 10.2174/0929867311320290002

[53] Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science*. 2005;**309**:1577-1581. DOI: 10.1126/science.1113329

[54] Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, et al. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nature Medicine*. 2011;**17**:211-215. DOI: 10.1038/nm.2284

[55] Roep BO. The role of T-cells in the pathogenesis of Type 1 diabetes: From cause to cure. *Diabetologia*. 2003;**46**:305-321. DOI: 10.1007/s00125-003-1089-5

[56] Anderson MS, Bluestone JA. The NOD mouse: A model of immune dysregulation. *Annual Review of*

- Immunology. 2005;**23**:447-485. DOI: 10.1146/annurev.immunol.23.021704.115643
- [57] Richer MJ, Lavallee DJ, Shanina I, Horwitz MS. Immunomodulation of antigen presenting cells promotes natural regulatory T cells that prevent autoimmune diabetes in NOD mice. PLoS ONE. 2012;**7**:e31153. DOI: 10.1371/journal.pone.0031153
- [58] Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4⁺CD25⁺ immunoregulatory T cells that control autoimmune diabetes. Immunity. 2000;**12**:431-440. DOI: 10.1016/s1074-7613(00)80195-8
- [59] Gregori S, Giarratana N, Smioldo S, Adorini L. Dynamics of pathogenic and suppressor T cells in autoimmune diabetes development. Journal of Immunology. 2003;**171**:4040-4047. DOI: 10.4049/jimmunol.171.8.4040
- [60] You S, Belghith M, Cobbold S, Alyanakian MA, Gouarin C, Barriot S, et al. Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells. Diabetes. 2005;**54**:1415-1422. DOI: 10.2337/diabetes.54.5.1415
- [61] Tritt M, Sgouroudis E, d’Hennezel E, Albanese A, Piccirillo CA. Functional waning of naturally occurring CD4⁺ regulatory T-cells contributes to the onset of autoimmune diabetes. Diabetes. 2008;**57**:113-123. DOI: 10.2337/db06-1700
- [62] Richer MJ, Straka N, Fang D, Shanina I, Horwitz MS. Regulatory T-cells protect from type 1 diabetes after induction by coxsackievirus infection in the context of transforming growth factor-beta. Diabetes. 2008;**57**:1302-1311. DOI: 10.2337/db07-1460
- [63] D’Alise AM, Auyeung V, Feuerer M, Nishio J, Fontenot J, Benoist C, et al. The defect in T-cell regulation in NOD mice is an effect on the T-cell effectors. Proceedings of the National Academy of Sciences of the United States of America. 2008;**105**:19857-19862. DOI: 10.1073/pnas.0810713105
- [64] Feuerer M, Shen Y, Littman DR, Benoist C, Mathis D. How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets. Immunity. 2009;**31**:654-664. DOI: 10.1016/j.immuni.2009.08.023
- [65] Nishio J, Feuerer M, Wong J, Mathis D, Benoist C. Anti-CD3 therapy permits regulatory T cells to surmount T cell receptor-specified peripheral niche constraints. The Journal of Experimental Medicine. 2010;**207**:1879-1889. DOI: 10.1084/jem.20100205
- [66] Thayer TC, Wilson SB, Mathews CE. Use of nonobese diabetic mice to understand human type 1 diabetes. Endocrinology and Metabolism Clinics of North America. 2010;**39**:541-561. DOI: 10.1016/j.ecl.2010.05.001
- [67] Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4⁽⁺⁾CD25⁽⁺⁾ T-cells from patients with type 1 diabetes. Diabetes. 2005;**54**:92-99. DOI: 10.2337/diabetes.54.1.92
- [68] Cantrell DA. T-cell antigen receptor signal transduction. Immunology. 2002;**105**:369-374. DOI: 10.1046/j.1365-2567.2002.01391.x
- [69] Larsson EL, Coutinho A. The role of mitogenic lectins in T-cell triggering. Nature. 1979;**280**:239-241. DOI: 10.1038/280239a0
- [70] Swingler S, Mann A, jacque JM, Brichacek B, Sassaville VG, Williams K, et al. HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. Nature Medicine. 1999;**5**:997-1003. DOI: 10.1038/12433

[71] Trickett A, Kwan YL. T cell stimulation and expansion using anti-CD3/CD28 beads. *Journal of Immunological Methods*. 2003;**275**:251-255. DOI: 10.1016/S0022-1759(03)00010-3

[72] Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *The New England Journal of Medicine*. 2006;**355**:1018-1028. DOI: 10.1056/nejmoa063842

[73] Han T, Takita H. Immunologic impairment in bronchogenic carcinoma: A study of lymphocyte response to phytohemagglutinin. *Cancer*. 1972;**30**:616-620. DOI: 10.1002/1097-0142(197209)30:3%3C616::aid-cnrcr2820300304%3E3.0.co;2-q

[74] Maciel RM, Miki SS, Nicolau W, Mendes NF. Peripheral blood T and B lymphocytes, *in vitro* stimulation with phytohemagglutinin, and sensitization with 2,4-dinitrochlorobenzene in Grave's disease. *The Journal of Clinical Endocrinology and Metabolism*. 1976;**42**:583-587. DOI: 10.1210/jcem-42-3-583

[75] Nisbet NW, Simonsen M, Zaleski M. The frequency of antigen-sensitive cells in tissue transplantation. A commentary on clonal selection. *The Journal of Experimental Medicine*. 1969;**129**:459-467. DOI: 10.1084/jem.129.3.459