

# Differential Leukocyte miRNA Responses Following Pan T Cell, Allorecognition and Allosecretome-Based Therapeutics Activation

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## Research

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# Abstract

**Background:** Effective immunomodulation of T cell responses is critical in treating both autoimmune diseases and cancer. Our previous studies have demonstrated that nanoscale bioengineering of cell surfaces with methoxypolyethylene glycol (mPEG) induces a potent tolerogenic immunomodulatory effect. Moreover, secretomes derived from mPEG- or control mixed lymphocyte alloactivation assays also exerted potent immunomodulatory activity that was mediated by microRNAs (miRNA). In this study, the immunomodulatory effects of Pan T cell activators (PHA and anti-CD3/CD28), alloactivation (MHC-disparate donors;  $\pm$  mPEG grafting) and biomanufactured miRNA-based allo-secretome therapeutics (SYN, TA1, IA1 and IA2) were examined on T cell proliferation, subset differentiation and leukocyte miRNA expression profiles of resting human PBMC.

**Results:** In contrast to Pan T cell activation, allorecognition and the pro-inflammatory IA1 secretome product induced increasingly controlled proliferation of resting PBMC. The differential effects of the activation strategies were also apparent in T cell differentiation and the Teff:Treg ratio and in the miRNA expression profiles noted in the treated PBMC. In contrast, the mPEG-PBMC and TA1 secretome products inhibited alloproliferation. Importantly, the activation strategies exerted significantly different miRNA expression in the treated leukocytes that was associated with differences in proliferation and cellular differentiation.

**Conclusions:** Immunomodulatory secretome-derived, miRNA-enriched, therapeutics can be reproducibly biomanufactured that will induce the specific bioregulatory events necessary to induce the differentiation of naïve T cells to produce a tolerogenic (TA1) or inflammatory (IA1) response both *in vitro* and *in vivo*. The successful development and biomanufacturing of immunomodulatory, miRNA-enriched, secretome biotherapeutics may provide potent tools for the systemic treatment of autoimmune diseases or enhancing the endogenous immune response to cancer while reducing the potential adverse risks of more non-specific immunomodulatory approaches.

## Background

Effective modulation of the T cell immune response is critical in treating both autoimmune (*e.g.*, multiple sclerosis, arthritis, type I diabetes) and immunodeficient (*e.g.*, cancer) diseases. (1–4) Treatment of autoimmune disease have commonly focused on cytotoxic agents (*e.g.*, cyclosporine) to remove self-reactive immune cells and more recently, cytokine absorptive approaches (*e.g.*, etanercept). (5–8) These ‘anti-inflammatory’ approaches have been highly successful in preventing graft rejection and in modulating disease severity in a number of autoimmune diseases. In contrast, in diseases characterized by a weak immune response (*e.g.*, cancer), enhancement of the inflammatory response have attempted to use mitogens (*e.g.*, phytohemagglutinin; PHA), monoclonal antibodies (mAb; *e.g.*, anti-CD3/CD28), cytokines (*e.g.*, IL-2) and, less commonly, induction of an alloresponse (*e.g.*, Coley’s toxins and Graft versus Leukemia effects) to strengthen the immune response. (9–18) However, these pro-inflammatory

approaches have been much less successful due to overly robust responses resulting in adverse events such as cell injury and cytokine release syndrome.

In contrast to these traditional approaches, bioengineering of cell surfaces have also been shown to induce an immunomodulatory effect. The immunocamouflage of the lymphocyte membrane by the covalent grafting of biocompatible polymers (*e.g.*, methoxypolyethylene glycol; mPEG) has been demonstrated to induce a pro-tolerogenic environment both *in vitro* and *in vivo*. (19–24) Surprisingly, these studies also found that the secretome from the control and mPEG allorecognition-based mixed lymphocyte reactions (MLR) also exerted potent immunomodulatory activity that was mediated by microRNAs (miRNA). (3, 4, 24, 25)

Mammalian miRNA are short [~22-nucleotide long] RNA molecules that regulate messenger RNA (mRNA) expression at a posttranscriptional level. Currently, more than 2,000 miRNA have been identified in humans. (26) Since their discovery in 1993 in the nematode *C. elegans*, the role of miRNA has transitioned from being '*junk nucleic acids*' to being recognized as key regulators of a multitude of biological processes including the immune response. (27, 28) However, to date, the vast majority of research into the role of miRNA in immune response has been largely observational, with specific miRNA being used as biomarkers of immunological and/or pathogenic disease states. (29, 30) Indeed, the profiles of cellular miRNA expression can actively reflect the systemic alterations in immune activity. (31–33) More recently, due to their rapid response and sensitivity to changing cellular environment, miRNA have also been used as potential biomarkers for drug efficacy prediction and therapeutic approaches. (29)

However, despite their vast potential as biomarkers, relatively little research has been done on miRNA as therapeutic agents largely due to the complexity of miRNA-based bioregulation. (34, 35) It is important to note that miRNA are 'low fidelity' in nature in that a single miRNA can interact with potentially hundreds of genes and a single gene can be regulated by hundreds of miRNA. (36, 37) Hence, replicating the 'pattern of miRNA expression' is key to exerting a desired bioregulatory effect. To reproduce the miRNA patterns necessary to induce either a pro-inflammatory or tolerogenic immune response, our laboratory has utilized an alloresponse-based biomanufacturing approach. In this strategy, alloreaction-derived cell-free secretomes are manufactured from resting PBMC or control- or mPEG-MLR. (3, 4, 24, 25) As previously demonstrated, the miRNA-based secretomes systemically re-orientate the immune system towards either a pro-inflammatory (IA1) or pro-tolerogenic (TA1) state. (3, 4, 25) Importantly, miRNA prepared from resting immune cells [human peripheral blood mononuclear cells (PBMC) or murine splenocytes] exhibited minimal immunomodulatory activity. (4, 25)

In this study, we examined the differential effects of our previously described miRNA-based secretome therapeutics to pan T cell (PHA; anti-CD3/CD28) and allorecognition-based activation of resting human PBMC. Cell proliferation and T cell subset differentiation were determined. Moreover, cross-species efficacy of the human-derived, but evolutionarily conserved, secretome-products on murine splenocytes was examined. Per the observation of Hayes et al, (29) we examined the 'immediate' effects (72 hours) of

the PBMC activators on intracellular miRNA expression to assess similarities and differences between the modes of activation. Intracellular miRNA expression was examined via clustergram, log<sub>2</sub> fold change analyses and volcano plots. To assess the anti-cancer effects of IA1 and IA2, *in vitro* HeLa cell proliferation and PBMC-HeLa cell interactions were examined. As will be demonstrated, highly distinct differences between the pan T cell activators, allorecognition and secretome therapeutics on intracellular miRNA expression were observed. Indeed, these studies suggest that pan T cell activators such as PHA and anti-CD3/CD28 do not model normal allorecognition-based stimulation, and a reliance of these agents may lead to artifactual conclusion regarding immune responses and could underlie bystander toxicities. In contrast, the secretome miRNA therapeutics (TA1 and IA1) are more 'biologically' relevant to allorecognition pathways. IA2 specifically involves in cell apoptosis pathways and exhibits direct cancer-killing efficacy.

## Results

miRNA-based secretome therapeutics (SYN, TA1, IA1 and IA2) have been successfully produced in our laboratory based on allorecognition ( $\pm$  polymer-mediated immunocamouflage) based MLR. (3, 4, 25) In order to compare the effects of our acellular secretome products to mitogen or monoclonal antibody mediated pan T cell activation and alloactivation, cell proliferation and CD4<sup>+</sup>/CD8<sup>+</sup> subset composition among the CD3<sup>+</sup> population were measured (**Figure 2**). As shown, resting PBMC demonstrated minimal proliferation ( $1.5 \pm 0.4\%$ ) and a CD4:CD8 ratio of  $1.7 \pm 0.1$ . In contrast, the pan T cell activators anti-CD3/CD28 and PHA induced massive CD3<sup>+</sup> cell proliferation ( $78.1 \pm 1.8\%$  and  $94.4 \pm 0.3\%$ , at days 3 and 4, respectively,  $p < 0.0001$ ) and altered the CD4:CD8 ratio (**Figure 2A**). Within the proliferating CD3<sup>+</sup> T cells, a significant increase (1.8% to 6-7%) in CD4<sup>+</sup>CD8<sup>+</sup> cells were noted. These poorly studied cells have been speculated to play a role in autoimmune and chronic inflammatory disorders.(38) Despite both PHA and anti-CD3/CD28 being Pan T cell activators, there were differences in how these agents modulated the CD4/CD8 differentiation. PHA, but not anti-CD3/CD28, significantly increased the CD8<sup>+</sup> population while simultaneously decreasing the CD4<sup>+</sup> population resulting in a significant ( $p < 0.0001$ ) decrease of CD4:CD8 ratio relative to resting PBMC (1.7 to 0.9). mAb activation also decreased the ratio but not as dramatically as PHA. Alloactivation, in comparison to the highly potent pan T cell activation, induced a more moderate proliferation of CD3<sup>+</sup> cells ( $30.9 \pm 3.4\%$  at Day 10;  $p < 0.0001$ ) relative to resting PBMC. The reduction in proliferation arose consequent to  $<10\%$  of T cells within a population typically being capable of allorecognition (**Figure 2B**). (39, 40) Nonetheless, alloactivation similarly increased the CD4<sup>+</sup>CD8<sup>+</sup> population and decreased CD4<sup>+</sup> cells leading to a significantly ( $p < 0.01$ ) decreased CD4:CD8 ratio.

The secretome products showed significant variability (at Day 10) in their effects when used to activate resting PBMC. As expected, resting PBMC treated with the SYN secretome were virtually identical to the resting PBMC with regards to both proliferation, subset analysis and CD4:CD8 ratio. Similarly, the tolerogenic TA1 preparation showed minimal proliferation and no substantive changes in the subset differentiation or the CD4:CD8 ratio. Of note, the small increase in CD3<sup>+</sup> proliferation ( $2.3 \pm 0.1\%$ ) supports earlier observations showing increased proliferation/differentiation of Treg (CD4<sup>+</sup>Foxp3<sup>+</sup>) cells in TA1-treated PBMC.(21) However, in contrast to the SYN and TA1 products, IA1 and IA2 showed significant variation from both the resting PBMC and from each other (**Figure 2C**). IA1, derived from a control MLR, significantly increased the total CD3<sup>+</sup> cell proliferation ( $12.2 \pm 1.2\%$ ,  $p < 0.001$ ) and decreased the relative abundance of CD4<sup>+</sup> cells ( $38.8 \pm 3.4\%$  versus  $56.0 \pm 1.5\%$  for resting PBMC). Consequent to this change, the IA1 CD4:CD8 ratio was significantly reduced ( $1.0 \pm 0.1$ ;  $p < 0.001$ ) suggestive of a pro-inflammatory state and similar to that noted with the pan T cell activators. Interestingly, the cancer cell (HeLa) stimulated biologic IA2 while inducing a similar level of CD3<sup>+</sup> cell proliferation ( $10.7 \pm 0.5\%$ ,  $p < 0.01$ ), showed dramatically different phenotype distribution (**Figure 2C**). In contrast to IA1 which reduced the CD4:CD8 ratio, IA2 significantly increased the ratio relative to both IA1 and the resting PBMC ( $2.1 \pm 0.4$ ,  $1.0 \pm 0.1$ , and  $1.7 \pm 0.1$ ; respectively) consequent to a decrease in CD8<sup>+</sup> cells and a dramatic increase ( $p < 0.05$ ) in CD4<sup>-</sup>CD8<sup>-</sup> cells. These double negative cells, while poorly studied, have been implicated in both inflammation and as regulatory cells.(41) Interestingly, our previous study suggested that the anti-HeLa effects of IA2 treated PBMC were different from that of IA1 treated PBMC. (4, 25)

To further investigate the effects of the secretome products, CD4<sup>+</sup> Th1, Th17, Treg and CD3<sup>+</sup>6B11<sup>+</sup> iNKT cell were examined 10 days post activation and compared to a human MLR (**Figure 3A**). As expected, the SYN product had minimal effect on the differentiation of any subset relative to the resting PBMC (grey bar; *a*) while the tolerogenic TA1 product increased Treg cells (**Figure 3A**). (21) In contrast, the TA1, IA1 and IA2 secretome products variably affected the immune cell subsets. Most notably, IA1 very significantly increased Th17 cells and, to a lesser extent, Th1 and iNKT cells. The IA1-mediated increase in Th17 cells was similar to that observed within an MLR. Further showing the biological disparity between the IA1 and IA2 products, IA2 exhibited less effect on Th17 and Th1 cells but a greater effect on iNKT cells. Consequent to the differential changes within the T cells, the Th17:Treg ratio was affected (**Figure 3B**). As shown the SYN product had no effect while TA1 slightly increased the ratio while IA1 significantly increased the ratio similar to that seen in an MLR; perhaps indicative of the preparation of IA1 from the MLR secretome. IA2 slightly, but not significantly, increase the ratio – perhaps due to the expansion of the CD4<sup>-</sup>CD8<sup>-</sup> population (**Figure 2C**). Due to the evolutionary conservation of miRNA, the cross-species efficacies of the human and murine sourced secretomes on murine splenocytes was examined relative to control splenocytes (grey bar; *a*) and a murine MLR (**Figure 3C-D**). As shown, human sourced SYN exerted no effect on the levels of Th17 and Treg cells relative to resting splenocytes. In contrast, both human and murine IA1 preferentially depressed Treg cells relative to Th17 cells (**Figure 3C**). This resulted in a significant increase in the Th17:Treg ratio for both human ( $p < 0.05$ ) and murine

( $p < 0.0001$ ) IA1 and similar to that seen in a murine MLR (**Figure 3D**). Hence, these data demonstrated that the different secretomes (SYN, TA1, IA1 and IA2) exerted differential effects on T cell subsets but that the evolutionarily conserved miRNA composition of the human and murine IA1 exhibited cross species efficacy.

To determine how these differential T cell activation strategies (i.e., Pan T cell, Allo and Secretome) affected resting human PBMC, intracellular miRNA expression was measured 72 hours post activation and the expression of 84 miRNA involved in immunopathology pathways were assessed profiled via clustergram,  $\log_2$  fold change and volcano plot analyses. As shown in **Figure 4A**, clustergram analysis demonstrated that PBMC activation by pan T cell activators (PHA and anti-CD3/CD28; **P**) resulted in significantly different miRNA expression profiles relative to resting (i.e., unactivated) PBMC with a general trend towards a complete inversion (i.e., green to red and red to green) of the miRNA signals. Alloactivation (**Allo**) also induced distinct miRNA expression profiles relative to resting PBMC, but to a lesser extent than pan T cell activators. Moreover, the Control MLR and mPEG-MLR, from which IA1 and TA1 are derived, showed similarities in the overall pattern of miRNA expression but some distinct differences in specific miRNA that results in dramatically different biological responses. (3, 4, 25) In comparison to pan T cell and allo activations, the secretome products (**S**) induced miRNA expression patterns that were very distinct from that of the Pan T cell activators. (4, 25) To further illustrate differences between pan T cell (**P**) and secretome (**S**) activation, the  $\log_2$  fold change of thirteen differentially expressed miRNA, as well as the relative patterns of expression, are shown (**Figure 4B**). These miRNA were selected from the clustergram analysis as well as previous studies and their putative/described functions are summarized in **Table 1**. (3, 4, 25) It is important to note however, that the 'putative' functions of the distinct miRNA can vary significantly depending on the model used and are, typically, best estimates of their function in the absence of examining the miRNA expression pattern as a whole. Based on the low fidelity of miRNA, perhaps the most informative approach, is the analysis of the differential effects that these agents had on the relative pattern of miRNA expression (**Figure 4C**). As noted, both PHA and anti-CD3/CD28 showed similarities in their patterns of expression and in the extent of proliferation induced (**Figure 2**;  $94.4 \pm 0.3$  and  $78.1 \pm 1.8\%$ , respectively). In contrast, the secretomes induced a more subtle change in miRNA expression. The IA1 secretome pattern of expression was the most similar to the pan-T cell activators – though it differed significantly in the magnitude of changes in miRNA expression and T cell proliferation ( $12.2 \pm 1.2\%$ ). In contrast, the tolerogenic TA1 secretome pattern varied significantly from IA1 and most closely resembled the miRNA expression of unactivated PBMC. IA2 similarly varied significantly from IA1 despite inducing a similar level of T cell proliferation ( $10.7 \pm 0.5\%$ ). Importantly, the differential patterns of expression of these miRNA was also associated with differential biological effects with TA1 inducing systemic tolerance in a murine model and IA1 enhancing PBMC-mediated inhibition of cancer cell growth and IA2 exhibiting direct toxicity (apoptosis) of cancer cells. (3, 4)

To further compare the miRNA expression profile of IA1 relative to the pan-T cell (anti-CD3/CD28) activation and the TA1 and IA2 secretomes, volcano plot analyses were conducted (**Figure 5**). Volcano

plot analyses visualizes the miRNA data based on log scale changes and allows for statistical comparison of the expression of discrete miRNA between samples – but largely misses out on the overall PATTERN of changes. As noted in **Figure 5A**, distinct differences are noted between IA1 and anti-CD3/CD28. IA1 significantly ( $p < 0.05$ ) upregulated the expression of miR-125b-5p and miR-451a relative to anti-CD3/CD28, while miR-18a-5p, miR-17-5p, miR-20a-5p and miR-135b-5p were downregulated. Similar to **Figure 4** multiple other miRNA were also differentially expressed between IA1 and anti-CD3/CD28 activation though they did not reach significance in the volcano plot analyses (though if compared to resting PBMC they are different). Interestingly, the miRNA expression profiles between IA1 and TA1 were not statistically significantly different (**Figure 5B**), though, as also seen in **Figure 4**, miR-298, miR-214-3p, miR-302a-3p and miR-206 were over-expressed in IA1 relative to TA1. Finally, the expression of miR-149-5p and miR-18b-5p were significantly ( $p < 0.05$ ) upregulated in PBMC treated with IA1 when compared to the same donor PBMC treated with IA2 (**Figure 5C**).

In sum, clustergram, log<sub>2</sub> fold change and volcano plot analyses demonstrate the differential activation strategies yielded dramatically different miRNA expression profiles that in turn resulted in significant differences in T cell activation and subset differentiation. In order to better understand these differences, an integrative Venn diagram analysis was done using all three sets of data (**Figure 6**) in order to differentially compare the pan T cell, allorecognition and secretome activation. As demonstrated, Pan T cell activation using PHA and anti-CD3/CD28 yielded similar, though not identical, changes in miRNA expression (solid circles = over expression; dashed circles = reduced expression; overlap are miRNA in common). For further comparison purposes, we averaged the miRNA expression profile and proliferation rates of PHA and anti-CD3/CD28 to represent the efficacy of pan T cell activation strategy. In contrast to Pan T cell activation, the miRNA changes induced by allorecognition were much more discrete (relative to resting PBMC) and highly limited when compared to the Pan T cell activators. Moreover, allorecognition resulted in a significant reduction in cell proliferation (Pan T: 86.3% versus 30.9% for Allorecognition). Similar to the allorecognition response, the allo-derived IA1 secretome also reduced the miRNA response pattern relative to Pan T cell activation and, not surprisingly, was similar to the pattern of expression observed in the alloresponse but with the increased expression of miR-298 and decreased expression of miR-206 and miR-214-3p. While some miRNA are in common to the Pan T cell, Allo, IA1 and IA2 pro-inflammatory responses (overlaps in Venn diagrams), some of these (*e.g.*, miR-155-5p) are also implicated in the tolerogenic TA1 and mPEG-Allo responses. This again argues that the '*pattern of miRNA expression*' (**Figure 4C**) encompassing increases, decreases and static levels of multiple, rather than a specific (single or small number), of miRNA is crucial.

Importantly, the biomanufacturing process is of importance. This is most obvious in comparing the MLR vs mPEG-MLR miRNA patterns, but it is also observed within the pro-inflammatory IA1 and IA2 secretome products (**Figure 7**). While IA1 and IA2 stimulate similar proliferative effects ( $12.2 \pm 1.2$  and  $10.7 \pm 0.5\%$ ; **Figure 2**), their biological activity was dramatically different. As shown in **Figure 7A**, control HeLa cells treated with either IA1 or IA2 secretome-activated PBMC showed similar inhibitory effects on HeLa cell proliferation over 7 days as evidenced by quantification of Area Under Curve (AUC). However,

the effect of IA2 was not predicated on the presence of PBMC. When the seeded HeLa cells were overlaid with the secretomes themselves (absent PBMC), IA2 exerted direct inhibitory effects on HeLa cell proliferation while SYN, TA1 and IA1 had no inhibitory effects (**Figure 7A**). Indeed, PBMC pre-treated with IA1 and IA2 induced entirely different miRNA expression (**Figure 7B**) that resulted in vastly different responses to the HeLa cells. As shown in **Figure 7C**, the IA1 treated PBMC resulted in a significantly enhanced cell:cell interaction between the PBMC and HeLa cells following 72 hours co-culture. In contrast, minimal cell:cell interaction of IA2-treated PBMC with the HeLa cells was noted at 72 hours, though the HeLa cells themselves showed evidence of apoptotic/necrotic blebbing suggestive of non-contact dependent killing. The same morphological picture was observed with the direct overlay of IA2 in the absence of PBMC. These findings agreed with our previous hypothesis that IA2 inhibited cancer cells proliferation via non-PBMC-mediated pathways and/or apoptotic mechanisms. (4)

In sum, these studies demonstrate that pan T cell activators, alloactivation and secretome therapeutics exerted differential efficacies on leukocyte proliferation/differentiation and miRNA expression. Pan T cell activators induced massive T cell proliferation and miRNA alteration profiles relative to resting cells. Allo-MLR demonstrated a more discriminatory activation of resting PBMC relative to pan T cell activators, while mPEG-MLR diminished allorecognition. Importantly, IA1 and TA1 secretome derived from allo- and mPEG-MLR respectively, exerted similar immunomodulatory efficacies to its origin MLR response. In contrast, resting cell generated secretome SYN had minimal effects on recipient resting PBMC. Of interest, The HeLa-MLR derived IA2 therapeutic exhibited distinct alterations to the leukocyte miRNA expression profile, suggesting an apoptosis-mediated immunomodulatory and anti-cancer pathway.

## Discussion

Systemic, and in some cases localized, immunomodulation of the immune response is proving to be a potent weapon in the treatment of both autoimmune disease and cancer. While multiple drugs (*e.g.*, cyclosporine, PHA) and biologics (*e.g.*, etanercept and anti-CD3) have been explored and, in some case used successfully in the clinic, these agents do not fundamentally reset the immune response. However, research from our laboratory has demonstrated that polymer-based bioengineering of immune cells can be used either directly or indirectly, via secretome miRNA, to systemically modify the immune response.(3, 4, 19–24, 42–44) Of potential therapeutic value, the bioreactor-based manufacturing of secretome therapeutics (*i.e.*, TA1 and IA1) have been explored more in depth and compared to existing Pan T cell activators (PHA and anti-CD3/anti-CD28) as well as the MHC-dependent alloresponse.

In this study we demonstrated that controlled and reproducible immunomodulation (cell proliferation and T cell subsets; **Figures 2-3**) could be accomplished using the TA1 and IA1 secretome biotherapeutics. Moreover, as shown in **Figure 3**, these findings demonstrated that the TA1 and IA1 therapeutics had inverse effects on the Teff:Treg ratio with the TA1 decreasing it (*i.e.*, enhancing the tolerogenic response) and IA1 increasing it (*i.e.*, enhancing the pro-inflammatory response). Mechanistically, the effects of the

Pan T cell, allorecognition and secretome-based activators on the cells was examined via proliferation, subset differentiation and miRNA-expression profiles in resting PBMC (**Figures 2-5**). In these studies, as well as our previous publications, PEGylated-PBMC as well as TA1, derived from an mPEG-MLR, was shown to induce a systemic and persistent tolerogenic state with an upregulation of Treg cells and the downregulation of multiple Teff subsets.(3, 19, 21–25) In contrast, the allorecognition-based IA1 increased Teff subsets and decreased Treg; importantly, however, IA1 induced a highly controlled inflammatory response when compared to either Pan T Cell activators or the alloresponse itself. This controlled modulation is important because a problem with other pro-inflammatory approaches has been an overly robust response leading to bystander cell injury and, in worst case scenarios, cytokine release syndrome.(3, 4, 19, 21–25) In aggregate, the results shown here and in our previous publications suggest that secretome-based therapeutics could be potent, but controllable, tools for immunomodulation.

Immune cell activation results in multiple changes within the cell itself and is ultimately defined by cell proliferation and differentiation. Since their discovery, miRNA have been increasingly found to be key bioregulatory messengers for both externally (*i.e.*, extracellular miRNA) effecting change as well as key intracellular effectors of gene activation and mRNA expression. Indeed, the importance of miRNA in immune cell proliferation and differentiation has been extensively studied.(31–33, 45–52) Moreover, the utility of miRNA in cancer diagnosis (*i.e.*, biomarkers) and treatment (siRNA) has gained increasing attention during the past decade.(29, 53–64)

As documented in this study, Pan T cell, allorecognition and secretome activation of resting PBMC induce dramatically different miRNA expression patterns within the treated PBMC (**Figures 4-6**) that lead to different proliferation and differentiation responses. Typically, due to the inherent reductionist nature of science, large increases or decreases of a single miRNA have been focused on as being responsible for the observed changes in proliferation and differentiation in both autoimmune diseases and in proinflammatory states. However, our understanding of miRNA-mediated bioregulation is still in its infancy. In light of the fact that a single miRNA can interact with hundreds of genes, and a single gene can interact with tens to hundreds of miRNA, this 'low fidelity' is suggestive that '***distinct patterns of miRNA expression***' rather than changes in a single miRNA are key to inducing complex (*e.g.*, tolerance or controlled inflammation) bioregulatory changes. (3, 4, 25, 46, 65) Indeed, examining the miRNA expression profiles (**Figures 4-6**) induced by Pan T cell activators (PHA or anti-CD3-CD28), allorecognition, or our secretome-derived TA1 and IA1 products shows significant variations in both cell proliferation and the miRNA expression profiles. Singling out a single miRNA is difficult to do. For example, miR-155-5p is a crucial component of both tolerogenic and pro-inflammatory responses, but other supporting miRNA differ (*e.g.*, **Figure 6**). However, if one appreciates the complexity of miRNA, one can begin to look at 'patterns of expression' as governing the bioregulatory response. Schematically this bioregulatory process is described in **Figure 8A** in which the unique pattern of miRNA expression observed in **Figure 4B** are presented and govern the biologic response in a '*lock and key*' manner. Hence, the '*pattern of expression*' of TA1 replicates the expression profile to induce a tolerogenic response (*i.e.*, increased Treg, decreased Teff; **Figure 8B**) while the IA1 expression profile cannot induce tolerance due to its '*pattern disparity*'. In contrast, IA1 effectively unlocks a controlled inflammatory response (**Figure 8C**) that is in stark contrast

to the Pan T cell or even alloresponse induced inflammation which are characterized by significantly more expansive miRNA expression profiles (**Figures 4-6**). Moreover, it is possible to also hypothesize that partial pattern homology could induce a partial response (**Figure 8D**); indeed, while TA1 is shown as the example, IA1 is a clear subset of the Pan T cell and Alloresponse miRNA response patterns (**Figure 6**). The complexity of this *'lock and key'* regulatory mechanism readily explains the often highly disparate functions assigned to a single miRNA in the literature (see **Tables 1** and **the additional file 1** table). Hence, despite the reductionist nature of science, understanding miRNA bioregulation may need to become more focused on the overall expression patterns including not just large changes (up/down), but more modest increases or decreases, and even miRNA that remain static responses during complex biological responses. By mimicking the *'pattern of miRNA expression'* it may be possible to produce secretome, or purified miRNA, products that can replicate the desired biological response. Indeed, the TA1 therapeutic induced a systemic *in vivo* tolerance resulting in disease attenuation in the NOD mouse model of Type 1 diabetes.(3) In contrast, treatment of resting PBMC with the IA1 therapeutic significantly enhanced their anti-cancer efficacy *in vitro*.(4) Of note, secretome-derived miRNA-based therapeutics could be used either independently or in conjunction with other cell-based therapies (*e.g.*, CAR T cells).

The use of secretomes, but not the derived complex pattern of miRNA in the TA1 and IA1 therapeutics, is not entirely new. Previous studies have examined the therapeutic use of exosome and demonstrated some clinical efficacy in this approach. (66–68) For example, Zhang *et al*/utilized mesenchymal stem cell exosomes to enhance cartilage repair in rats by enhancing chondrocyte proliferation, attenuating apoptosis and modulating the immune response.(69) However, these studies did not utilize a clearly defined biomanufacturing system differentially capable of generating tolerogenic and pro-inflammatory products nor had they deduced the important role of miRNA in mediating the immunomodulatory effects. Moreover, all of these approaches bear some historical resemblance to Coley's toxins (introduced in 1891) which were used to induce an inflammatory response that enhanced the anti-cancer response and were clinically used until the early 1960s.(70–73) Indeed, William Coley is often recognized as *"Father of Immunotherapy"*.(14, 70–73)

Finally, an important finding of this study is that Pan T cell activation bears little resemblance to allorecognition with regards to the miRNA induced, which, in turn, will govern the regulation and differentiation of resting T cells. While many publications have utilized pan T cell activators (*e.g.*, PHA or anti-CD3/anti-CD28) to model T cell-mediated pathologies (*e.g.*, allorecognition, transplant rejection, graft-versus-host disease (GvHD), autoimmunity and inflammation),(12, 74) our results clearly indicate that there are very significant differences in the miRNA response pattern of human PBMC at 72 hours (well into the proliferation and differentiation cycle) between the activation strategies (**Figure 6**). Indeed, pan T cell stimulators (~90% activation) induce massive miRNA alterations (**Figures 2-6**), that yield non-specific overactivation and significant bystander injuries. (74–76) In contrast, allorecognition generates a more discriminatory T cell response (proliferation/differentiation and miRNA expression; **Figures 2-6**) as only 1-10% of an individual's T lymphocytes are alloreactive. (39, 40) Despite the 'low' number of potentially reactive cells, the alloresponse is still quite potent as exemplified by the severity of GvHD. (77) Not surprisingly, the alloresponse has been studied in the context of cancer immunity for decades. (18,

40, 78–80) Although mitogens and anti-CD3/CD28 have been widely used to activate and expand immune cells *ex vivo* in cancer immunotherapies, our findings suggest that pan T cell activators do not model normal allorecognition-based stimulation and a reliance of these agents may lead to artifactual conclusion regarding immune responses, and could underlie the cytokine release syndrome commonly seen in overly robust T cell responses to ‘non-self’. (74–76)

## Conclusions

The biomanufacturing of immunomodulatory, miRNA-enriched, secretome biotherapeutics may provide potent tools for the systemic treatment of both autoimmune diseases (TA1) and cancer (IA1). This cost-effective approach yields a stable (freeze-thaw) product that can be used either *in vivo* or *in vitro*. (3, 4, 25) Moreover, due to the conserved nature of miRNA, cross species efficacy was noted in the IA1 and TA1 therapeutics (**Figure 3D**). Our findings also highlight the disparity between alloactivation and mitogen induced proliferation and the importance of the ‘*pattern of miRNA expression*’ at inducing inflammation or tolerance. It is proposed that the ‘*pattern of miRNA expression*’, in contrast to altered expression of one or even a few miRNA, is crucial for the induction of a desired immunological response. The successful development of secretome-derived, miRNA therapeutics that replicate specific bioregulatory events may prove useful in the treatment of autoimmune diseases or enhancing the endogenous immune response to cancer while reducing the potential adverse risks of more non-specific immunomodulatory approaches.

## Methods And Materials

*Human PBMC:* All human experiments were done in accordance with the University of British Columbia Clinical Research Ethics Board and the Code of Ethics of the World Medical Association (Declaration of Helsinki). The PBMC layer were isolated from donor whole blood using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) as described before. (3, 4, 21, 24) Human PBMC were washed and resuspended in AIM V media (research grade; ThermoFisher Scientific, Grand Island, NY).

*Murine Splenocytes:* All murine studies were done in accordance with the Canadian Council of Animal Care and the University of British Columbia Animal Care Committee guidelines and were conducted within the Centre for Disease Modeling at the University of British Columbia. Two MHC (*i.e.*, mouse H-2 haplotype complex) disparate mouse strains were used: C57BL/6, H-2b; and BALB/c, H-2d. Murine spleens were freshly harvested and homogenized into a cell suspension in phosphate buffered saline (PBS) as described before. (3, 21) Red blood cells were removed from the cell suspension using BD Pharm Lyse buffer (BD Pharmingen, San Diego, CA). Purified mouse splenocytes were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products, West

Sacramento, CA), 1% L-glutamine, 1% penicillin-streptomycin and 50  $\mu$ M  $\beta$ -mercaptoethanol (all from Invitrogen by Life Technologies, Carlsbad, CA).

*Cancer Cell Line:* Human epithelial HeLa cell line (CCL-2TM) was purchased from ATCC and cultured under 5% CO<sub>2</sub> in Dulbecco's modified eagle's medium (DMEM; high-glucose contains 4.5 g/L D-glucose, without L-glutamine or sodium pyruvate; Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2 mM GlutaMAX, 10 mM HEPES, 100 U penicillin and 100  $\mu$ g streptomycin (all from Invitrogen, Carlsbad, CA). HeLa cells were used at ~80% confluency.

*Differential Effects of Activation Strategies on Resting Leukocytes:* The effects of pan T cell activators [*i.e.*, anti-CD3/anti-CD28 and mitogen (phytohemagglutinin; PHA)], alloactivators (*i.e.*, control MLR and camouflaged MLR) and secretomes (*i.e.*, SYN, TA1, IA1 and IA2) on the activation of resting leukocytes were compared (**Figure 1**). In pan T cell activation, freshly isolated human PBMC were stimulated with plate-bound (24-well plate precoated with antibody) anti-human CD3e [5  $\mu$ g/ml; BD Pharmingen, San Diego, CA] in the presence of soluble anti-human CD28 (1  $\mu$ g/ml; BD Pharmingen) for 3 days, or with PHA (Sigma-Aldrich, St. Louis, MO) at an amount of 2  $\mu$ g per  $1 \times 10^6$  total cells for 4 days incubation (**Figure 1A**). (4) Alloactivation was conducted in an MLR system with or without succinimidyl valerate activated (SVA) mPEG (Laysan Bio Inc. Arab, AL) for 10 days (**Figure 1B**). In mPEG-MLR, one population of donor PBMC were grafted with 20 kDa mPEG at a 2mM concentration as described previously. (3, 24) Control two-way MLR were conducted by mixing two PBMC populations at a final cell density of  $2 \times 10^6$  cells/ml as described previously. (3, 21, 24) Effects of alloactivation were compared to untreated resting PBMC. To explore the immunomodulatory effects of alloactivation-secretome-derived therapeutics, cell-free TA1 and IA1 biologics were produced from mPEG-MLR and MLR respectively. Cell secretions from untreated resting PBMC were collected as SYN. (4) A lymphocyte-cancer cell (HeLa) biotherapeutic IA2 was concurrently developed from a HeLa-MLR as previously described. (4) All secretomes were collected at 5 days post plating, followed by centrifugation and ultrafiltration to remove cells and cell debris. In allo- and secretome activation studies, proliferation and phenotyping of treated PBMC were measured at day 10 (**Figure 1C**). For all activation strategies, PBMC miRNA expression profiles were measured at 72 hours post treatment.

*T Cell Proliferation and Phenotyping:* *In vitro* cell proliferation was assessed via flow cytometry using the CellTrace CFSE (carboxyfluorescein diacetate succinimidyl ester) Cell Proliferation Kit (Cat. No. C34554i; CellTrace, Molecular probes, Invitrogen, Eugene, OR) as described before. (3, 4, 21, 23, 24) The T cell lymphocyte subpopulations (CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>) were measured using fluorescently labeled anti-CD3, CD4 and CD8 monoclonal antibodies (mAb; BD Pharmingen, San Jose, CA). Human Th17 and Treg subsets were measured using the BD Th17/Treg Phenotyping Kit (Cat. No. 560762; BD Biosciences, San Jose, CA). Because of interrelationship between Th17 and Treg cells, the ratio (Th17:Treg) of the two populations serves as a surrogate for assessing the inflammatory state. (3, 4, 81–83) Other human inflammatory T cell subsets such as Th1 lymphocytes and type I or invariant NKT (iNKT) cells were stained as CD3<sup>+</sup>/CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> and CD3<sup>+</sup>/6B11<sup>+</sup> respectively. All samples were acquired using the

FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences, San Jose, CA) for both acquisition and analysis.

*Leukocyte miRNA Expression:* Total RNA was extracted from resting PBMC  $\pm$  treatment (anti-CD3/anti-CD28, PHA, MLR  $\pm$  mPEG, SYN, IA1, IA2 and TA1,) following 72 hours incubation using the mirVana™ PARIS™ kit (Ambion, Life Technologies, Grand Island, NY). Following processing, the highly enriched small RNA fraction containing miRNA was prepared using RNase/DNase free water. To partially characterize and quantify the relative abundance the miRNA species present in the resting and differentially activated PBMC, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was done using the miScript miRNA PCR Array system (Qiagen, Frederick, MD) for the human immunopathology pathway as described before. (3, 4) The data shown represent three biological replicates analyzed independently by qRT-PCR.

*Inhibition of Secretomes on HeLa Cell Proliferation:* *In vitro* cytotoxicity of activated PBMC and secretomes against HeLa cells was analyzed using the ACEA iCELLigence system as described previously. (4) All studies were done with an initial seeding density of 5,000 HeLa cell in DMEM medium with an acclimation time for initial adherence for 1 hour. PBMC activated (24 hours) with differential secretomes (SYN, TA1, IA2 and IA2) were overlaid on HeLa cells to achieve an PBMC:HeLa ratio of 50:1. Importantly, following 24 hours of pretreatment, the PBMC were extensively washed to remove the residual secretome, leaving only the activated leukocytes. Cells were incubated in the ACEA instrument maintained at 37° C in a humidified 5% CO<sub>2</sub> incubator for 7 days (168 hours). The immune cell-induced alterations in HeLa cell attachment were monitored via the current impedance index and presented in real-time HeLa cell proliferation curves and the Area Under Curve (AUC). Direct toxicity of the differential secretomes (added 1:1 into DMEM) was also assessed by measuring HeLa cells proliferation in the absence of PBMC.

*PBMC-HeLa Cell Conjugation Microscopy:* PBMC (activated with IA1 or IA2 for 24 hours) and HeLa cells (seeded at 5,000 total) were co-cultured in RPMI 1640 media, at a ratio of PBMC:HeLa = 50:1, in a heated (37° C) humidity chamber (Becton Dickinson, Franklin Lakes, NJ) for 72 hours. Photomicrographs were taken at 20X magnification using a Nikon Eclipse Ti microscope (Digital sight DS-U3) as described previously. (4)

*Statistical Analysis:* All data were expressed as mean  $\pm$  standard error mean (SEM). A minimum of three independent experiments were performed in duplicates for all studies. Data analysis was conducted using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA). For comparison of means, a one-way analysis of variance (ANOVA) was performed. When significant differences were found, a post-hoc Tukey test was conducted for pair-wise comparison of means. For significance, a minimum p value of < 0.05 (labeled with '\*') was used. The symbols '\*\*', '\*\*\*' and '\*\*\*\*' were used for labeling p value of < 0.01, < 0.001 and < 0.0001, respectively.

## Abbreviations

**miRNA:** microRNA; **PBMC:** Peripheral Blood Mononuclear Cells; **CD3<sup>+</sup>:** Pan T lymphocyte marker; **CD4<sup>+</sup>:** CD3<sup>+</sup> subset (CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>); **CD8<sup>+</sup>:** CD3<sup>+</sup> subset (CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup>); cytotoxic T cells; **Treg:** CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> T cell; promotes tolerance; **Teff:** CD3<sup>+</sup>CD4<sup>+</sup> T effector cell encompassing subsets such as Th17 and Th1; **iNKT:** invariant NKT cells heterogeneous group of T cells that CD3<sup>+</sup>6B11<sup>+</sup> and share properties of both T cells and natural killer cells; **mPEG:** methoxypolyethylene glycol; **PHA:** phytohemagglutinin; activates ~90% of T cells; **Anti-CD3/anti-CD28:** monoclonal antibodies directed against CD3 and ; activates ~80% of T cells; **CFSE:** carboxyfluorescein diacetate succinimidyl ester; used to measure proliferation; **AUC:** Area Under Curve; used to analyze cell proliferation/killing; **Control-MLR:** Mixed Lymphocyte Reaction (MLR); proliferation occurs due to allorecognition.; **mPEG-MLR:** MLR reaction in which one donor population has been modified via the covalent grafting of mPEG; **PEGylation:** covalent grafting of mPEG to cell membrane

**HeLa:** Epithelial cancer cell line; **SYN:** control secretome product derived from resting PBMC; **TA1:** Tolerance Agent 1; derived from mPEG-MLR; **IA1:** Inflammatory Agent 1; derived from a the control-MLR; **IA2:** Inflammatory Agent 2; derived from PBMC-HeLa cell co-culture

## Declarations

### Ethics approvals:

All human experiments (i.e., blood donations) were reviewed and approved by the University of British Columbia Clinical Research Ethics Board (H02-70215) and done in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All murine studies were done in accordance with the Canadian Council of Animal Care and approved by the University of British Columbia Animal Care Committee (A17-0220) and were conducted within the Centre for Disease Modeling at the University of British Columbia.

### Consent for publication

All authors have read and approved the paper. If the paper is accepted, all authors will observe the terms of the license to publish.

## Availability of data and materials

All data analyzed in this study are included within the included figures and tables, **additional file 1**, or are available from the authors upon reasonable request.

## Competing interests

MDS and WMT are inventors on multiple issued and pending patents related to the disclosed technology. These patents are assigned to their primary employer: Canadian Blood Services (Ottawa, ON, Canada). Canadian Blood Services is a not-for-profit organization whose primary mission since its organization in 1998 is to collect, manufacture and manage blood and blood products for all of Canada (except Quebec). Canadian Blood Services is funded solely by the federal, provincial and territorial governments of Canada.

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## Authors' contributions

MDS and XY were responsible for the design and analysis of the study. XY and WMT were responsible for the execution of the studies experiments. MDS, XY and WMT all participated in the writing of the manuscript.

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## Table 1

Table 1. Putative Functions of Key miRNA

hsa-miR-	Key Activator(s)	Putative Function(s)
155-5p	in Pan T, IA1, TA1	miR-155 expression inhibits malignant growth <i>in vivo</i> . (84) <i>see also</i> miR-147a.
298	in Pan T, IA1	Implicated (along with miR-296-3p) in increased resistance of mammalian pancreatic $\alpha$ cells to cytokine-induced apoptosis. (85)
135b-5p	in Pan T, IA1	Reported to be an oncogenic miRNA enhancing cancer cell invasive and metastasis <i>in vivo</i> . (86) Important in the regulation of inflammation. miR-135b expression in inflammation is regulated by IL-1R1 in a regulatory feedback mechanism to resolve inflammation. (87)
149-5p	in Pan T	Expression is inversely associated with inflammation and has regulatory effects on TNF- $\alpha$ , IL-1 $\beta$ and IL-6 and is involved in the regulation of apoptosis. (88, 89)
214-3p	in Pan T	Predicted to target a number of genes regulating apoptosis, immune cell proliferation and angiogenesis. Upregulation in Hela cells reduced cell growth. In contrast, elevated expression has also been shown to inhibit chemotherapy effectiveness. (90–92)
302a-3p	in PHA, IA1	Shown to be able to reprogram human cancer cells to human embryonic stem cell (hESC)-like pluripotent cells with a slow cell cycle rate and dormant cell-like morphology. (93, 94) Inhibits CDK2 and CDK4/6 cell cycle pathways. (95)
335-5p	~ in Pan T	Has been identified as a cancer suppressor and found to be downregulated in gastric cancer and involved in tumorigenesis. (96, 97) Overexpression of miR-335-5p promotes osteogenic differentiation (98) and bone formation and regeneration in mice. (99)
379-5p	~ in Pan T	Is reported to be a tumor suppressor involved in multiple cancers, especially in glioblastoma multiforme. (100) Reported to (with miR-374-5p and miR-503-5p) regulate proliferation and hypertrophic differentiation of growth plate chondrocytes in male rats. (101)
147a	IA1	Implicated along with miR-155 ( <i>see this table</i> ) and miR-21 ( <i>see additional file 1</i> ) in regulation of Toll-like receptors (TLRs). The dysregulation of these miRNAs may be involved in inflammatory diseases and cancers. (102)
9-5p	IA1	Induced by TLR4 activation as well as TLR2 and TLR7/8 agonists and by the proinflammatory cytokines (TNF- $\alpha$ and IL-1 $\beta$ , but not by IFN- $\gamma$ ). (103) However, other studies have shown that miR-9 expression is reduced in some cancers. (104)
206	~ in TA1 versus IA1	Inverse relationship between miR-206 expression and Th17 cells in an inflammatory disease (dermatomyositis). (105)
29b-3p	in IA2	miR-29 family has diverse roles in cancer. Can function as a tumor suppressor, attenuating cancer progression via promoting tumor cell apoptosis and by reducing cell proliferation. However, as a tumor promoter, miR-29 mediates epithelial-mesenchymal transition (EMT) and promotes metastasis in breast cancer and colon cancer. (106)
181a-5p	~ in IA2	Promotes proliferation and invasion and inhibits apoptosis of cervical cancer cells. (107) Expression is upregulated in gastric cancer and correlated with invasion and facilitates cell proliferation in acute lymphoblastic leukemia. (108, 109) In contrast, other studies report that it inhibits cancer cell proliferation, migration, invasion and angiogenesis via downregulation of matrix metalloproteinase-14 (MMP-14) and/or in targeting Kras. (110, 111).

# Figures

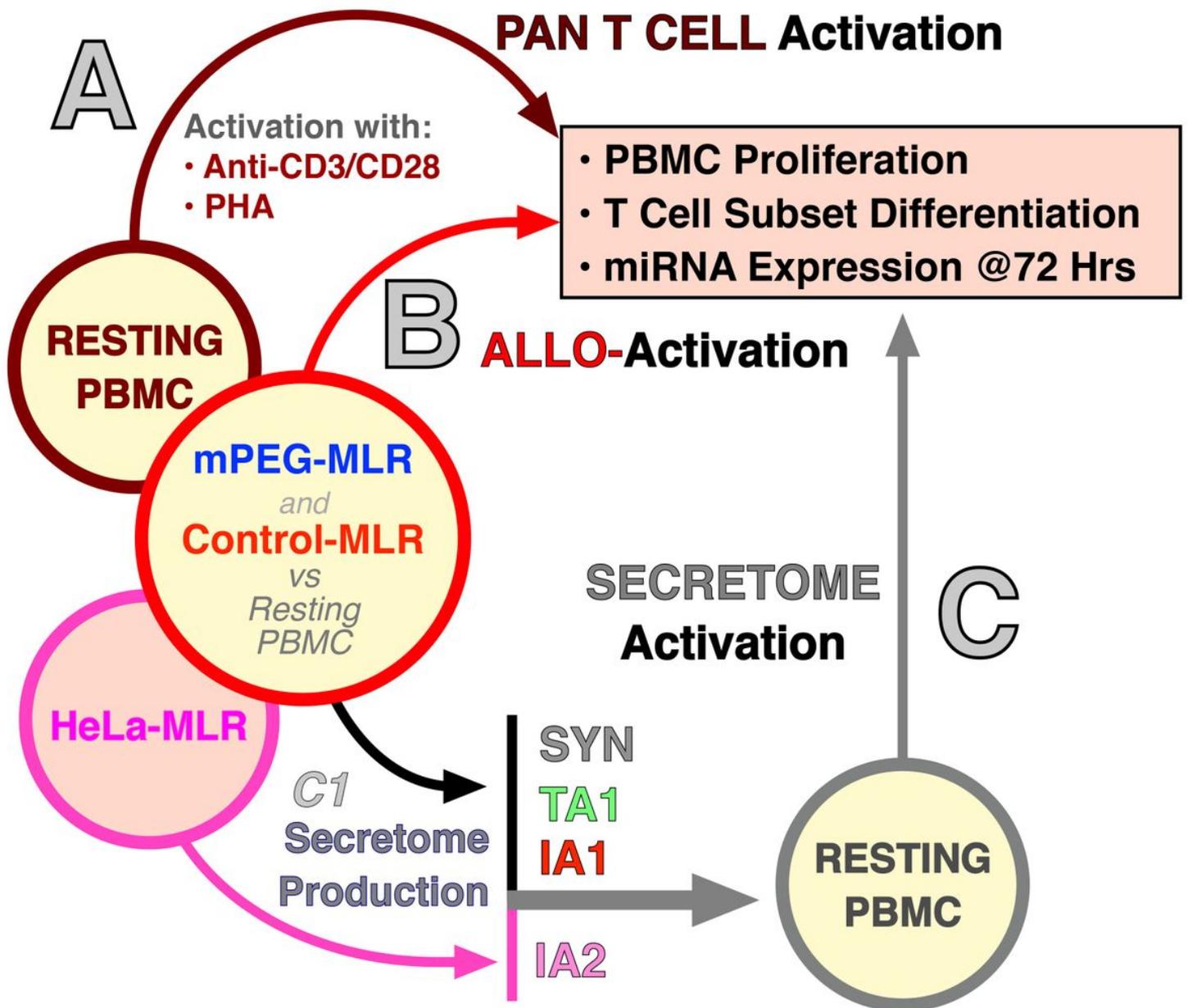
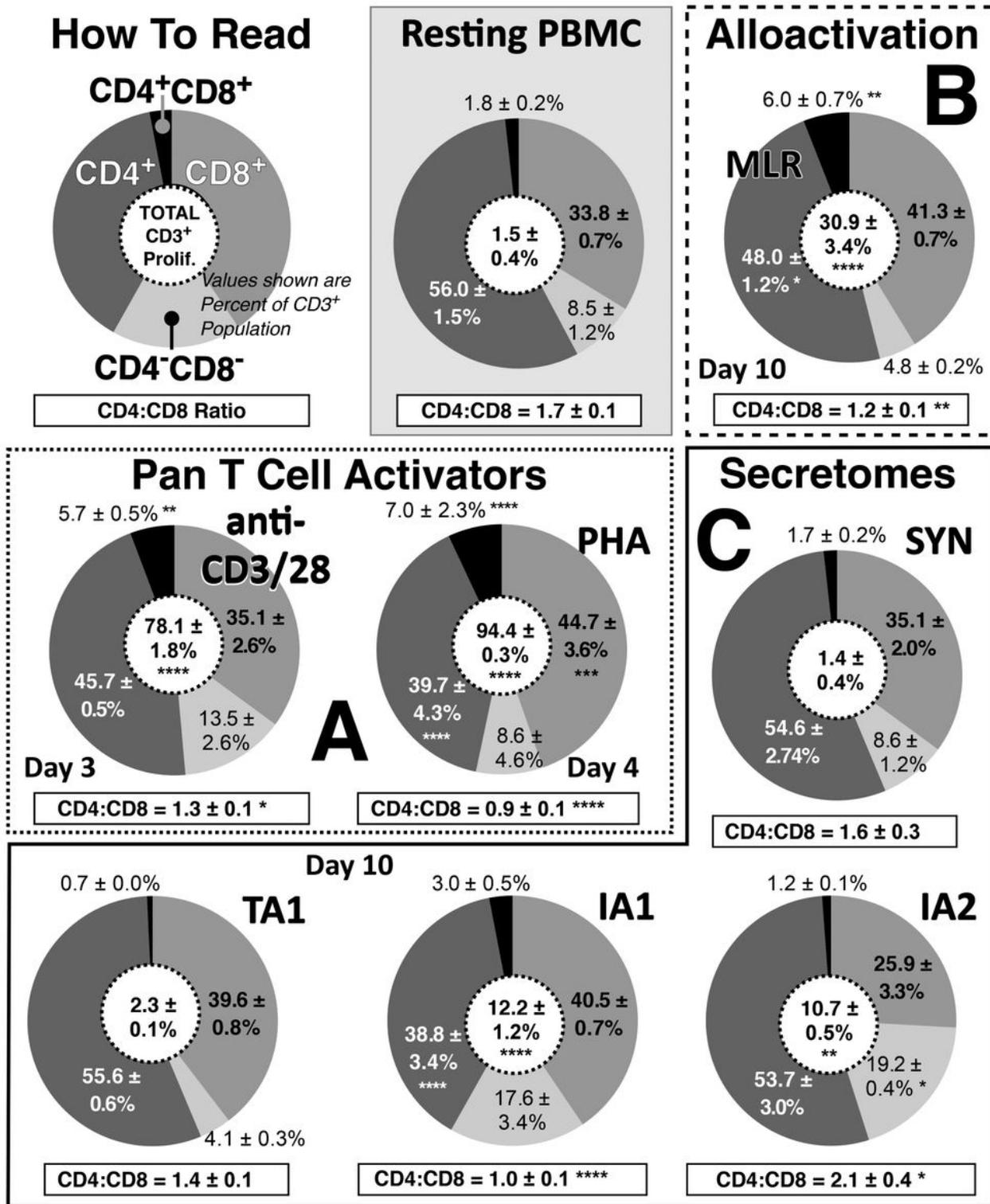


Figure 1

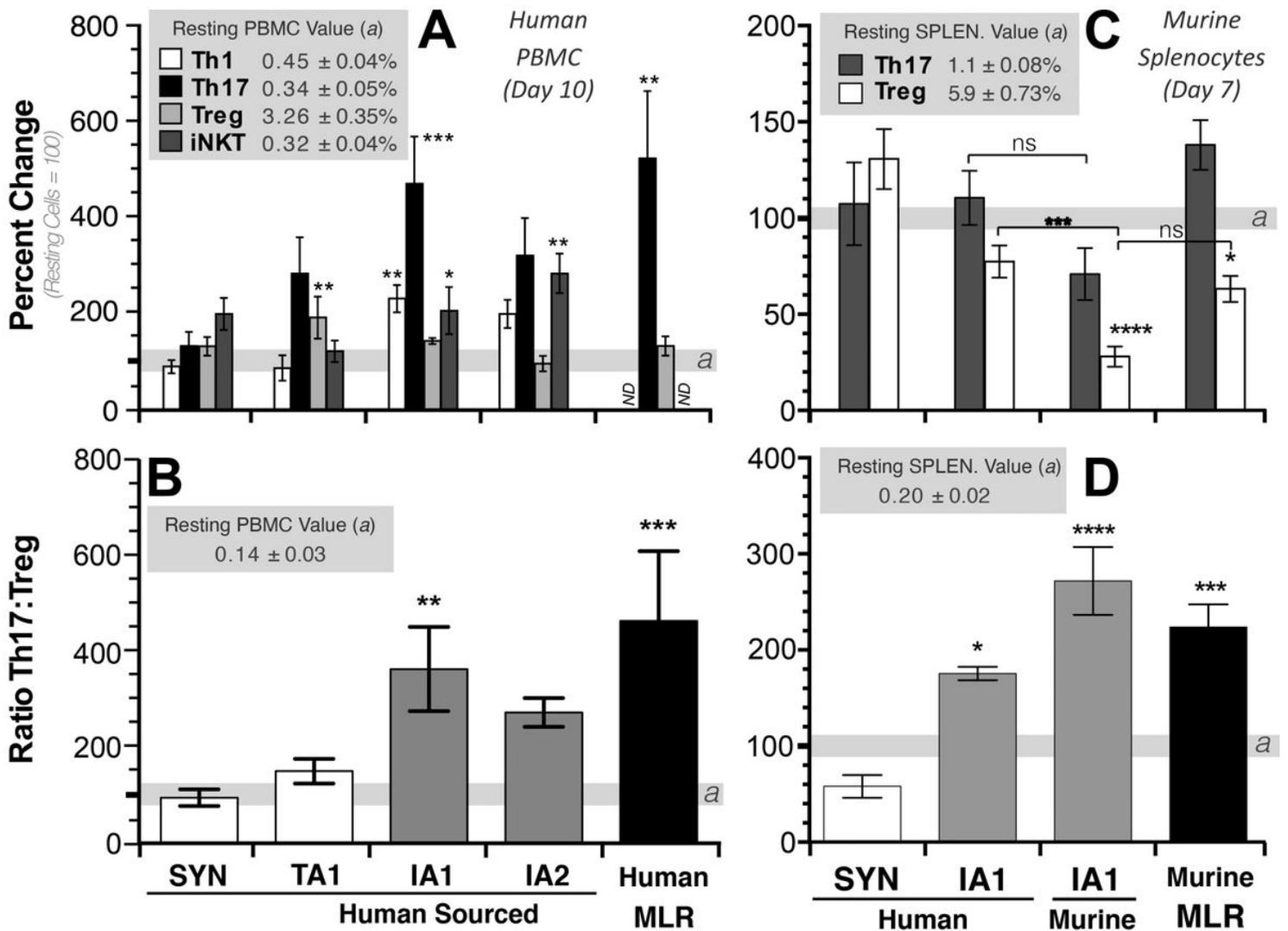
. Schematic presentation of the three differential activation strategies (A-C: Pan T; Allorecognition; and Secretome) of PBMC activation studied. T cell proliferation and subset differentiation were measured via flow cytometry and leukocyte miRNA expression levels which were compared to untreated resting PBMC. Pan T cell activation (A) of freshly isolated human resting PBMC utilized anti-CD3/anti-CD28 or PHA. Alloactivation (B) was conducted via an MLR with or without mPEG (20 kDa mPEG; grafting concentration of 2 mM) immunocamouflage of one MHC-disparate donor. The negative control consisted of the untreated resting PBMC. Secretome activation consisted of two phases: production of the secretome therapeutics (C1) and the activation (C) of freshly collected donor PBMC with the manufactured secretome therapeutic. The SYN, TA1 and IA1 was derived from resting PBMC, mPEG-MLR and control MLR respectively. The IA2 secretome product was produced using an PBMC-cancer cell (HeLa) MLR model (HeLa-MLR). Proliferation and subset analysis of the different activation strategies were optimized to their day of maximum proliferation (PHA, Day 4; Anti-CD3/CD28, Day 3; Alloactivation, Day 10; and Secretome Activation, Day 10). miRNA profiles of the activated PBMC of the differential strategies (A-C) was done at 72 hours for and compared to untreated resting PBMC.



**Figure 2**

Effects of differential activation strategies on CD3<sup>+</sup> T cell proliferation, CD4<sup>+</sup> and CD8<sup>+</sup> subsets. Upper left panel describes the data presentation. Briefly the centre circle indicates total CD3<sup>+</sup> proliferation and the slices of the pie-chart denote percentages of the indicated CD4/CD8 subsets of the total CD3<sup>+</sup> cells at the indicated day. Resting CD3<sup>+</sup> PBMC are shown for comparison at 10 days incubation. Panel A: Results of the Pan T cell activators Anti-CD3/CD28 at Day 3 and PHA at Day 4. Panel B: Control MLR

alloactivation at Day 10. Panel C: Secretome therapeutics at Day 10 post activation. Of note, the increase in proliferation by IA1 was more controlled than pan T cell activators or alloactivation while the SYN and TA1 products showed no significant difference from the resting cells. Of interest however, TA1 induces a tolerogenic effect. (3, 4, 25) Statistics (all relative to resting PBMC):  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*) and  $p < 0.0001$  (\*\*\*\*),  $n = 4$ .



**Figure 3**

Effects of secretome-derived therapeutics on Th1, Th17, Treg and iNKT differentiation of human PBMC and murine splenocytes. Human or murine Th1, Th17, Treg and iNKT differentiation was measured via flow cytometry. Percent population of resting donor cells was normalized to 100 and shown by the grey bar labelled as a. Panels A-B: Human resting PBMC were treated with human sourced secretomes for 10 days. For comparison, a two-way human control-MLR was also conducted. The pro-inflammatory IA1 and the allo-MLR induced a similar Teff activation pattern and both increased Th17:Treg ratio. In contrast, the tolerogenic TA1 increased the Treg population. Panels C-D: Murine splenocytes were treated with human sourced SYN and human or mouse sourced IA1 secretomes for 7 days and compared to a control two-way murine MLR. Due to miRNA sequence conservation, human IA1 induced an increase in the murine

Th17:Treg ratio demonstrating a cross-species activity. The Th17:Treg ratio (D) of the murine sourced IA1 was statistically identical to the murine control-MLR. Statistics (all relative to resting cells a):  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*) and  $p < 0.0001$  (\*\*\*\*),  $n = 4$ .

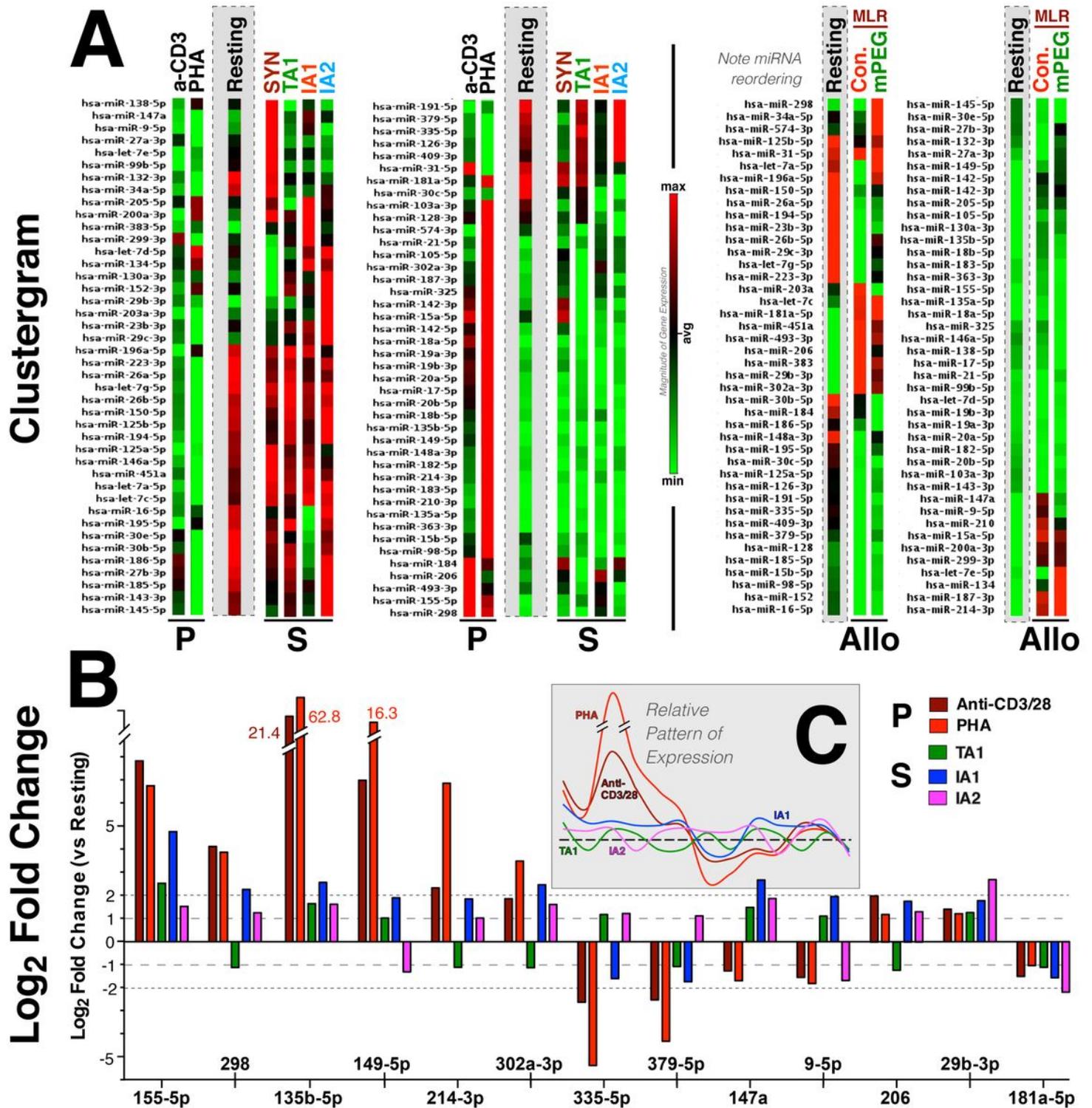
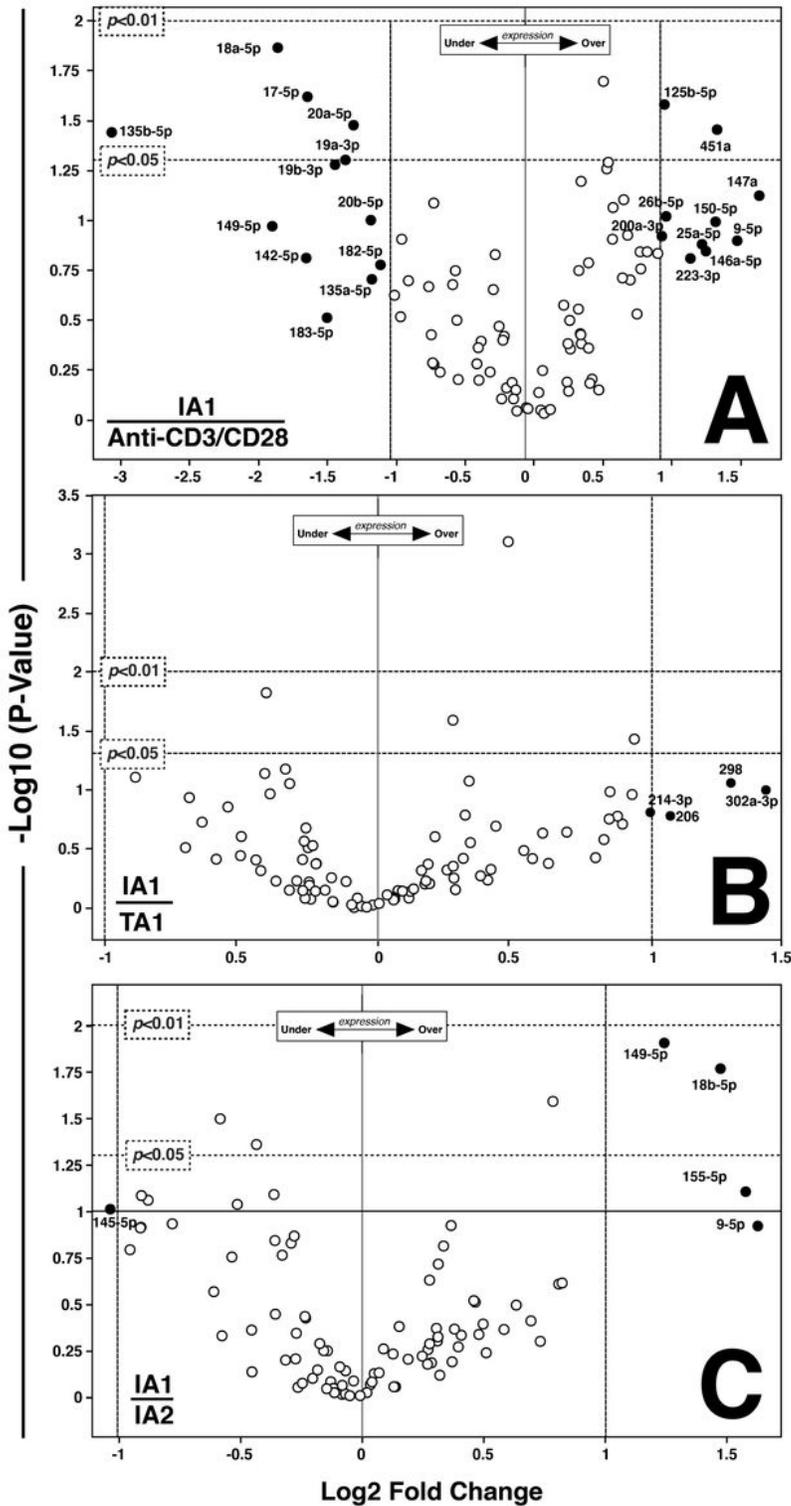


Figure 4

Effects of differential activation strategies on PBMC miRNA expression. Panel A: Clustergram analysis demonstrated very significant differences between the Pan T cell (P) and secretome activators (S) on the

expression profiles of 84 immunopathology pathway miRNAs at 72 hours post treatment. Similarly, dramatically different profiles were observed between the miRNA expression profiles of the control- and mPEG-MLR (Allo). Indeed, the mPEG-MLR expression profile exhibited similarities to both the control-MLR and the resting cells. Panel B: Log<sub>2</sub> fold change analysis similarly demonstrated the differential effects of Pan T cell activators (P) and secretome therapeutics (S) on miRNA expression relative to resting PBMC (solid line at 0). Grey dashed lines of -2, -1, 1 and 2 values are shown for comparison purposes. Pan T cell activators altered the PBMC miRNA expression to a much larger magnitude than the pro-inflammatory IA1 secretome. The SYN, pro-tolerogenic TA1 and cancer-derived IA2 secretome exerted significantly different effects on miRNA expression as would be expected based on their differential effects on cell proliferation and differentiation. Panel C: Relative pattern of expression of key miRNA from Panel B. Putative/described functions of these miRNA in biological studies were presented in Table 1. Data shown represent three independent experiments using PBMC collected from the same donor at 3 different intervals. Please note the reordering of sequence of the clustergram miRNA listings between (Allo) and P/S.



**Figure 5**

Volcano plot analysis of the secretome agents demonstrated differential PBMC miRNA expression profiles. Volcano plot analysis of selected secretome products was done to better compare the effects of these agents on resting PBMC miRNA expression 72 hours post activation. Panel A: IA1 versus anti-CD3/CD28. Panel B: IA1 versus TA1. Panel C: IA1 versus IA2. Vertical ( $\text{Log}_2 \text{Fold Change}$ ) and horizontal ( $-\text{Log}_{10}$ ;  $p$  value  $< 0.05$  and  $0.01$ ) dashed lines denote miRNA over- or under- expressed. Unchanged (and

unlabeled) miRNA are open circles while miRNA exhibiting significant changes are black circles and labeled. Data shown represent three independent experiments using PBMC collected from the same donor at 3 different intervals.

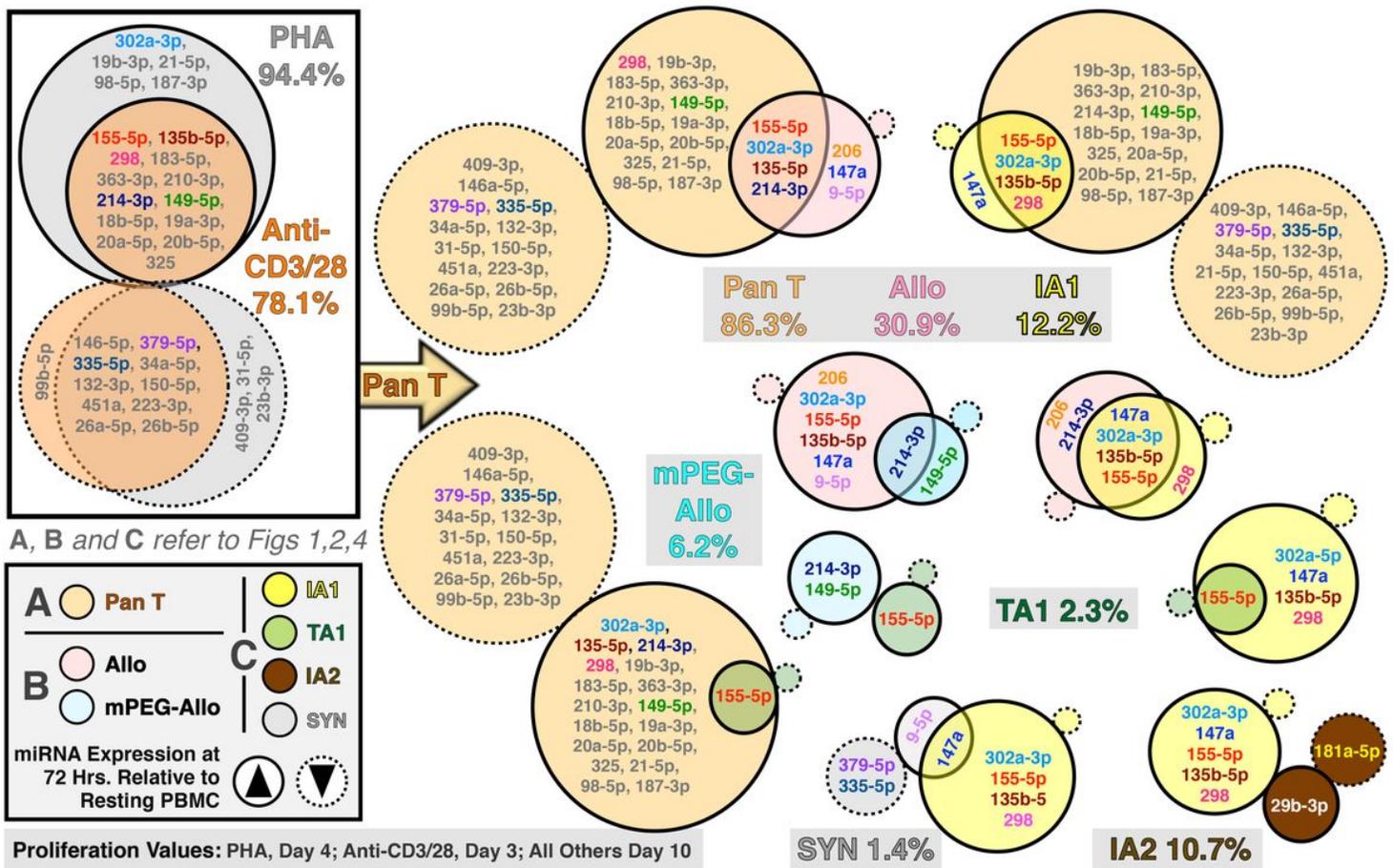
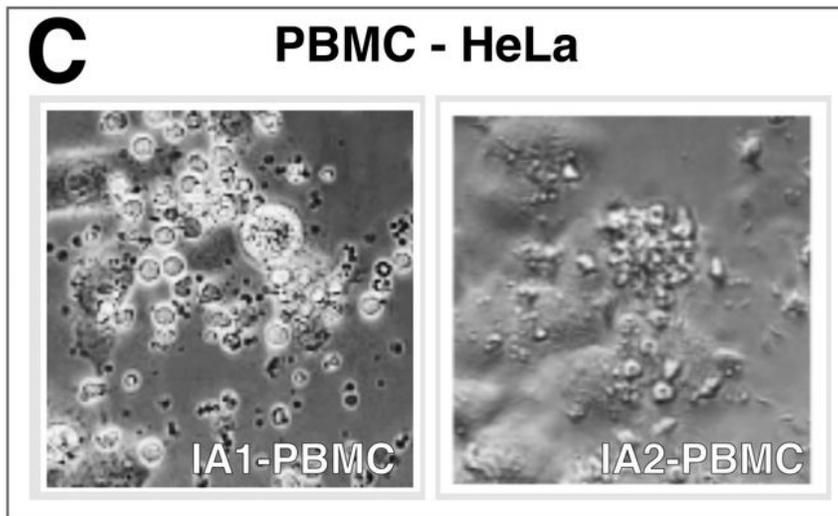
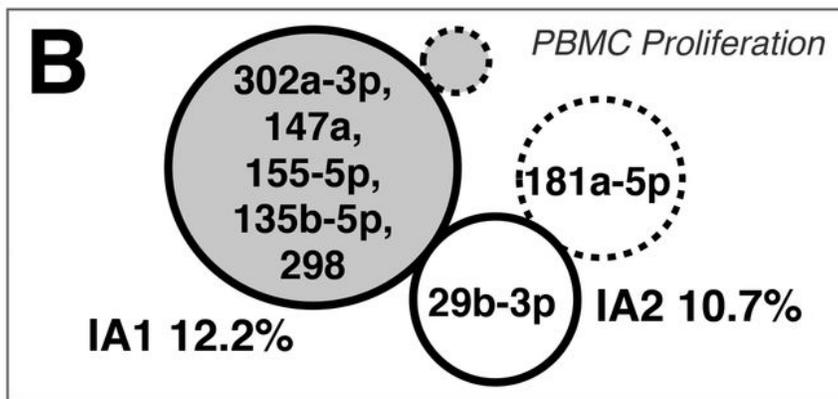
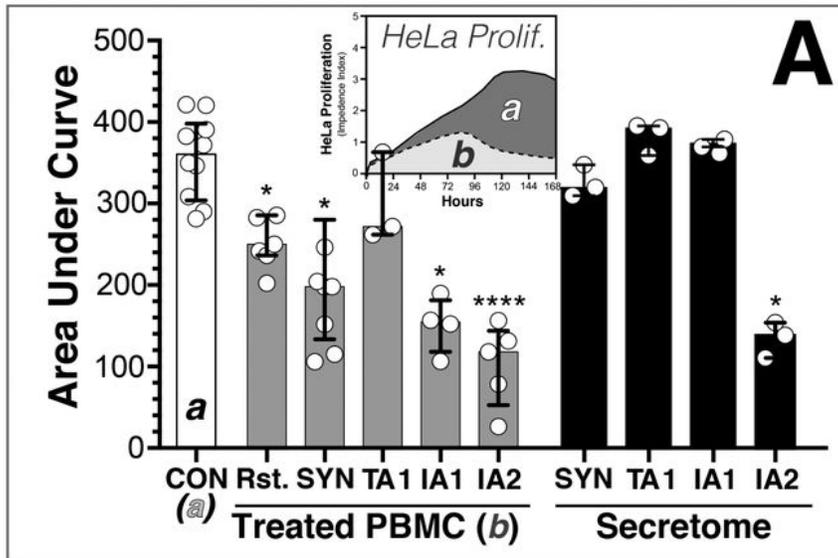


Figure 6

Venn diagram comparison between Pan T Cell (A), Allo (B) and Secretome (C) activation of resting human PBMC. Over-expressed miRNA are listed in solid line bubbles, while under-expressed miRNA are in dashed line bubbles. Overlapped areas indicate miRNAs with similarly increased/decreased miRNA expression. The bubble color indicates different activation treatments. miRNA of interest (relative to Figures 4-5; Table 1) are in color. As shown, pan T cell, allo- and secretome activation exerted distinct patterns of miRNA expression in treated resting PBMC. As shown, Pan T cell activators exhibit huge changes (up/down) in a large number of miRNA. In contrast, alloactivation (both the control- and mPEG-MLR) showed dramatically reduced miRNA changes and the secretome products showed an even further reduction in the differentially expressed miRNA. Proliferation values for each approach/agent are provided within the figure. Data shown are from 3 independent replicates.



**Figure 7**

Despite similarities in biomanufacturing, secretome products exhibited significant differences in their abilities to inhibit HeLa cell proliferation. Panel A: HeLa cell proliferation was measured using an ACEA iCELLigence which monitors cell proliferation/killing via changes in electrical impedance. HeLa proliferation curves were graphed and the Area Under Curve (AUC) was determined as indicated in the INSERT. The shaded grey areas of the INSERT (a and b) correspond, respectively, to the control HeLa cells

and HeLa cells overlaid with IA1-treated PBMC (50:1; PBMC:HeLa). (4, 25) Shown are the effects of secretome treated-resting PBMC (24 hours; grey bars) on HeLa cell proliferation/killing. The direct effects of the secretomes on HeLa cells are shown by the black bars. As noted, both IA1 and IA2 treated PBMC exhibited significant inhibition of HeLa cell proliferation; however, IA2 exhibited direct toxicity potentially negating any direct PBMC role. Panel B: As shown in Figure 6, IA1 and IA2 promoted a similar magnitude of resting PBMC proliferation but induced distinct leukocyte miRNA expression patterns. IA1 specifically upregulated pro-inflammatory miRNA while IA2 solely altered the expression of apoptosis-related miRNA. Panel C: PBMC-HeLa cell conjugation was examined via microscopically. Photomicrographs obtained at 72 hours post co-culture (PBMC:HeLa cell = 50:1) demonstrated that IA1-activated PBMC exhibited a great degree of interactions with the HeLa cell monolayer resulting in reduced HeLa cell proliferation. In contrast, IA2-treated PBMC did not show significant cell:cell interaction. However, the HeLa cells exhibited significant blebbing and vesiculation. The observation of Panel A and C suggest that IA2 exerts its anti-HeLa effect via soluble factors. Indeed, as shown in Table 1, the increase in miR-29b-3p and decrease in miR-181a-5p have been reported to be involved in apoptosis. Statistics (all relative to control HeLa a):  $p < 0.05$  (\*) and  $p < 0.0001$  (\*\*\*\*),  $n \geq 3$ .

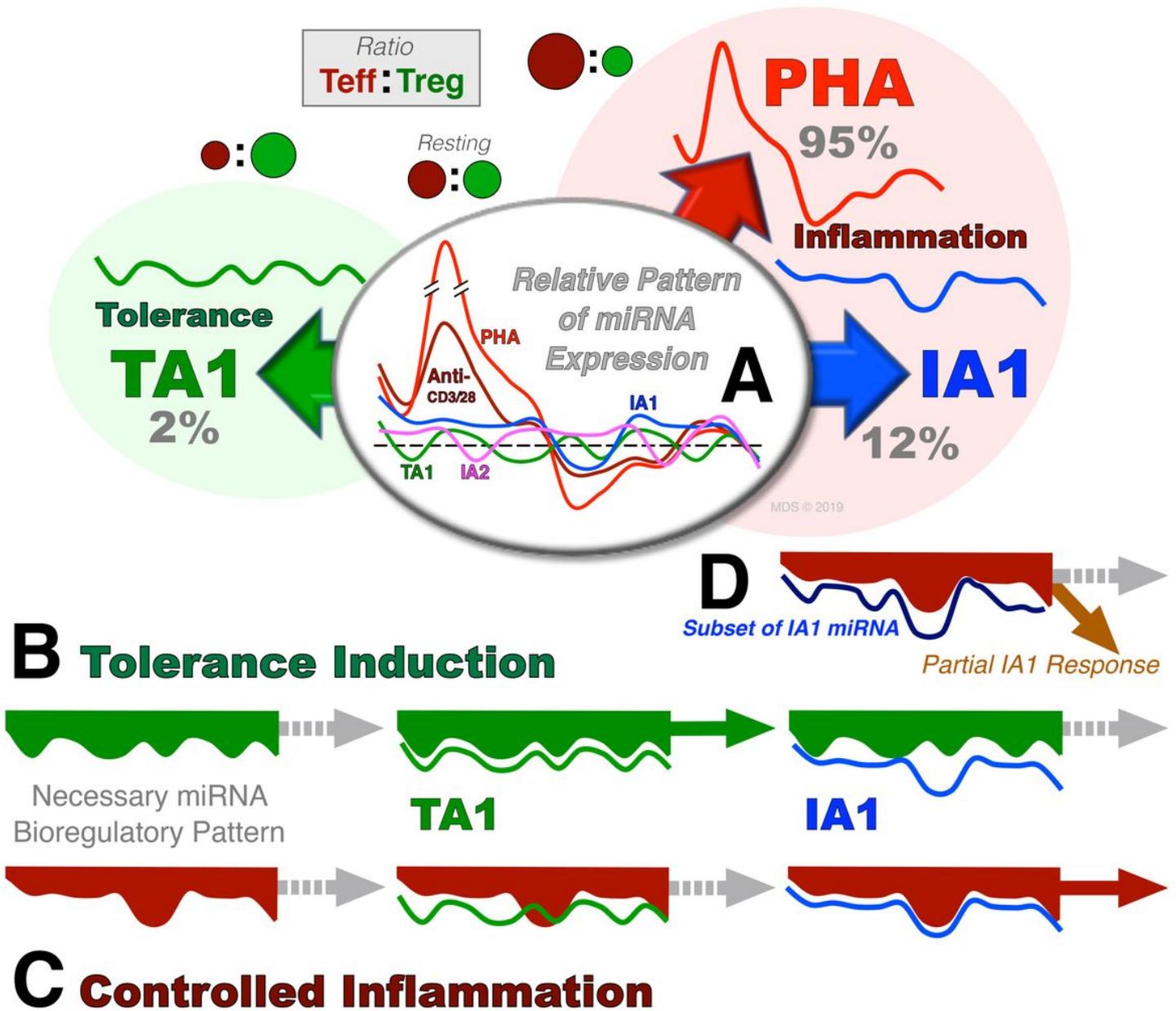


Figure 8

Bioregulation occurs consequent to pattern specificity of miRNA expression. miRNA are 'low fidelity' due to the promiscuous nature of miRNA: a single miRNA can interact with hundreds of genes and a single gene can interact with multiple miRNA. Hence, the 'Pattern of miRNA' (both species and relative abundance) govern the biologic response in a 'lock and key' manner. Panel A: PBMC activation with Pan T cell, Allorecognition-driven, and secretome products yield dramatically different miRNA expression patterns, proliferation and effects on the Teff:Treg ratios. Panel B-C: Using a 'lock and key' analogy, TA1 (B) and IA1 (C) induce miRNA expression profiles that induce exclusively induce either a tolerogenic (TA1) or pro-inflammatory (IA1) effect in treated PBMC. Panel D: However, if partial pattern parity exists, aspects of the biological response may be retained. As shown a similar IA1-like pattern may induce a partial response. Indeed, per Figure 6, IA1 may be viewed as a subset of the alloresponse which itself is a subset of Pan T cell activators. The disparity in the miRNA expression between these three pro-

inflammatory responses is similarly reflected in the reduction of proliferation noted between the activators.

## Supplementary Files

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