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14. ABSTRACT
This project addresses the hypothesis that elevated and/or altered IFN-γ signaling within selective subsets of mononuclear cells promotes disease severity in RA. This study developed from our novel observation that in peripheral blood the expression levels of interferon gamma receptor 1 (IFNGR1) is associated with RA and the expression levels of IFNGR2 correlates significantly with the degree of radiographic damage in RA patients. The aims of this proposal are: (1) To identify the specific circulating cell type in which IFNGR expression is elevated in RA. Using a combination of molecular biological and immunological approaches, we will analyze the expression levels of IFNGR1 and IFNGR2 in monocytes, naïve and memory B cell populations, naïve and memory T cell populations including T-follicular helper cells, Treg cells and T helper effector subpopulations (Th1, Th17 and Th17/1). (2) To determine the outcome of IFNGR signals by assaying the activation of IFN-γ induced STAT1 and changes in activation of STAT3 and STAT5 in RA versus healthy controls, at basal level and following stimulation with cytokines such as IL-2, IL6 etc. (3) To determine the molecular mechanism and outcome of attenuated IL-2 induced activation of STAT5 in specific subpopulations of T cells in RA. The information to be gained can potentially help to identify new cell signaling targets, perhaps cell-type specific, for RA and other autoimmune diseases, and perhaps malignancies. This in turn may help to develop new drugs that are more targeted, either to particular cell types or patients in whom these cell types are most important to the disease. Ultimately, this may lead to more effective, and safer drugs with fewer adverse effects.

15. SUBJECT TERMS
Rheumatoid arthritis; Autoimmunity; T lymphocyte subsets; Cell Signaling; Interferon-gamma; STAT1; STAT3; STAT5: Interleukin-2

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TABLE OF CONTENTS

1. INTRODUCTION

This project addresses the role of STAT signaling in circulating immune cells on susceptibility to rheumatoid arthritis, its severity, and potentially response to treatment. This study developed from our novel observation that in peripheral blood the expression levels of interferon gamma receptor 1 (IFNGR1) is associated with RA and the expression levels of IFNGR2 correlates significantly with the degree of radiographic damage in RA patients. The aims of this proposal and our progress toward them, are laid out in Section 3 below.

2. KEYWORDS

Rheumatoid arthritis; Autoimmunity; T lymphocyte subsets; Cell Signaling; Interferon-gamma; STAT1; STAT3; STAT5: Interleukin-2

3. ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1 (specified in proposal)	**Timeline**	**Status**
Major Task 1 - To identify the circulating cell types in which IFNGR expression is upregulated in RA and determine how it relates to disease activity.	Months	**Completed, % complete, or Future Work**
Subtask 1 – To recruit 250 participants for Major Tasks 1, 2, and 3. This includes 150 with RA (50 each with low disease activity/remission; moderate disease activity; high disease activity); 50 with multiple sclerosis; 50 healthy controls. Collect data, including disease activity, medications, demographics, etc.	Begin Month 4 (after IRB approval); end Month 27	Completed. Unfortunately, due to COVID-19, our recruitment was stopped in March 2020 and did not resume. We have screened ~225 RA participants and enrolled 148, and have enrolled ~70 participants with MS and ~30 healthy controls.
Subtask 2 – Perform FACS and quantitative real-time PCR (qRT-PCR) to measure IFNGR1 and IFNGR2 expression in multiple T cell and B cell populations and monocytes.	Begin Month 4 and proceed in batches; end Month 27	Completed. We have analyzed ~80% of participants enrolled
Subtask 3 - Assess IFN-γ receptor protein levels in cell subsets (including Th1, Th17, Th17/1, etc.) in RA with different disease activity (remission/low; moderate; high), MS, and controls.	Begin Month 4 and proceed in batches; end Month 27	Completed. We have analyzed ~75% of participants enrolled.

Subtask 4 - We will compare results among patients with RA with different disease activity (remission/low; moderate; high), MS, and controls.	Begin Month 7 and proceed throughout the funding period	Completed. Comparison of results among these three groups has been done on ~50% of participants enrolled.
Milestone(s) Achieved		
Local IRB Approval		NCE: xx-xx-2020
HRPO Approval		NCE:HRPO Log Number A-19648 - approved on xx-xx-2019
Present results at scientific meetings	18, 24	Completed. See details in corresponding sections of this report.
Publish results in scientific journals	24, 30	Completed, plus additional manuscripts are being finalized for publication.
Specific Aim 2 (specified in proposal)		
Major Task 2 - To determine the effect of upregulated IFNGR expression on IFN-γ- induced activation of STAT1, STAT3, and STAT5 signaling in peripheral blood cell subsets in RA.		
Subtask 1 - Compare the level of activation of STATs (as assessed by the degree of phosphorylation) in peripheral blood naïve and memory CD4+ T cells, Th effector populations, Treg, naïve and memory B cells, and monocytes at baseline and following stimulation with IFN- γ in RA (n=150) using phospho-flow cytometry.	Begin Month 4 and proceed in batches; end Month 27	Completed. Unfortunately, due to COVID-19, our recruitment was stopped in March 2020 and did not resume. We have completed analysis of peripheral blood populations on ~50% of participants. In addition, we have added analysis of an existing dataset from the TETRAD Study. We have determined several RA CD4 but not CD8 T cell populations have lower IFN-γ induced STAT1 activation than HC (see appropriate sections of this report below.
Subtask 2 - Determine if altered STAT1 (or STAT3 or STAT5) activation leads to differences in nuclear localization of STAT1 (or STAT3 or STAT5) followed by changes in cellular morphology in different mononuclear subpopulations using quantitative image analysis and flow	Begin Month 4 and proceed in batches; end Month 27	This subtask was not performed because our studies showed that IFN-γ induced STAT1 activation in RA is lower than HC.

cytometry (Imagestream).		Thus pursuit of this aim would not provide meaningfully to our understanding of RA.
Subtask 3 - Determine if IFN-γ signals alter the ability of other cytokines (IL-2, GM-CSF, IL-6, IL-23) to activate their respective STATs.	Begin Month 4 and proceed in batches; end Month 27	Completed. We have analyzed IFN-γ induced STAT1 activation in HC, RA and MS. Due to COVID-19, we did not examined responses to other cytokines. As part of this study, we identified a dataset (TETRAD) that would help address the role of cytokine-stimulated STAT activation in T and B cells in RA. See details in below.
Present results at scientific meetings	Mos. 18, 24	Completed.
Publish results in scientific journals	Mos. 24, 30	Completed, plus additional manuscripts are being finalized for publication.
Specific Aim 3 (specified in proposal)		
Major Task 3 - To determine the molecular mechanism and outcome of attenuated IL-2 induced activation of STAT5 in specific subpopulations of T cells in RA.		
Subtask 1 – Determine whether altered IL-2 mediated activation of STAT5 in subpopulations of T cells in RA contributes to disease pathogenesis.	Begin Month 4 and proceed in batches; end Month 27	Completed. We analyzed peripheral blood from 50% of participants. We discovered that in MS, IL-2 induced activation of STAT5 is much greater than HC for several CD4 and CD8 T cell populations, but not Treg. We have also determined that the Treg population in MS is distinct from that in HC.
Subtask 2 – Determine the outcome of attenuated IL-2 mediated activation of STAT5 on Th effector cell and regulatory cell expansion and function.	Begin Month 4 and proceed in batches; end Month 27	We did not pursue this subtask as additional data from our lab suggests IFN-γ attenuated STAT-5 activation is not a

		consistent finding.
Present results at scientific meetings	18, 24	Completed.
Publish results in scientific journals	24, 30	Completed, plus additional manuscripts are being finalized for publication.

1. Major activities

Collection of Blood samples from RA, MS (multiple sclerosis) and healthy controls (HC).
As stated in the Table above, we enrolled more than 200 subjects with RA, MS, and healthy controls.

Training of graduate students:
Two graduate students, Mr. Vishal Sharma (Ph.D. Immunology program) and Mr. Brandon Pope (MD/Ph.D. program) were trained as part of these studies. Mr. Pope will be defending his PhD thesis on Sept. 3 and graduate in December 2020. He will continue his Medical training leading to a MD degree in May 2022. Mr. Sharma is expected to complete his Ph.D. by Dec 2022.

2. Specific objectives.

Aim 1. To identify the circulating cell types in which IFNGR expression is upregulated in RA and determine how it relates to disease activity.

IFNGR expression in mononuclear cell subpopulations is not different between RA and MS. Our results show no differences in expression of IFNGR1, IFNGR2, IL2RA, IL2RB, IL2RG in CD4 and CD8 naïve, effector, central memory and effector memory cells T cells and regulatory T cells between RA and MS (Figure 1). Similarly, we found no significant differences in expression in these same analytes between RA and MS B cells and monocytes (data not shown).

Altered proportions of CD4 and CD8 T cell populations in RA. We found that the proportion of $CD4^+$ T cells in the peripheral blood was elevated by ~1.5-fold compared to HC. This difference was attributable to expansion of the T_{EM} and T_E $CD4_+$ T cell populations (Figure 1A). Unlike for $CD4^+$ T cells, the proportions of total peripheral $CD8^+$ T cells in RA and HC were similar. However, there was expansion of the T_{EM} and T_E populations and a contraction of the T_N population in RA compared to HC (Figure 1B).

IL-2 enhances IFN-γ induced STAT1 in RA CD4 populations. Based on preliminary data, we expected that IFN-γ attenuated IL-2 induced activation of STAT5. This data was not sustained. However, our results reveal a novel biology related to integration of IFN-γ and IL-2 signals. We found that IL-2 co-stimulation enhanced IFN-γ induced activation of STAT-1 in RA CD4 naïve, central memory and effector populations but not CD4 effector memory populations. Co-stimulation of CD4 populations from HC and MS did not increase STAT-1 activation over that if IFN-γ alone. However, co-stimulation resulted in sustained IFN-γ induced STAT1 activation in

HC and MS for a period up to 4h (Figure 4). IL-2 had no effect on IFN-γ induced activation of STAT-1 in any CD8 T cell population (data not shown). IFN-γ had no effect on IL-2 induced STAT5 or STAT-3 (data not shown).

<u>Aim 2. To determine effect of upregulated IFNGR expression on IFN-γ-induced activation of STAT1, STAT3, and STAT5 signaling in peripheral blood cell subsets in RA.</u>

IFN-γ induced activation of STAT1 is lower in CD4 and CD8 T cells populations for RA compared to HC and MS. Our original prediction was that IFN-γ induced activation of STAT-1 will be greater in RA CD4 and CD8 T cell populations than HC. Remarkably, we find that RA T cell populations respond less efficiently to IFN-γ than both HC and MS (Figure 2 and data not shown). The lower activation of IFN-γ induced activation of STAT1 in RA was observed for all time points of stimulations from 15 mins to 4 h. The lower response in RA compared to MS is not due to levels of receptor expression.

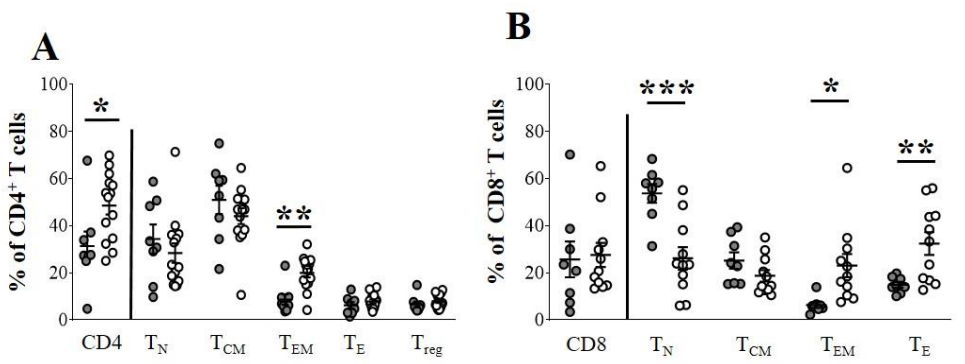

Figure 1: $CD4^+$ and $CD8^+$ T cell populations in RA compared to HC. Proportion of total ***A,*** $CD4^+$ T cells and ***B,*** and $CD8^+$ T cell subpopulations in HC and RA. Peripheral blood mononuclear cells (PBMC) from HC (solid circles) and RA (open circles) were characterized for proportion of $CD4^+$ T cells and its subpopulations by flow cytometry. T_N = naïve T cells ($CD45RA^+$ $CCR7^+$); T_{CM} = central memory T cells ($CD45RA^-$ $CCR7^+$); T_{EM} = effector memory T cells ($CD45RA^-$ $CCR7^-$), T_E = effector T cells ($CD45RA^+$ $CCR7^-$), Treg ($CD25^+$ $CD127^{lo}$). Each circle represents an individual donor and data shown is mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; for unpaired student's *t*-tests (A & B).

Higher IFN-γ induced activation of STAT1 in RA patients with active disease. We examined IFN-γ induced activation of STAT1 after stratifying patients into remission RA (CDAI <2.8) and active RA (CDAI>2.9). Overall, the activation of IFN-γ induced STAT1 was slightly higher in CD4 and CD8 T cell populations patients with active disease, but the difference was not significant (Figure 3 and data not shown). IFN-γ efficiently stimulated STAT1 in both remission and active RA patients.

TCR co-engagement enhances IFNγ induced STAT1^{Y701} phosphorylation in RA CD4$^+$ T cells. We previously showed in mice that TCR engagement enhances IFNγ induced activation of

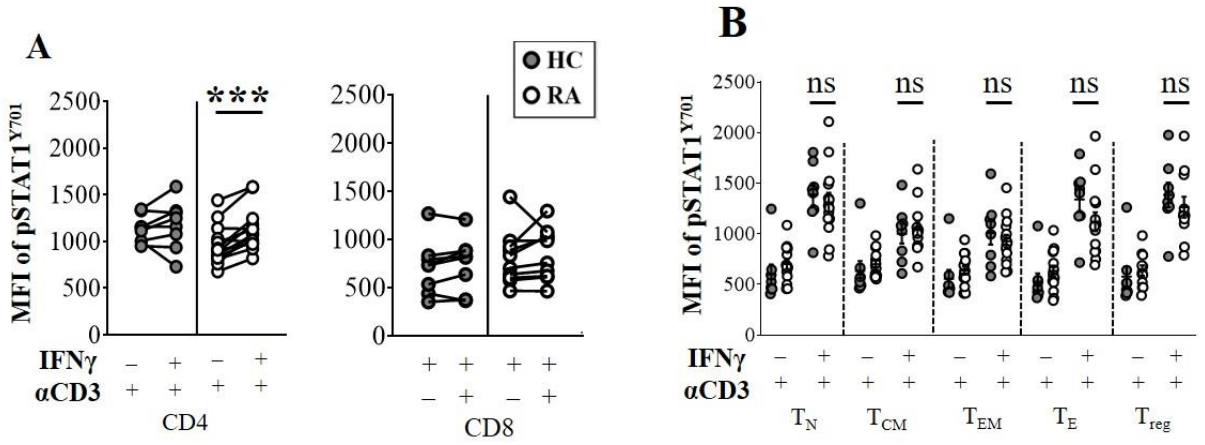

Figure 2: TCR co-engagement enhances IFNγ induced activation of STAT1 in RA CD4$^+$ T cell populations. ***A,*** Pairwise analysis of pSTAT1^{Y701} MFI following stimulation of total CD4$^+$ and CD8$^+$ T cell with IFNγ or IFNγ + αCD3 in HC and RA. ***B,*** Levels (MFI) of pSTAT1^{Y701} in CD4$^+$ T cells subpopulations from HC and RA following stimulation with αCD3 or IFNγ + αCD3. The levels of pSTAT1^{Y701} were determined at basal, following stimulation with IFNγ, αCD3, or IFNγ + αCD3. Each circle represents an individual donor and data shown is mean ± SEM*** = $P < 0.001$; unpaired student's *t*-tests (A), or paired student's *t*-tests (E).

STAT1^{Y701} in CD4$^+$ T cells (Rowse et al, PLoS One, 2012, 7(12):e52658). We therefore investigated whether engaging the TCR would enhance the reduced IFNγ induced activation of STAT1 in RA T cells. Importantly, co-stimulating CD4$^+$ T cells with αCD3 significantly enhanced IFNγ induced STAT1 activation from patients with RA but not from HC (Figure 2A). Anti-CD3 had no effect on IFNγ induced activation in CD8 T cells from RA or HC. The enhancing effect of αCD3 co-stimulation on IFNγ induced activation of STAT1 was not limited to any particular CD4$^+$ T cell subset in patients with RA (data not shown). Co-stimulation with αCD3 raised the levels of pSTAT1^{Y701} in RA T cells to the levels seen in HC T cells (Figure 2B).

CD4$^+$ and CD8$^+$T cells from RA have lower levels of STAT1. We investigated whether differences in the levels of total STAT1 might explain the differences in STAT1 activation seen in RA and HC CD4$^+$ and CD8$^+$ T cell subsets. Surprisingly, we found significantly lower levels of total STAT1 in all but one T cell population from patients with RA (CD8$^+$ T_N being the exception) compared to HC (Figure 3). It appears that total STAT1 levels do not explain the observed differences in IFNγ induced activation of STAT1 seen in RA and HC CD4$^+$ and CD8$^+$ T cell subpopulations. This conclusion is supported by the finding that in RA, co-stimulation with αCD3 restored IFNγ induced activation of STAT1 to the levels seen in HC.

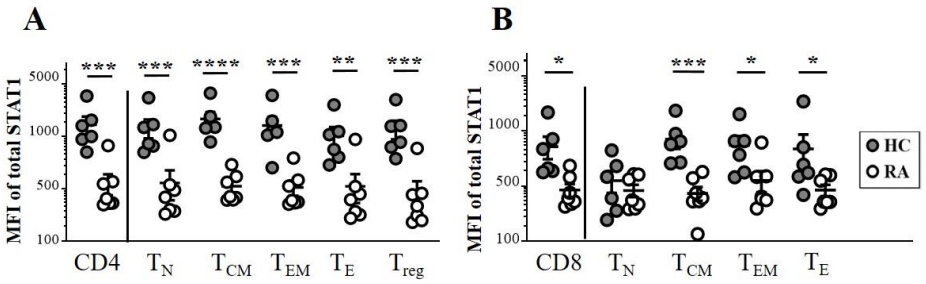

Figure 3: Total STAT1 levels in $CD4^+$ and $CD8^+$ T cell populations in RA compared to HC.**A**, Levels (MFI) of total STAT1 (unphosphorylated + phosphorylated) in $CD4^+$ T lymphocytes in HC and RA. **B,** Levels (MFI) of total STAT1 (unphosphorylated + phosphorylated) in $CD8^+$ T lymphocytes in HC and RA. Peripheral blood mononuclear cells (PBMC) from HC (solid circles) and RA (open circles) were characterized by flowcytometry. The levels of Total STAT1was determined at baseline (-) without IFNγ stimulation (+). T cell subpopulations are as described in Figure legends 1 and 2. Each circle represents an individual donor and data shown is mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; and **** = $P < 0.0001$, for one-way ANOVA with uncorrected Fishers least significant difference (A and B).

Associations of cytokine-induced STAT activation in RA susceptibility, severity, and treatment response. As part of the current DoD PRMRP project, we gained access to a wealth of data to analyze through the TETRAD (Treatment Efficacy and Toxicity in Rheumatoid Arthritis Database and repository) cohort– see Appendix for details. We studied functional immune signaling capacity in multiple pathways to examine disease mechanism and treatment response in RA using single-cell network profiling (SCNP).

We used existing data from this innovative systems immunology approach to help address the hypotheses proposed in this CDMRP grant. We assessed existing data obtained simultaneously on signaling nodes (Jak/STAT signaling readouts modulated by cytokines and other stimuli) in 21 immune cell subsets. We studied data from 194 RA patients and 41 controls, including 146 well-characterized RA patients prior to, and 6 months after, initiation of methotrexate or biologic agents. Our analyses found strikingly attenuated signaling capacity in RA patients in IFNα stimulation followed by measurement of phosphorylated STAT1 [IFNα stimulation using phosphorylated STAT1 as a readout] in six immune cell subsets (see Appendix 1 for details). Multiple nodes showed negative association with disease activity, including IFNα→STAT5 signaling in naive and memory B cells. In contrast, IL-6-induced STAT1 and STAT3 activation in central memory CD4-negative T cells showed a positive association with disease activity. Multiple nodes were associated with treatment response, including IFNα→STAT1 in monocytes and IL-6→STAT3 in CD4+ naive T cells. These findings demonstrate that IFN stimulated STAT activation in immune cells plays an important role in RA, a key hypothesis of this CDMRP grant. This manuscript is under review.

<u>Aim 3. To determine the molecular mechanism and outcome of attenuated IL-2 induced activation of STAT5 in specific subpopulations of T cells in RA</u>

IL-2 induced activation of STAT5 is equivalent in HC and RA. The results above show that IL-2 enhanced IFN-γ induced STAT-1 in RA, but not in HC and MS. We therefore asked if there were differences in activation of STAT-5, the major STAT activated by IL-2. Our results show that in both CD4 and CD8 T cells, the activation of STAT5 (pY694) is equivalent in HC and RA for all time points (15 min to 4h). Remarkably, IL-2 induced activation of STAT-5 was much greater in MS than either HC or RA. This result indicates to us that the enhancement of IFN-γ induced STAT-1 by IL-2 co-stimulation could represent a biology associated with RA disease.

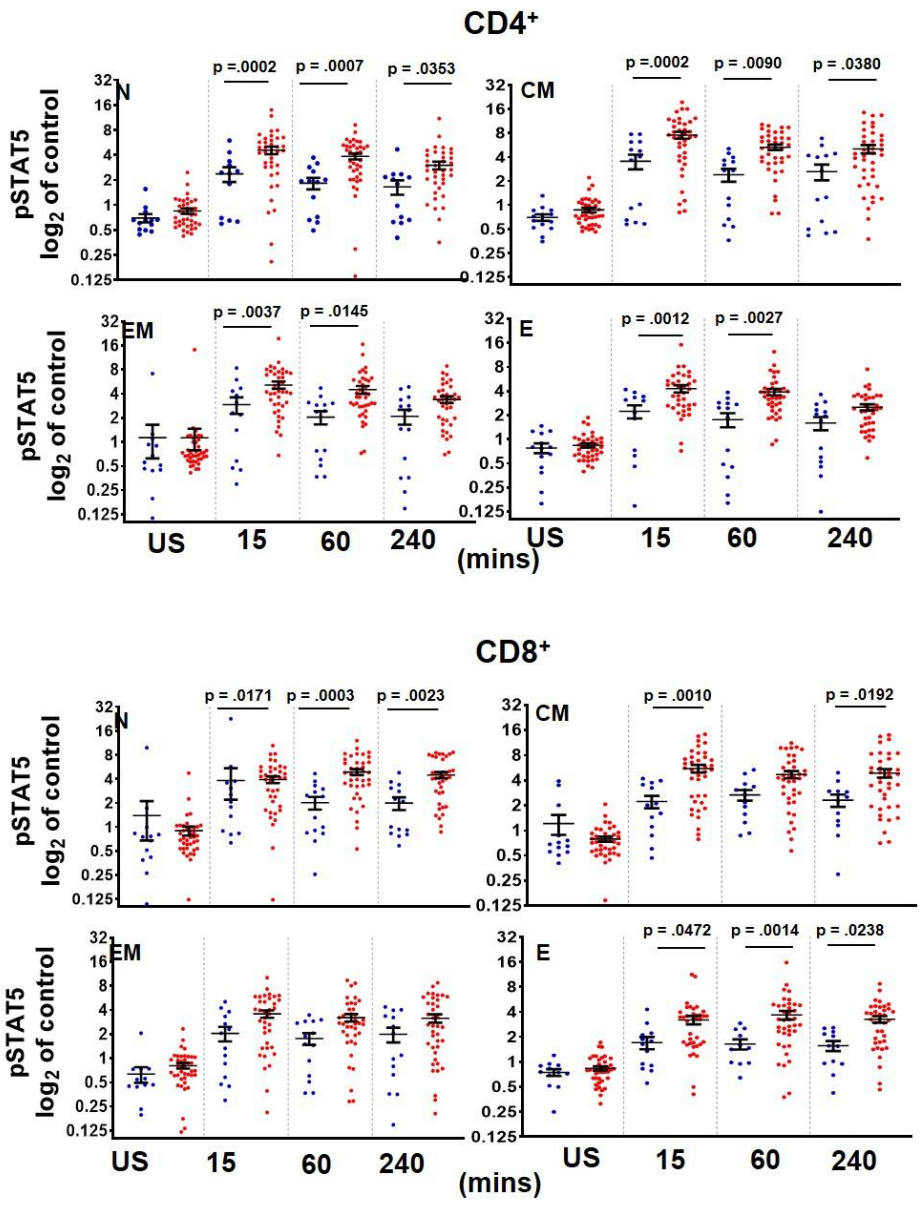

Figure 4. Enhanced IL2-induced activation of STAT5 in RRMS CD4 and CD8 T cell populations. Flow cytometry analysis of pSTAT5 levels in gated subpopulations of $CD4^+$ and $CD8^+$ T lymphocytes from HC (blue) or RRMS (red). PBMC from RRMS or HC were unstimulated (US) or stimulated with IL-2 for various time periods (15 – 240 min) and levels of pSTAT5 (pY694) was determined in naïve (N), central memory (CM), effector memory (EM) and effector (E) populations. The activation of STAT5 was normalized to isotype control for each individual and data represented as Log_2. Each dot represents and individual control or patient. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the effect of disease, stimulation time, and the interaction of these two factors as they relate to pSTAT5 expression. Post hoc Student's t tests were performed using Bonferroni's correction to approximate the appropriate familywise error rate. p values were listed exactly as calculated.

Enhanced IL-2 induced activation of STAT5 in $CD4^+$ and $CD8^+$ T cell populations from MS. PBMC from from RRMS and HC were unstimulated or stimulated for various time

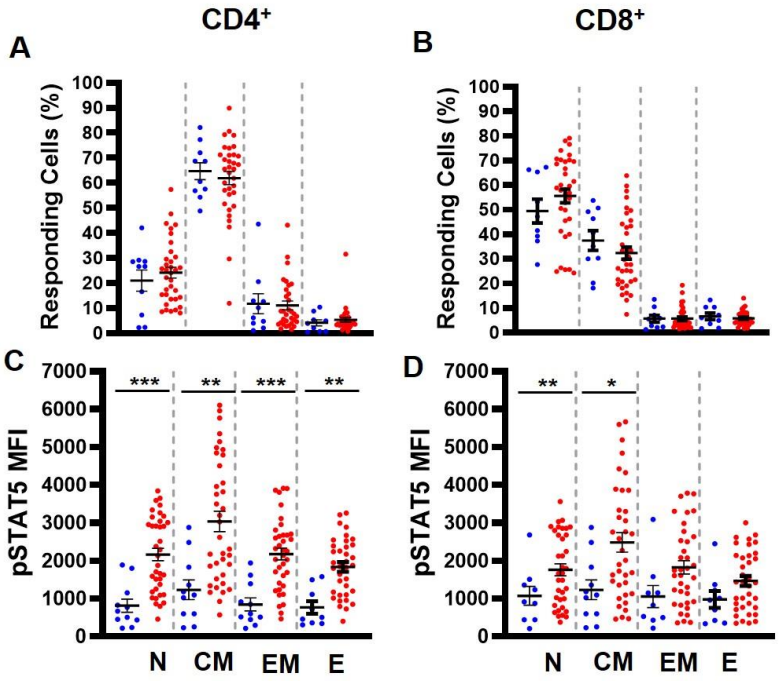

Figure 5: IL-2 high responder and STAT5 activation in CD4 and CD8 T cell populations from RRMS and HC. The frequency of high responding $CD4^+$ (*A*) and $CD8^+$ *(B)* T lymphocytes at 15 min post IL-2 stimulation in gated populations from RRMS (red) and HC (blue). The levels of pSTAT5 within high responders from $CD4^+$ (*C*) and $CD8^+$ (*D*) T cell populations from RRMS and HC. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the effect of disease, stimulation time, and the interaction of these two factors as they relate to responder frequency. Post hoc Student's t tests were performed using Bonferroni's correction to approximate the appropriate familywise error rate. p values calculated for subset pSTAT5 MFI are presented as: *= p<.05, ** = p <.01, *** = p<.001.

periods and activation of STAT5 (pSTAT5-Y694) was determined in naïve, memory (CM and EM) and effector CD4 and CD8 T cell populations. The activation of STAT5 at all time points in naïve and CM and at 15 and 60 min post IL-2 stimulation in EM and effector CD4 T cells was significantly greater in RRMS than HC. Within CD8 T cell populations, enhanced IL-2 induced activation of STAT5 was observed in naïve, CM and effector populations from RRMS compared to HC; in EM cells the activation of STAT5 was similar in RRMS and HC (Figure 4). IL-2 continued to activate STAT5 up to 24 h of stimulation, but by this time point the levels of pSTAT5 in all CD4 and CD8 populations were similar in RRMS and HC (data not shown). The proportion of IL-2 responding naïve, memory and effector CD4 and CD8 T cell populations from RRMS and HC were similar (Figure 5A and 5B). However, the level of IL-2 induced activation of STAT5 was significantly greater within the responder T cells from RRMS compared to HC in all CD4 T cell populations; in CD8, significant difference between the cohorts was observed only in naïve and CM T cells (Figure 5C and 5D).

The levels of expression of IL2RA (CD25) and IL2RB (CD122) on all CD4 and CD8 T cell populations were not significantly different between RRMS and HC (Figure 6). This indicates to us that enhanced IL-2 induced activation observed in several CD4 and CD8 T cell populations from peripheral blood of RRMS compared to HC is independent of IL2R levels. Overall, these results indicate that in newly confirmed treatment naïve RRMS, enhanced response to IL-2 is frequently observed in several non-regulatory CD4 and CD8 T cell populations.

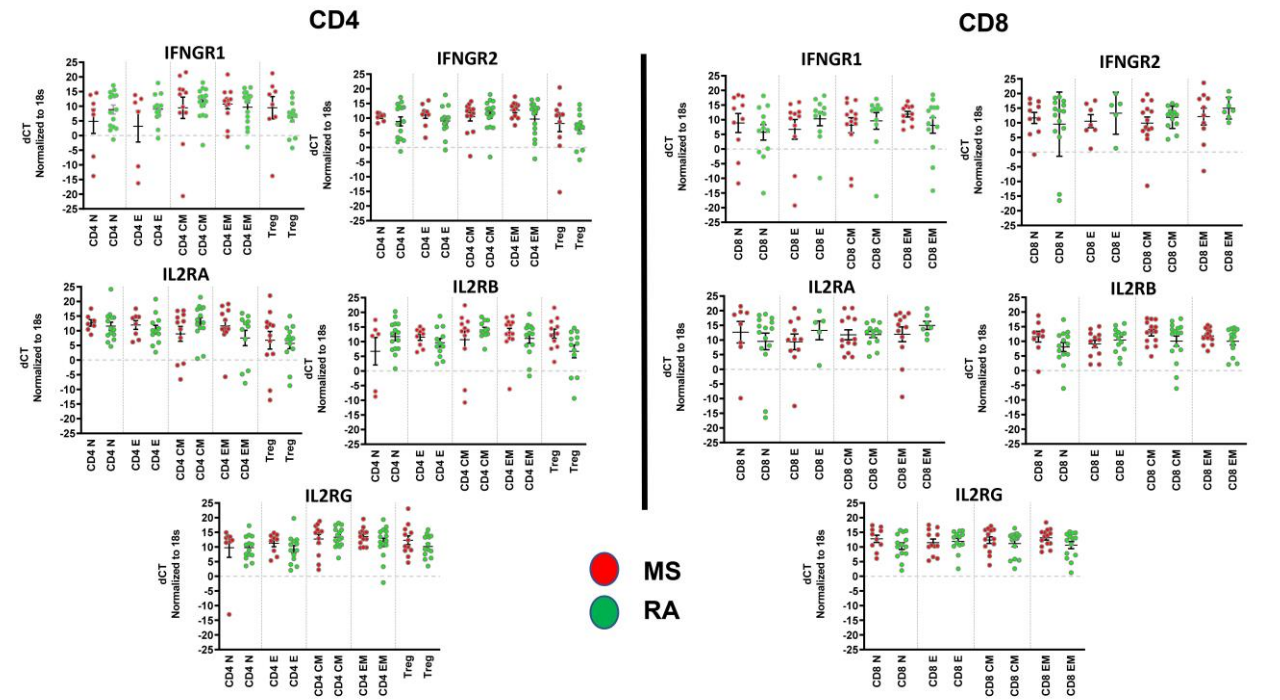

Figure 6. Expression of IFNGR and IL2 receptor on CD4 and CD8 T cell populations is not different between RA and MS. The expression of IFNGR1, IFNGR2, IL2RA, IL2RB and IL2RG was determined in CD4 and CD8 T cell populations (N = naïve; E = Effector; CM = Central Memory; EM = Effector Memory). and Treg cells as indicated. The results show no difference in expression of transcripts to any of the receptor chains between MS and RA.

Overlapping and distinct populations of CD4 and CD8 T cells in RRMS and HC.
Conventional flow cytometry analysis unavoidably requires biased sequential gating based recognized expression of phenotypic markers. The approach does not allow for simultaneous expression analysis of several proteins in multiple individuals. Because of the visualization of heterogeneity that define subpopulations are easily overlooked. To overcome this, we performed t-SNE to visualize simultaneously the expression of CD45RA, CCR7 and pSTAT5 on CD4 and CD8 T cell populations, basal and stimulated with IL-2. At basal, overlapping and distinct clusters of RRMS (red) and HC (blue) CD4 and CD8 T cell populations (Figure 7A and 7B). Such differences were also observed in cells following activation with IL-2 for 15 min or 240 min.

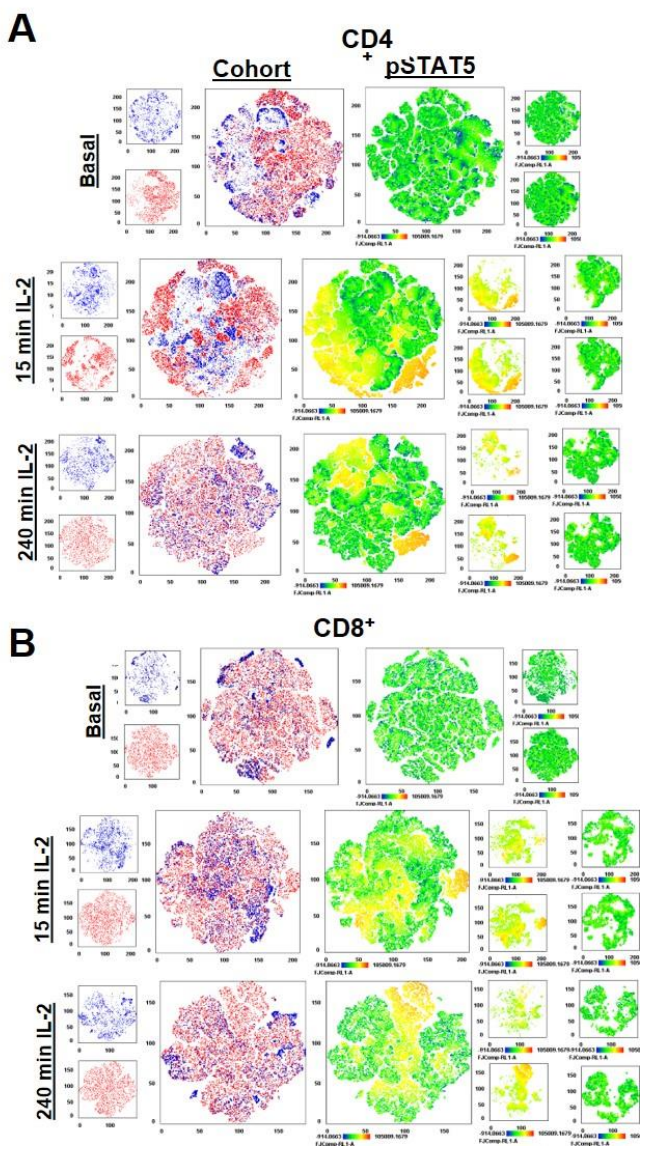

Figure 7: Unbiased automated algorithm based data analysis of CD4$^+$ and CD8$^+$ T cells shows non-overlapping populations RRMS and HC that are activated by IL-2. Barnes-Hut t distributed stochastic neighborhood embedding (tSNE) was performed on concatenated files of either CD4$^+$ or CD8$^+$ T lymphocytes from individuals with RRMS (red) or healthy individuals (blue) that were either unstimulated or stimulated with IL-2 for 15 minutes or 240 minutes. pSTAT5 expression was superimposed as a mapping overlay over the tSNE plot specific to each stimulation condition. Insets represent pSTAT5 expression for high responding/low responding populations organized by disease cohort. Intensity of pSTAT5 expression is represented on a scale plotting the lowest mean pSTAT5 MFI (dark blue/green) to the highest pSTAT5 MFI (orange) in superimposed plots.

Although the t-SNE analysis is a powerful data visualization tool to identify heterogeneity in populations from multi-dimensional data, the location of the clusters do not offer any insight into relatedness between neighboring clusters. To determine if distinct expression patterns of phenotypic markers and activation of STAT5 exists between RRMS and HC, we employed FlowSOM, which is a machine-learning neural-network based algorithm. The data reveals distinct patterns between RRMS and HC both in populations of cells and following activation by IL-2 (Figure 8). Overall, these data identify CD4 and CD8 T cell populations in RRMS that are distinct from that in HC.

Distinct populations of IL-2 responding Treg cells between RRMS and HC

It is well established that IL-2 signals are essential for human Treg stability and function. The enhanced IL-2 induced activation of STAT5 in non-regulatory CD4 T cell populations from RRMS led us to question if this also was a phenotype of RRMS Treg cells. Remarkably, we found no difference in IL-2 induced activation of STAT5 in Treg between RRMS and HC (Figure 9A). Similarly, the proportion of IL-2 responders or activation of STAT5 in responders were not different between RRMS and HC (Figure 9B and 9C). We further analyzed data using t-SNE. Unexpectedly, at basal and following 15 min of stimulation with IL-2, we found that distinct Treg cells from RRMS and HC clustered independently into distinct clusters indicating that they represented populations with different phenotypic characteristics (Figure 10). The clusters that showed maximal activation of STAT5 following 15 min IL-2 stimulation in RRMS and HC

did not overlap. Following stimulation for 240 min with IL-2 we observed remarkable overlap between RRMS and HC (Figure 10). We further analyzed the data employing FlowSOM. Here again we observed striking differences between Treg cell populations from RRMS and HC (Figure 11). Specifically, we observed complete absence of nodes in RRMS compared to that in HC. Overall these data have identified phenotypic differences Tregs from RRMS and HC. The functional significance of this finding needs to be elucidated.

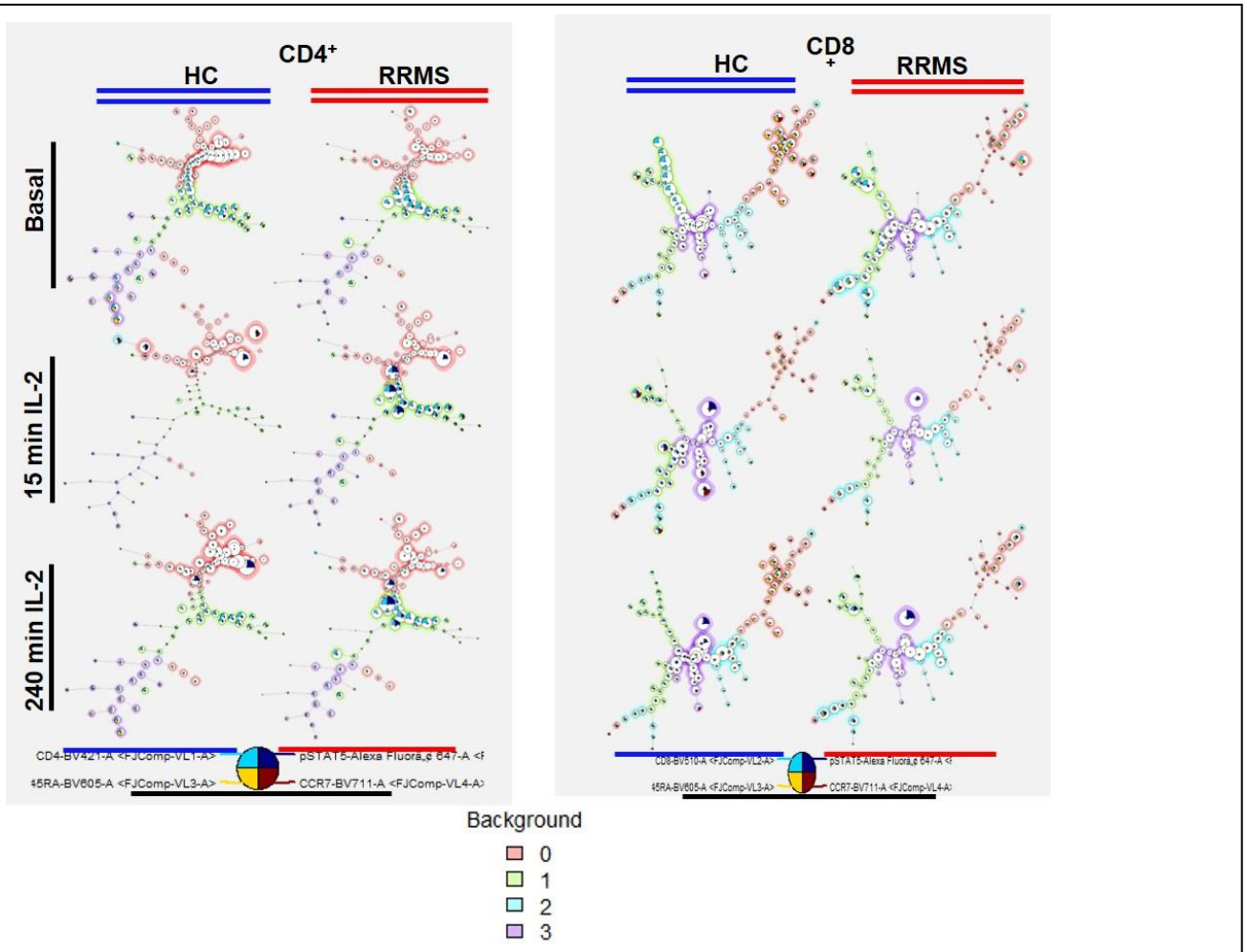

Figure 8: Automated clustering nodes show difference in cell populations and IL-2 induced activation of STAT5 in RRMS and HC. FLOWSOM analysis of the expression of CD4 (*left*) and CD8 (*right*) along with CD45RA, CCR7, and pSTAT5 in HC and RRMS at basal and following stimulation with IL-2 for 15 min or 240 min. Minimum spanning tree structure were compared qualitatively to assess the dynamics of fluctuations in the contraction and/or expansion of groups of nodes and marker profile of specific nodes clusters. Phenotypically related clusters of nodes are emphasized by the corresponding background color surrounding each node.

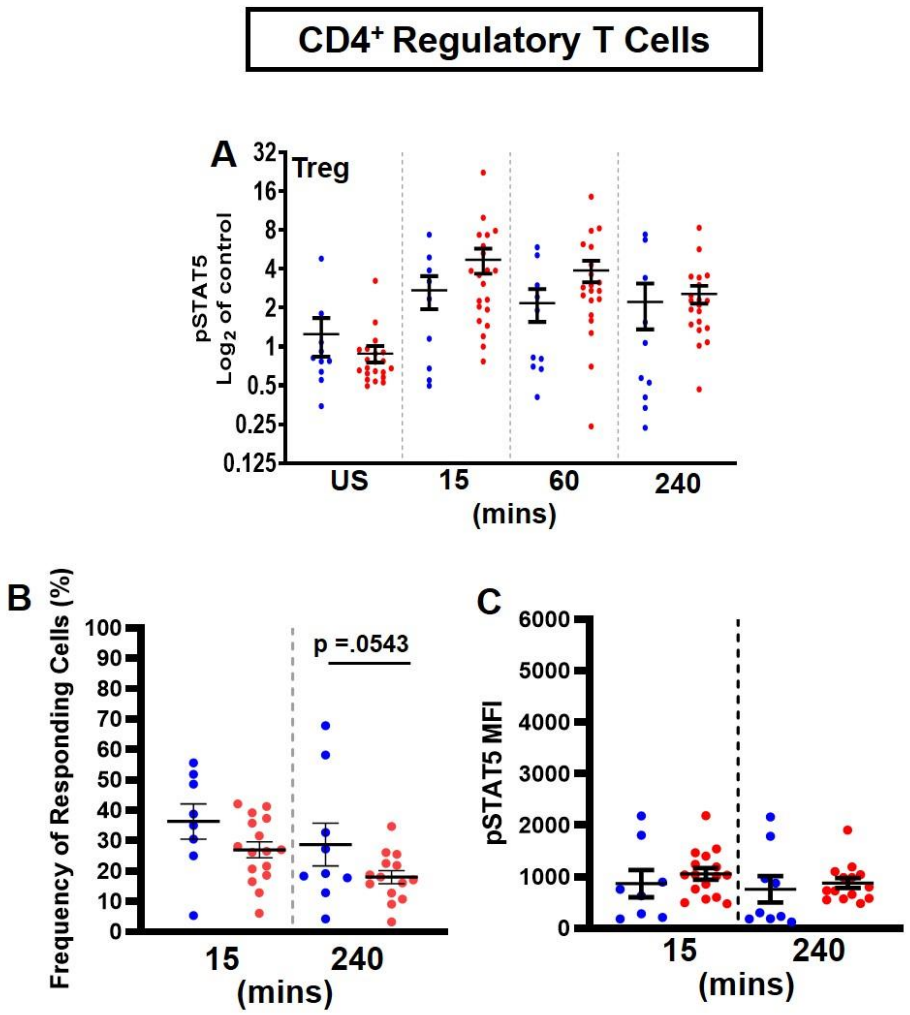

Figure 9: IL-2 induced activation of STAT5 in Treg cells from RRMS and HC are similar. . A. pSTAT5 levels in Treg cells ($CD25^{hi}$, $CD127^{lo}$) from HC (blue) or RRMS (red) at basal (US) or following stimulation with IL-2 for various time points as indicated. B. Proportion of high IL-2 responders and C. Levels of pSTAT5 in high responders. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the effect of disease, stimulation time, and the interaction of these two factors as they relate to pSTAT5 expression. Post hoc Student's t tests were performed using Bonferroni's correction to approximate the appropriate familywise error rate. p values were listed exactly as calculated.

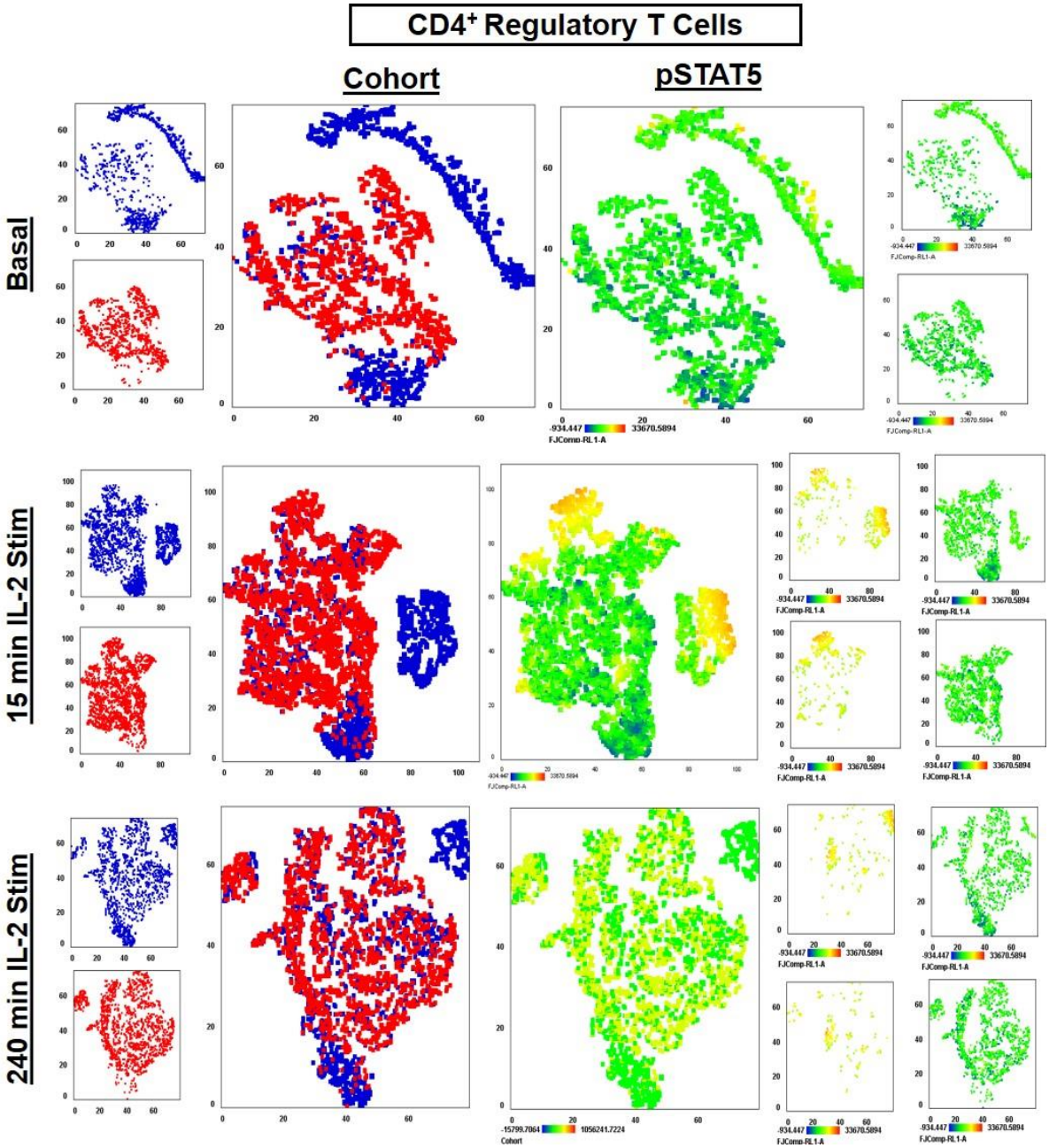

Figure 10: IL-2 induced activation of STAT5 in Treg cells from RRMS and HC are similar. . A. pSTAT5 levels in Treg cells ($CD25^{hi}$, $CD127^{lo}$) from HC (blue) or RRMS (red) at basal (US) or following stimulation with IL-2 for various time points as indicated. B. Proportion of high IL-2 responders and C. Levels of pSTAT5 in high responders. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the effect of disease, stimulation time, and the interaction of these two factors as they relate to pSTAT5 expression. Post hoc Student's t tests were performed using Bonferroni's correction to approximate the appropriate familywise error rate. p values were listed exactly as calculated.

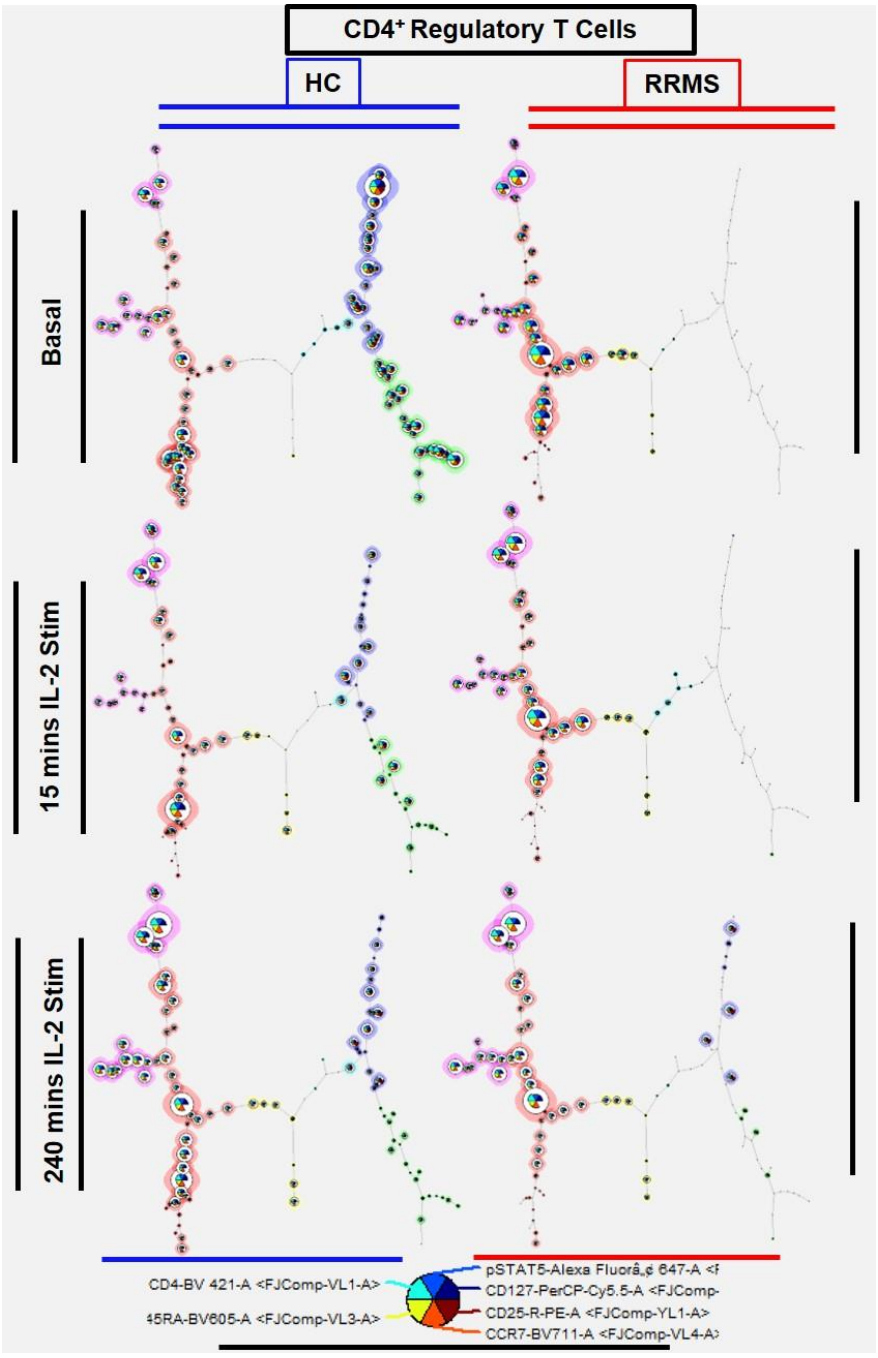

Figure 11: Contraction of specific populations of clustered nodes of Tregs in RRMS. FLOWSOM analysis of the expression of CD4, CD45RA, CCR7, CD25, CD127, and pSTAT5 in HC and RRMS at basal and following stimulation with IL-2. Minimum spanning tree structure were compared qualitatively to assess the dynamics of fluctuations in the contraction and/or expansion of groups of nodes and marker profile of specific nodes clusters. Phenotypically related clusters of nodes are emphasized by the corresponding background color surrounding each node.

3. Significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative)

Major findings:

1. IFNγ receptor expression in CD4 and CD8 T subsets in RA and MS are not different.
2. IFN-γ induced activation of STAT-1 in RA CD4 T cell populations is lower than HC and RA.
3. IFN-γ induced activation of STAT-1 in RA may be different between patients with active disease and those in remission. The difference may become more evident following using unbiased computational analysis such as T-SNE and FlowSOM (see example in Figure 5 below).
4. IL-2 co-stimulation enhances IFN-γ induced activation of STAT1 in CD4 T cell populations from RA but not HC and MS.
5. In HC and MS, IL-2 co-stimulation contributed to sustained activation of IFN-γ induced activation of STAT-1.
6. Conventional analysis of flow cytometry data limits the ability to focus on heterogeneity of expression of markers common in human. This is because the analyses reflect analysis of expression of two markers at a time with sequential gating. Advanced computational analyses favor un-biased and favor visualization of multi-dimension marker expression profile. These include t-SNE (t-distributed scholastic neighbor embedding) and FLOW-SOM, an algorithm that clusters with Self-Organizing-Maps. We have begun to use these for analysis data obtained from the experiments performed in this proposal, an example of which is shown in Figure 6. As the representative analysis shows, t-SNE clustering reveals similarities and differences between HC and MS that would have been missed by standard flow cytometry analysis. Similarly, FLOW-SOM identified striking differences between HC and MS with respect to nodes that are activated by IL-2. We will be performing such analyses for all of the data from this study.
7. There is strikingly attenuated signaling capacity in RA patients in IFNα stimulation followed by measurement of phosphorylated STAT1 [IFNα stimulation using phosphorylated STAT1 as a readout] in six immune cell subsets (see Appendix 1 for details).
8. Multiple nodes showed negative association with RA disease activity, including IFNα→STAT5 signaling in naive and memory B cells. In contrast, IL-6-induced STAT1 and STAT3 activation in central memory CD4-negative T cells showed a positive association with RA disease activity.
9. Multiple nodes were associated with treatment response, including IFNα→STAT1 in monocytes and IL-6→STAT3 in CD4+ naive T cells.
10. IL-2 induced activation of STAT5 is greatly enhanced in MS non-regulatory CD4 and CD8 T cell populations but not in Tregs compared to HC.
11. MS Treg populations represent a distinct population from HC as determined by algorithm based data analysis approaches, t-SNE and FlowSOM.

4. What opportunities for training and professional development has the project provided?

While this project was not intended to provide training and professional development opportunities, it has been an excellent training vehicle for several learners. This project provided opportunities for training for two PhD students, Vishal Sharma, and Brandon Pope. These

trainees have benefited from mentorship from Drs. Raman and Bridges.

5. Describe how the results were disseminated to communities of interest.

As noted above, we have analyzed existing data from the TETRAD cohort as part of Specific aim 2. This was uploaded for public distribution through biorxiv, a pre-print server (see Appendix 1)).

6. What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

We have contributed to the field by delineating the mechanisms of cell signaling of lymphocytes in the autoimmune diseases RA and MS. In addition, we have identified potential biomarkers of RA disease activity or treatment response.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

As outlined in section 3, there were changes to several subtasks due to results of data obtained during this course of this grant. In addition, we acquired data from the TETRAD study which helped greatly to advance our goals.

Actual or anticipated problems or delays and actions or plans to resolve them

During the study, enrollment was slightly slower than anticipated. In addition, during the no-cost extension (Sep 1 2019 to Aug 31 2020), the pace of our studies was greatly influenced by the COVID-19 pandemic. All research deemed non-essential (any research not directed at COVID-19 was halted. When a partial re-opening occurred, we were unable to recruit

participants at the previous rate.

Changes that had a significant impact on expenditures

There were no changes that had a significant impact on expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

No significant changes.

Significant changes in use or care of vertebrate animals.

No significant changes.

Significant changes in use of biohazards and/or select agents

No significant changes.

6. PRODUCTS:

List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

Publications, conference papers, and presentations

Journal publications.
See Appendix 1 for biorxiv preprint which is now under review at PLoS ONE.

Books or other non-periodical, one-time publications.
Nothing to report.

Other publications, conference papers, and presentations.

Presentations

Pope, B. J., Sharma, V, Boland, M, Reynolds, R., Bridges, S.L. Jr., and Raman, C. Enhanced IFN-γ STAT1 signaling in CD4 T cell populations and attenuated IL-2 STAT5 signaling contribute to the pathogenesis of rheumatoid arthritis (RA). *Presented at the annual meeting of the American College of Rheumatology, San Diego, November 3-8, 2017.*

Pope, B.J., Sharma, V., Boland, M., Meador, W.S., Bridges, S.L. and Raman, C. IL-2 enhances IFNγ signals in subpopulations of T and B lymphocytes from treatment naïve relapsing remitting multiple sclerosis (RRMS) patients. *Presented at the annual meeting of the American Association of Immunologists, Austin, May 4-8, 2018.*

Sharma, V., Pope, B. J., Boland, M., Reynolds, R., Sun, D., Bridges, S. L. and Raman, C. Enhanced interferon gamma response contributes to disease remission in rheumatoid arthritis. *Presented at the annual meeting of the American Association of Immunologists, Austin, May 4-8, 2018.*

Sharma, V, Pope, B., Boland, M., Reynolds, R., Sun, D, Bridges, S. L. and Raman, C. Enhanced interferon gamma response contributes to disease remission in Rheumatoid *Presented at the 7th Annual Southeastern Immunology Symposium; June 16 to 18, 2018, Birmingham, AL.*

Pope, B., Sharma, V., Boland, M., Meador, W., Reynolds, R., Bridges, S.L., and Raman, C. IL-2 enhances IFNγ signals in subpopulations of T and B lymphocytes from treatment naïve relapsing remitting multiple sclerosis (RRMS) patients. *Presented at the 7th Annual Southeastern Immunology Symposium; June 16 to 18, 2018, Birmingham, AL.*

Pope, B., Sharma, V., Boland, M., Meador, W., Reynolds, R., Bridges, S.L., and Raman, C. IL-2 enhances IFNγ signals in subpopulations of T and B lymphocytes from treatment naïve relapsing remitting multiple sclerosis (RRMS) patients. *Presented at the* American Committee for Treatment and Research in Multiple Sclerosis (ACTRIMS) 2019 Forum*; February 28 – March 2, 2019, Dallas, TX.*

Pope, B., Sharma, V., Bridges, S.L., and Raman, C., Meador, W. IL-2 enhances IFNγ signals in subpopulations of T and B lymphocytes from treatment, naïve relapsing remitting multiple sclerosis (RRMS) patients. *Presented at the Society for Neuroscience Annual Meeting; October 19 - 23, 2019, Chicago, IL.*

- Website(s) or other Internet site(s)
  Nothing to report.

- Technologies or techniques
  Nothing to report.

- Inventions, patent applications, and/or licenses
  Nothing to report.

- Other Products
  Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*S. Louis Bridges, Jr., MD, PhD*
Project Role:	*Principal Investigator*
Researcher Identifier:	*ORCID ID: 0000-0003-3785-1389*
Nearest person month worked:	*1.2 calendar months*
Contribution to Project:	*Dr. Bridges has provided overall guidance for this project.*

He has led the effort to identify patients to be enrolled, overseen all studies in Aim 1 and worked closely with Dr. Raman on all lab-based studies of the project. He has supervised the Laboratory Manager (Mr. Wanzeck) and all non-lab-based study personnel. He has overseen the collection of clinical data, processing of blood samples, and all data management aspects of the project.

Name: *Chander Raman, PhD*
Project Role: *Co-Investigator*
Nearest person month worked: *4.8 calendar months*
Contribution to Project: *Dr. Raman has been critical to the success of this project.*
He has directed and overseen the functional/mechanistic studies. He has directly overseen all lab-based research personnel except for Mr. Wanzeck. Dr. Raman has worked closely with the PI on all three Aims of this project and has been key to data analysis, manuscript preparation and submission.

Name: *Richard Reynolds, PhD*
Project Role: *Co-Investigator*
Nearest person month worked: *1.2 calendar months*
Contribution to Project: *Dr. Reynolds has provided direct input into the study design, overall analysis plan, and statistical analyses for the project.*

Name: *Keith Wanzeck, BS*
Project Role: *Laboratory Manager Nearest person month worked:* *1.2 calendar months*
Contribution to Project: *Mr. Wanzeck has been responsible for coordination of blood collection, processing, and routing of biospecimens. He has served as a liaison between Dr. Bridges' lab and Dr. Raman's lab.*

Name: *Stephanie Ledbetter Miller, MS*
Project Role: *Program Manager*
Nearest person month worked: *1.2 calendar months*
Contribution to Project: *Ms. Miller has been responsible for all regulatory issues, including the UAB IRB and HRPO submissions.. She has coordinated other aspects of the study, including laboratory meetings, and other logistic issues.*

Name: *Vishal Sharma*
Project Role: *Graduate student*
Nearest person month worked: *4 calendar months*
Contribution to Project: *As part of his PhD studies, Mr. Sharma has performed dissertation research on this project, focusing on RA. He has performed assays, data analysis, and presented results from this project at scientific meetings.*

Name: *Brandon Pope*
Project Role: *MD/PhD student*
Nearest person month worked: *4 calendar months*
Contribution to Project: *As part of his PhD studies, Mr. Pope has performed dissertation research on this project, focusing on MS. He has performed assays, data analysis, and presented results from this project at scientific meetings.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

9. APPENDICES

None.