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This Thesis, The Effect of Cytokines on Granzyme K and Granzyme B Expression in $CD8^+$ T Cells, presented by Karishma Vijay Rupani, and Submitted to the Faculty of The Harvard Medical School in Partial Fulfillment of the Requirements for the Degree of Master of Medical Sciences in Immunology has been read and approved by:

Michael Brenner

Dr. Michael B. Brenner, MD

Dr. Anna Helena Jonsson, MD PhD

Date: 29th April, 2020

The Effect of Cytokines on Granzyme K and Granzyme B Expression in $CD8^+$ T Cells

Karishma Vijay Rupani

A Thesis Submitted to the Faculty of

Harvard Medical School

in Partial Fulfillment of the Requirements

for the Degree of Master of Medical Sciences in Immunology

Harvard University

Boston, Massachusetts

May 2020

Dissertation Advisor: Dr. Michael Brenner, MD Karishma Vijay Rupani

The Effect of Cytokines on Granzyme K and Granzyme B Expression in $CD8^+$ T Cells

$CD8^+$ T cells make up nearly half of all T cells in the synovium of patients with rheumatoid arthritis (RA). To date, most studies have focused on the role of $CD4^+$ T cells in RA. Recent unbiased studies of T cell subsets in RA synovium using single cell RNA sequencing revealed that the majority of $CD8^+$ T cells expressed granzyme K (GzmK), either alone or in combination with granzyme B (GzmB). GzmK has pro-inflammatory effects on synovial fibroblasts, inducing them to produce IL-6, CCL2, and reactive oxidative species (ROS), all of which are up regulated in RA synovium. In this project, we studied whether cytokines regulated GzmK and GzmB expression.

To test the effects of selected candidate cytokines on GzmK and GzmB expression in $CD8^+$ T cells, we designed an experimental system. $CD8^+$ T cells from healthy control blood were cultured for up to 4 days in the presence of selected cytokines, either alone or in combination. The cytokines were tested with and without concurrent TCR stimulation, using plate-bound anti-CD3 antibodies and soluble anti-CD28 antibodies. On days 0, 1, 2, 3, and 4, the harvested cells were stained for surface activation markers (CD69 and HLA DR) and intracellular markers (GzmK, GzmB, Nur77, and Ki67). Data were collected on a BD Fortessa flow cytometer and analyzed using FlowJo 10.5 software.

Stimulating peripheral blood $CD8^+$ T cells via their TCR led to GzmB expression. Furthermore, $CD8^+$ T cells showed no increase in GzmK and GzmB co-expression following individual cytokine stimulation. However, stimulation with IL-12 in combination with IL-15 (in the absence of TCR stimulation) led to the maintenance of GzmK and GzmB expression, and an up regulation of the activation markers CD69, HLA DR, and Ki67. Studying the role of cytokines in the differentiation and activation of $CD8^+$ T cells can help us better understand the factors that drive RA pathogenesis.

Table of contents:

List of figures:

List of tables:

Acknowledgements:

I would first like to thank Dr. Michael Brenner for giving me the opportunity to join his lab. I will always be grateful for his mentorship, encouragement and kindness. I want to express my deepest gratitude to my mentor, Dr. Anna Helena Jonsson. It has been an honor and privilege to train under her guidance, and I am grateful to her for teaching me everything I know.

I am fortunate to have had the opportunity to be a part of the Brenner Lab. I would like to thank Emma Gomez-Rivas, Gerald Watts, Edy Kim, Alisa Mueller, Nelson LaMarche, Carlos Donado, Hung Nguyen, Deepak Rao, Aidan Cullen, Evelyn Montes, Daimon Simmons, Heather Faust, Byunghee Koh, Bill Apruzzese, Kurt Kongthong, and Kevin Wei.

Thank you to my HMS mentors, Dr. Shiv Pillai and Dr. Mike Carroll. I remain indebted to Dr. Pillai for really teaching me immunology, for his mentorship and for always advocating for my success. My thesis writing was guided by Dr. Carroll's constant guidance and direction. Thank you also to Dr. Gavin Porter and Selina Sarmiento. I would also like to express my gratitude to the Class of 2020, in particular Isaac Benque, Kanupriya Kusumakar, Michelle Dong, Aarti Srivastava and Diana Pena Nunez.

Finally, I would like to thank my parents and my brother for their unwavering support and encouragement.

This work was conducted with support from students in the Master of Medical Sciences in Immunology program of Harvard Medical School. The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard University and its affiliated academic health care centers.

Introduction:

Rheumatoid arthritis (RA) is an autoimmune disease that primarily affects the joints resulting in pain and stiffness. It involves chronic inflammation of the synovium leading to destruction of the joint cartilage and bone. Symptoms come on gradually over weeks to months. Extra-articular manifestations of RA include vasculitis and lung injury.[1] Like other autoimmune diseases, RA is a complex, multifactorial disorder in which genetic and environmental factors contribute to the breakdown of tolerance to self-antigens. With prevalence rates between 0.5 and 1%, RA affects about 24.5 million people in the United States. Onset is most frequent during middle age, and women are affected 2.5 times as frequently as men.[2] A positive family history increases the risk of rheumatoid arthritis roughly three to five times.[3] Furthermore, environmental insults, such as smoking and periodontal disease, induce modification of self-proteins, leading to the creation of new antigenic epitopes.[1,2] Because these modified epitopes are neo-antigens that are not usually present, there may not be existing tolerance to these antigens.

Both humoral and cell-mediated immune responses contribute to development of synovitis. Activated B cells and plasma cells are often present in the synovium of affected joints. Patients also frequently have circulating rheumatoid factor antibodies (IgM or IgG antibodies that react with the Fc portions of self-IgG molecules), and anti-citrullinated protein antibodies (antibody against proteins that are modified by the conversion of arginine residues to citrulline).[1] Individuals with HLA alleles that are capable of presenting these epitopes may mount T cell and antibody responses against the proteins.[4,5]

The strongest risk alleles detected in genome wide association studies (GWAS) fall in genes encoding the HLA class II molecule, HLA DR.[1, 6–10] While the mechanisms underlying RA are unclear, HLA DR4 is widely acknowledged as being associated with RA. Different MHC risk alleles share conserved features in the peptide-binding groove (DR4/DR1). These

MHC risk alleles can present arthritogenic antigens to T cells. While the association between the HLA DR locus and RA has led to the widely agreed hypothesis that $CD4^+$ T cells are directly implicated in the disease, several other findings also support the role of $CD4^+$ T cells in RA. For example, the presence of Th1 $CD4^+$ T cells and regulatory T cells (Treg) have been reported in the synovium of RA patients.[11] A recent study from our lab reported a new subset of $CD4^+$ T cells present in RA known as peripheral T helper cells (T_{ph}). T_{ph} cells express MHC class II, high levels of PD-1, and provide B-cell help in RA.[12]

In contrast to $CD4^+$ T cells, little is known about $CD8^+$ T cell subsets in RA. One reason for this may be that the prototypical effector function of $CD8^+$ T cells is cytotoxicity. These effector cells known as cytotoxic T lymphocytes (CTLs) carry granules containing cytotoxic proteins such as perforin and granzyme B (GzmB). Perforin is a membrane-pore forming molecule whose main function is to facilitate delivery of the granzymes into the cytosol of the target cell.[13,14] Once in the cytosol, the granzymes cleave various substrates leading to downstream effector functions. For example, GzmB cleaves and activates caspase-3, as well as the Bcl-2 family member Bid, which triggers the mitochondrial pathway of apoptosis.[15–19]

However, not all $CD8^+$ T cells are cytotoxic. In humans, five granzymes with differing substrate specificity have been identified.[20,21] Unlike GzmB, granzyme K (GzmK) does not activate cytotoxic caspases. Instead, GzmK induces synovial fibroblasts to produce IL-6, CCL2, and reactive oxygen species (ROS), all of which are elevated within RA synovium and lead to inflammation.[22,23]

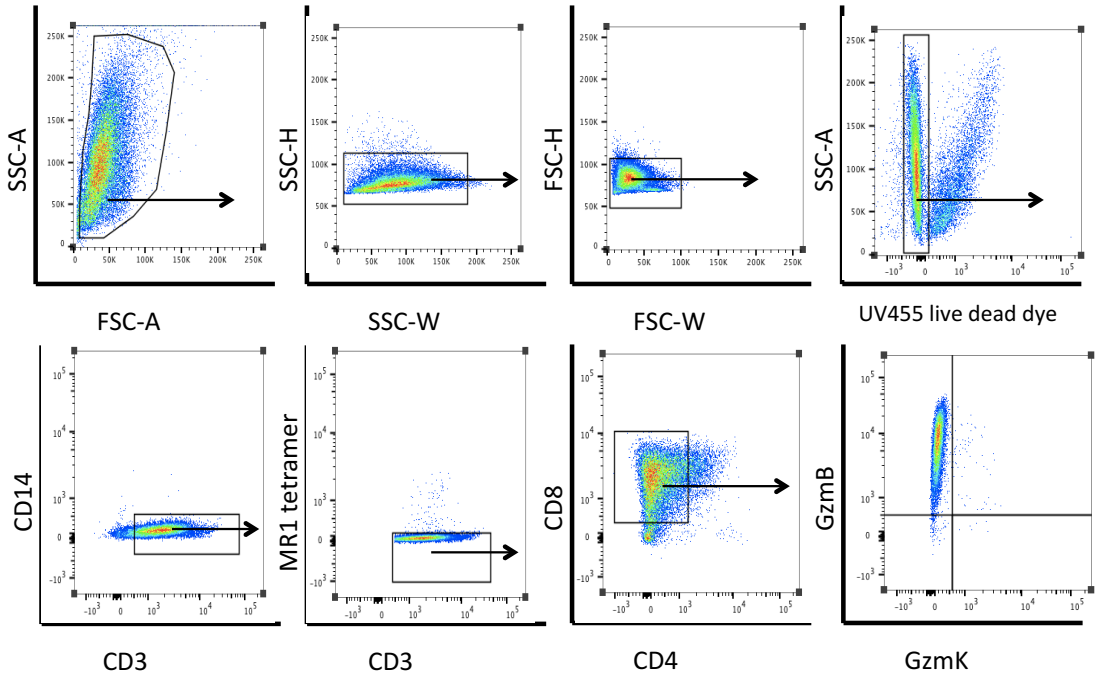


Figure 1: **Gating strategy** (left to right) to identify GzmK$^+$ GzmB$^+$ CD8$^+$ T cell populations by flow cytometry. We excluded mucosal-associated invariant T (MAIT) cells, innate T cells restricted by MHC-related molecule 1 (MR1), from our analysis because they are known to express high levels of GzmK.

Our understanding of GzmK at sites of inflammation is limited, but we know that GzmK and GzmB have different expression patterns during CD8$^+$ T cell differentiation. Different states of CD8$^+$ T cell differentiation can be identified on the basis of the expression of CD45RO and CCR7. Within the memory T cell compartment CD45RO$^+$ CCR7$^+$ central memory CD8$^+$ T (TCM) cells home to secondary lymphoid organs mounting robust recall responses, whereas the CD45RO$^+$ CCR7$^-$ effector memory CD8$^+$ T (TEM) cell subset is commonly found in non-lymphoid tissues.[24–30] Memory CD8$^+$ T cells express different combinations of granzymes at various stages of differentiation: TCM cells are GzmB$^-$GzmK$^+$ whereas cells at an intermediate stage of differentiation are GzmB$^+$GzmK$^+$ and finally, TEM cells are GzmB$^+$GzmK$^-$.[31]

While most studies on RA pathophysiology have focused on the role of CD4$^+$ T cells, there are indications in the literature that CD8$^+$ T cells also contribute to disease

pathogenesis. A recent paper from our lab showed that $CD8^+$ T cells comprise up to 40% of synovial leukocytes. Furthermore, $CD8^+$ T cells producing TNF and IFNγ are enriched in RA synovium and may contribute to the inflammatory phenotype. Interestingly, synovial $CD8^+$ T cells express IFNγ at a higher frequency, and TNF at an equivalent frequency compared to $CD4^+$ T cells.[32] Production of TNF, IFNγ and IL-17 positively correlates with the 28-joint disease activity score (DAS28), and the increased cytokine production by $CD8^+$ T cells in the blood of patients with RA normalizes in the remission phase of the disease.[33] $CD8^+$ T cells within RA synovium also express CD69 and HLA DR, indicating their activated state.[34,35]

The recent advent of unbiased large-scale single cell RNA sequencing technology has given us a new insight into the cells responsible for RA pathophysiology. The Accelerating Medicines Partnership (AMP) is an NIH-sponsored consortium that recently applied this technology to synovial tissue from patients with RA to complete an extensive study of the cells involved.[32] Among all T cells investigated in this study, half were $CD8^+$ T cells. These $CD8^+$ T cells in the synovium have an unexpected gene expression profile wherein only a small minority of $CD8^+$ T cells express genes encoding GzmB and high levels of perforin. Instead, the majority of $CD8^+$ T cells express GzmK, either alone or in combination with GzmB, a finding that has been confirmed at the protein level (Figure 2).

$CD8^+$ T cell differentiation is driven by multiple mechanisms including antigen stimulation, co-stimulatory signals and cytokine-mediated differentiation. $CD8^+$ T cells migrating into the joint may be further affected locally. The goal of my project was to determine the factors that promote blood $CD8^+$ T cells to co-express GzmK and GzmB. We hypothesized that migration through the blood into the inflamed joint, local differentiation at the site of tissue inflammation, TCR stimulation, cytokine stimulation, or TCR stimulation in the presence of cytokines, might contribute to the development of a $GzmK^+$ $GzmB^+$ phenotype.

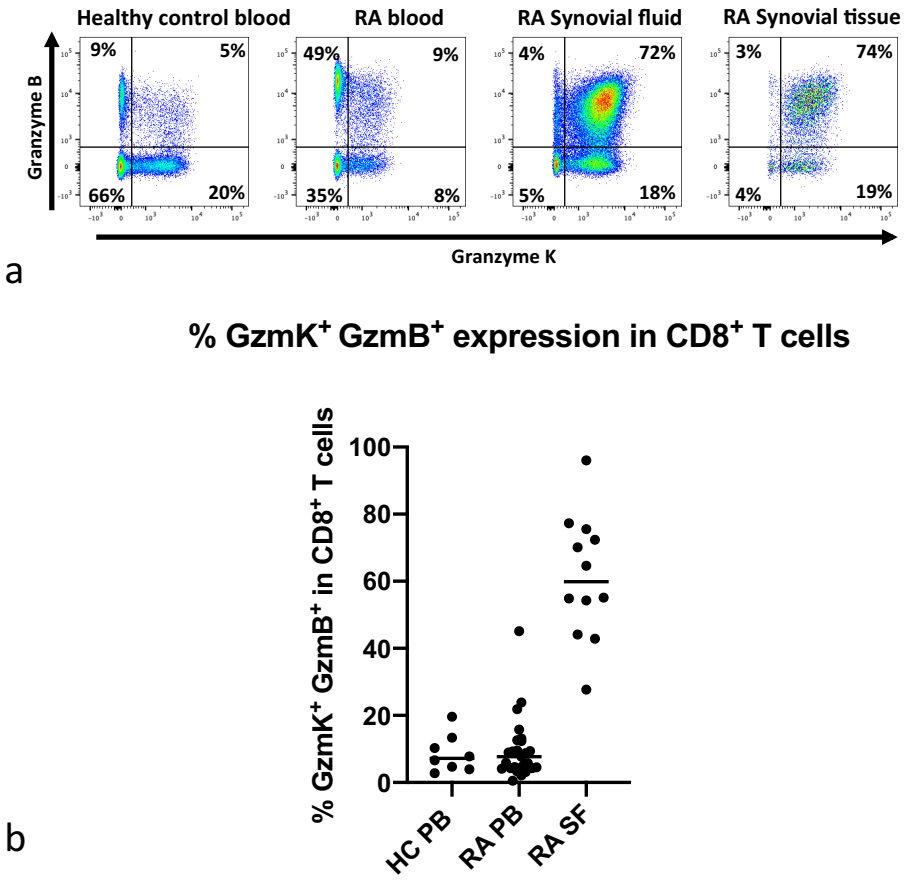


Figure 2: **Majority of the CD8$^+$ T cells in synovial fluid and synovial tissue of patients with RA express GzmK (with or without GzmB)**. a) Gzm K (x axis) and Gzm B (y axis) expression in CD8$^+$ T cells isolated from healthy control blood, blood from a patient with RA, synovial fluid from a patient with RA, and synovial tissue from a patient with RA. b) CD8$^+$ T cells isolated from RA synovial fluid (RA SF) express greater levels of GzmK GzmB compared to healthy control peripheral blood (HC PB) and RA peripheral blood (RA PB). *Figures 2a and 2b show previously unpublished data courtesy of Dr. Anna Helena Jonsson, MD PhD and Dr. Michael Brenner, MD.*

Preliminary experiments showed that stimulation of CD8$^+$ T cells through their TCR leads to a uniformly GzmB$^+$ phenotype (Figure 3). We used plate bound anti-CD3 antibodies and soluble anti-CD28 antibodies to stimulate CD8$^+$ T cells from healthy control blood, and saw a uniformly GzmB$^+$ phenotype. However, this is not the granzyme expression pattern we see in RA synovial fluid and synovial tissue. We therefore decided to test whether cytokines (with or without TCR stimulation) lead to GzmK and GzmB expression in CD8$^+$ T cells.

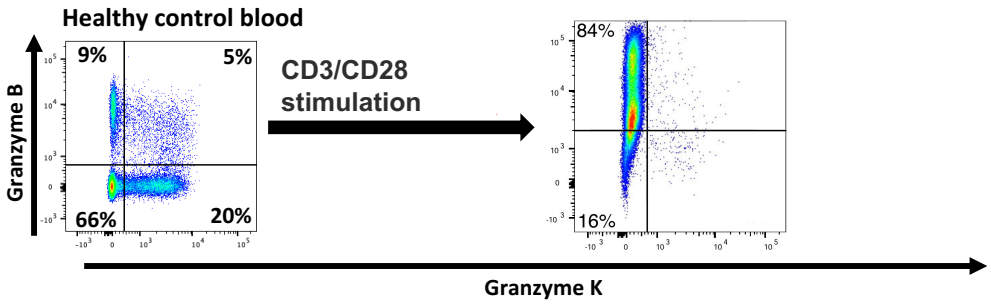


Figure 3: **TCR stimulation of CD8$^+$ T cells from healthy control blood leads to GzmB$^+$ expression.** CD8$^+$ T cells from blood were stimulated with plate bound anti-CD3 antibodies and soluble anti-CD28 antibodies, leading to uniformly GzmB$^+$ expression.

Results:

Materials and methods:

Blood bank leuko-reduction collars from Brigham and Women's Hospital, were subjected to density centrifugation using Ficoll to isolate mononuclear cells (PBMCs). The PBMCs were cryopreserved in liquid nitrogen using fetal calf serum (Gemini) containing 10% DMSO. After thawing into warm media, CD8$^+$ T cells were then purified from PBMCs using magnetic bead negative selection as per the manufacturer's protocol (Miltenyi). Flat-bottomed 96-well tissue culture plates were coated with low endotoxin, azide-free anti-CD3 antibodies (Biolegend, clone OKT3, Cat# 317326) at a concentration of 10ug/ml. After washing, 150,000 CD8$^+$ T cells were added to each well. We then added the indicated cytokine(s) at a final concentration of 100ng/ml and incubated the cells at 37C, 5% CO_2.

On days 0, 1, 2, 3, and 4, the harvested cells were incubated with Fixable Viability Dye UV455 (eBioscience) and human Fc blocking antibodies (eBioscience), followed by staining for surface markers in Brilliant Stain Buffer (BD Bioscience). Cells were then fixed and permeabilized using an intracellular staining kit (Biolegend), followed by intracellular staining for granzyme expression. Data were collected on a BD Fortessa flow cytometer and

analyzed using FlowJo 10.5 software. Staining was performed with the following antibodies (Table 1):

Table 1: Antibodies used for flow cytometry analysis

Marker	Fluorophore	Clone	Vendor
MR1 tetramer	BV421	N/A	NIH tetramer core
CD14	BV510	M5E2	BioLegend
Ki67	BV605	Ki67	BioLegend
CD3	BV650	UCHT1	BioLegend
HLA DR	BV785	L243	BioLegend
Granzyme K	FITC	GM26E7	BioLegend
CD69	PerCP eF710	FN50	BioLegend
Nur77	PE	12.14	Thermo Fisher
CD4	PE Cy7	RPA T4	BioLegend
Granzyme B	AF647	GB11	BioLegend
CD8	APC eF780	RPA T8	BioLegend

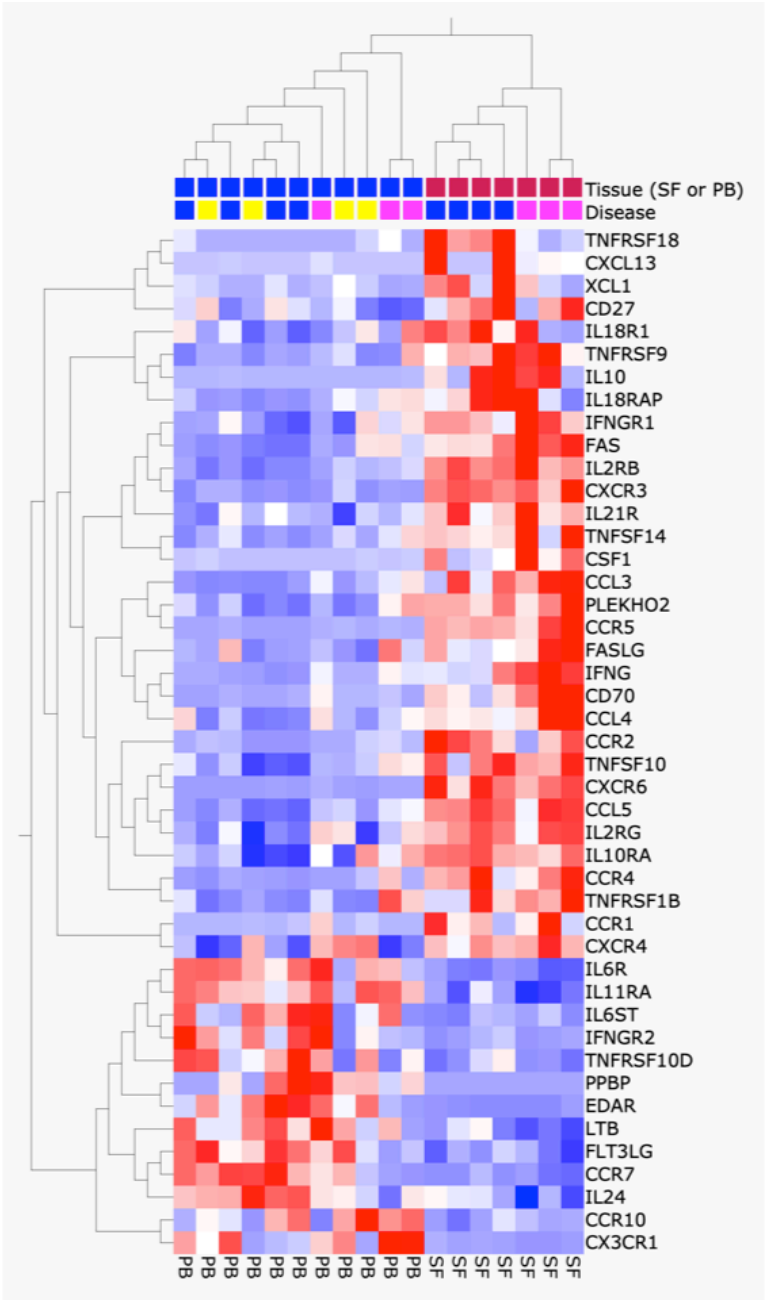


Figure 4: **Cytokine receptor expression by CD8$^+$ T cells in peripheral blood and synovial fluid.** CD8$^+$ T cells were sorted from peripheral blood (PB) or matched synovial fluid (SF) from patients with RA (pink), juvenile idiopathic arthritis (blue) and healthy controls (yellow). 1,000 sorted CD8$^+$ T cells from each sample were submitted for low-input RNA sequencing. Shown above is a heat map of expression of selected cytokine receptor genes. High relative expression is in red and low relative expression is in blue. *This figure shows previously unpublished data courtesy of Dr. Anna Helena Jonsson, MD PhD and Dr. Michael Brenner, MD*

Granzyme expression:

As discussed in the introduction, $CD8^+$ T cells in RA synovium have an unusual phenotype, in that the majority express GzmK (+/-GzmB). The percentage of cells positive for both granzymes was highest in RA synovial fluid (72%) and RA synovial tissue (74%). This is higher than RA blood, where only about 10% of cells are positive for both granzymes (Figure 2a). We hypothesized that cytokines may drive GzmK expression.

Based on bulk RNA sequencing data generated by Dr. Anna Helena Jonsson, MD, PhD (Brenner lab), genes encoding the following cytokine receptors are expressed by $CD8^+$ T cells in synovial fluid: IL18R1, IL18RAP, TNFRSF9 (4-1BB, CD137), IFNGR1, IL2RB (IL-2 and IL-15), IL2RG (common gamma chain), IL21R (with common gamma chain), IL10RA, and TNFRSF1B (TNFR2). In contrast, IL6R, IL6ST, IL11RA, IFNGR2, and TNFRSF10D (TRAILR4, DCR2) are enriched on $CD8^+$ T cells in peripheral blood. Using these data from the bulk RNA sequencing (Figure 4), we conducted a literature review to confirm the presence of the above cytokines and others, in the serum and synovial fluid of patients with RA (Table 2):

Table 2: Literature review of cytokine levels in RA synovial fluid

Cytokine	RA synovial fluid cytokine levels (pg/ml)	References
IL-2	38	Sanmarti, 2013[36]
IL-4	5	Krtinic, 2014[37]
IL-6	1260	Sanmarti, 2013[36]
IL-7	34	Ponchel, 2008[38]
IL-8	161	Sack, 2013[39]
IL-10	24	Moller, 1998[40]
IL-12	7	Kim, 2000[41]
IL-15	4	Guillen Nieto, 2015[42]
IL-17	13	Krtinic, 2014[37]
IL-18	2	Bresnihan, 2004[43]
IL-21	820	Sanmarti, 2013[36]
IL-23	311	Sanmarti, 2013[36]
IFNβ	Detected by immunohistology	Tak, 2005[44]
IFNγ	4	Krtinic, 2014[37]
TNF	100	Moller, 1998[40]

To test the effects of selected candidate cytokines on GzmK and GzmB expression in $CD8^+$ T cells, we designed an experimental culture system (Figure 5). In brief, $CD8^+$ T cells from healthy control blood were cultured for up to 4 days in the presence of selected cytokines, either alone or in combination. The cytokines were tested with and without concurrent TCR stimulation, using plate-bound anti-CD3 antibodies and soluble anti-CD28 antibodies. The pooled results of those experiments are discussed below.

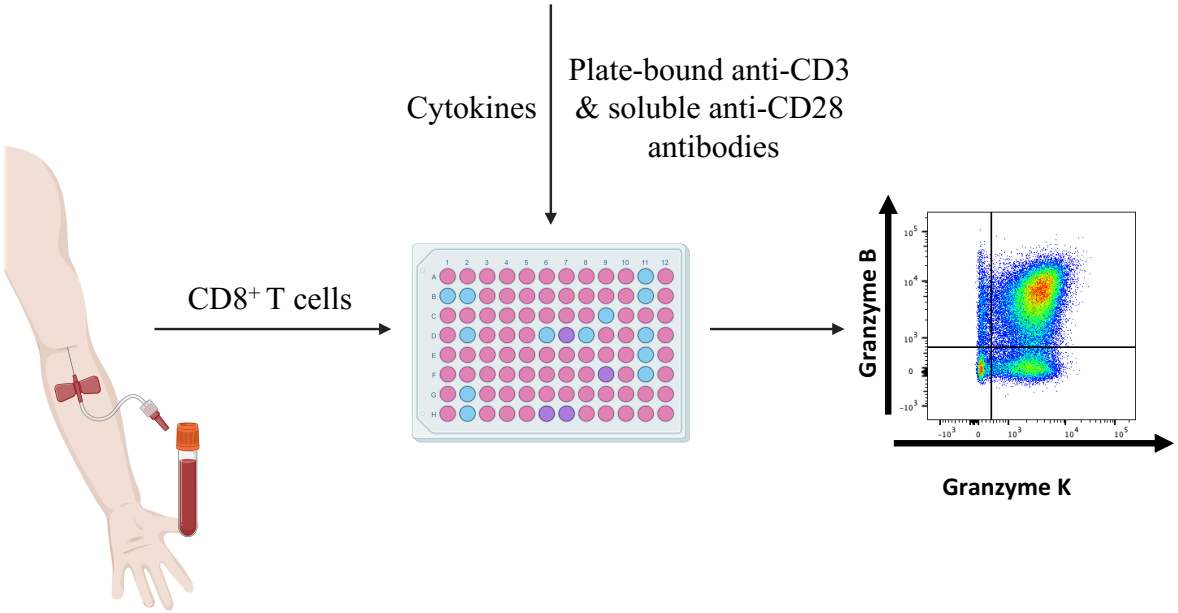


Figure 5: Experimental setup: $CD8^+$ T cells were cultured for up to 4 days in the presence of selected cytokines, either alone or in combination. The cytokines were tested with and without concurrent TCR stimulation (using plate-bound anti-CD3 antibodies and soluble anti-CD28 antibodies).

Addition of single cytokines alone to $CD8^+$ T cells led to no change in granzyme expression. Following treatment with indicated cytokines IL-6, IL-7, IL-10, IL-33, IFNβ, IFNγ, TNF and TGFβ for 4 days, $CD8^+$ T cells showed no increase in GzmK and GzmB co-expression, and instead remained unchanged from their unstimulated phenotype. We also tested these cytokines in the presence of concurrent TCR stimulation using plate-bound anti-CD3 antibodies and soluble anti-CD28 antibodies. Following treatment with cytokines in the presence of TCR stimulation for 4 days, $CD8^+$ T cells again showed no increase in GzmK

and GzmB co-expression (Figure 6). The $CD8^+$ T cells stimulated with cytokines in the presence of TCR stimulation instead became uniformly $GzmB^+$ (as seen in Figure 3).

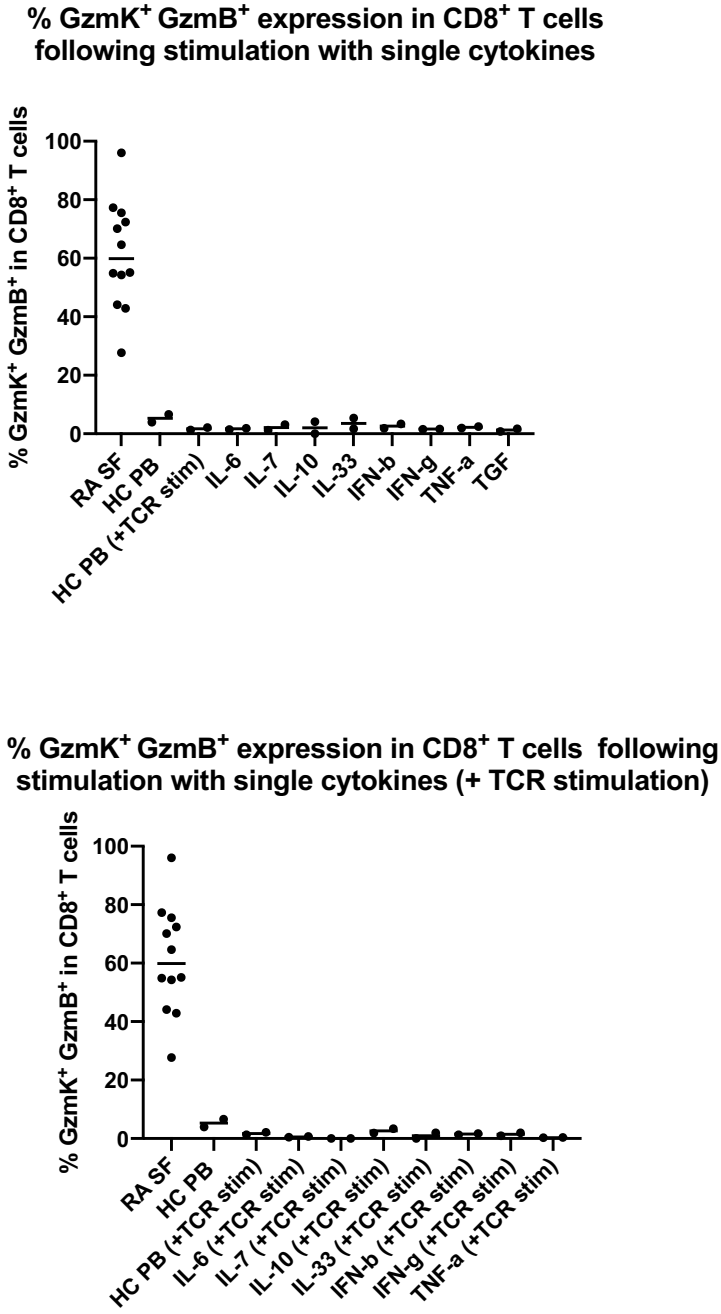
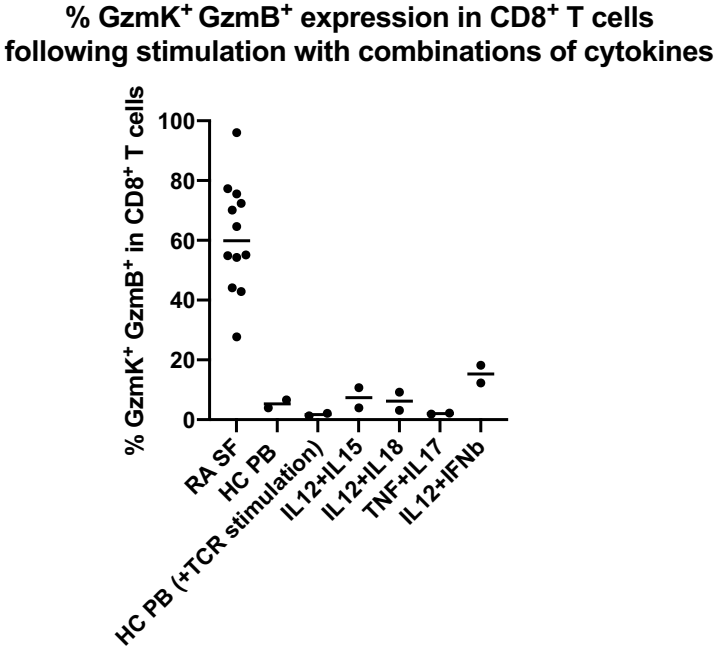


Figure 6: **Cytokine stimulation of $CD8^+$ T cells (+/- TCR stimulation) led to no change in $GzmK^+$ $GzmB^+$ expression.** Cells were cultured for 4 days in the presence of the indicated cytokines, and then stained for GzmK and GzmB expression. This graph shows the percentage of $CD8^+$ T cells in the $GzmK^+$ $GzmB^+$ quadrant gate. $CD8^+$ T cells from RA synovial fluid (RA SF), healthy control peripheral blood (HC PB), and HC PB in the presence of TCR stimulation are shown for reference.

In a study by Freeman et al, cytokine combinations were shown to be more effective than single cytokines at modulating the activation of $CD8^+$ T cells in the absence of stimulation through the T-cell receptor, with combinations that included IL-12 being the most effective.[46] Based on this study, we treated $CD8^+$ T cells with combinations of cytokines in the presence and absence of TCR stimulation. We prioritized combinations that were found to be particularly potent or combinations with special relevance in RA (e.g. TNF + IL-17).

Following treatment with cytokines IL-12 + IL-15, IL-12 + IL-18, TNF + IL-17, and IL-12 + IFNβ for 4 days, only $CD8^+$ T cells treated with IL-12 + IFNβ showed an increase in GzmK and GzmB co-expression (Figure 7). Although it was only a modest increase, it indicated that perhaps GzmK and GzmB co-expression increased at earlier time-points.

We next decided to vary the duration of cytokine stimulation given the possibility that GzmK expression was up regulated prior to day 4. Therefore, as a next step, we tested two sets of combination cytokines over 3 different time points: day 0, day 1 and day 4. Following treatment with cytokines IL-12 + IL-15 and IL-12 + IFNβ, we saw a modest increase in GzmK and GzmB expression in cells that were treated with IL-12 + IL-15 (Figure 8). We did not see an increase in $GzmK^+$ $GzmB^+$ $CD8^+$ T cells after treatment with IL-12 + IFNβ, which suggested that our initial observations (Figure 7) were not reproducible.



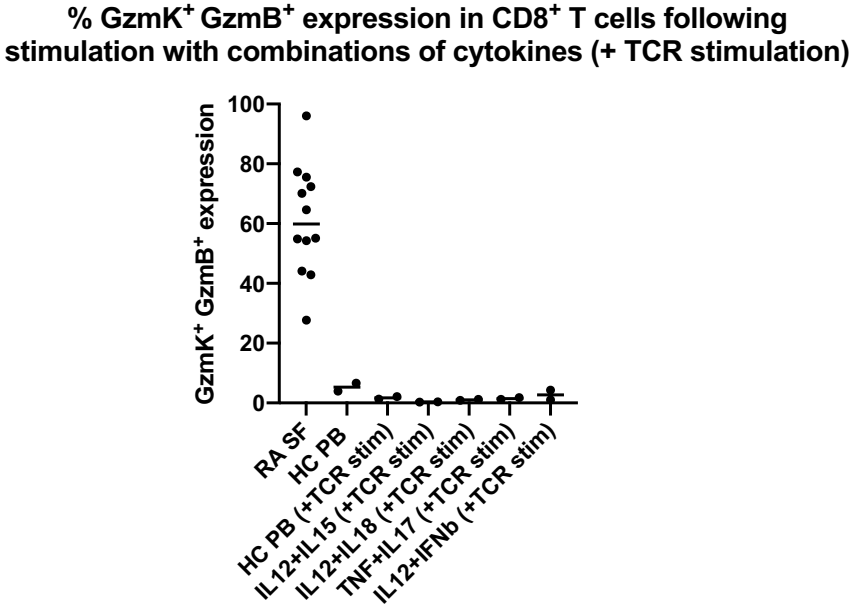


Figure 7: Combination cytokine stimulation of CD8$^+$ T cells (+/- TCR stimulation) led to modest change in GzmK$^+$ GzmB$^+$ expression following treatment with IL-12 + IFNβ. Cells were cultured for 4 days in the presence of the indicated cytokines, and then stained for GzmK and GzmB expression. This graph shows the percentage of CD8$^+$ T cells in the GzmK$^+$ GzmB$^+$ quadrant gate. CD8$^+$ T cells from RA synovial fluid (RA SF), healthy control peripheral blood (HC PB), and HC PB in the presence of TCR stimulation are shown for reference.

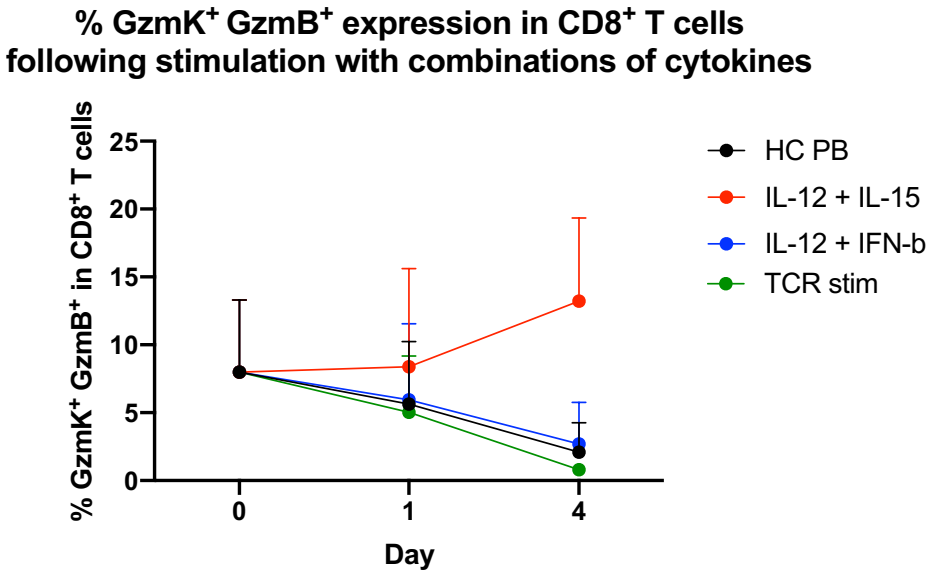


Figure 8: Stimulation of $CD8^+$ T cells with IL-12 + IL-15 led to the maintenance of $GzmK^+$ $GzmB^+$ expression. Cells were cultured for 1 or 4 days in the presence of the indicated cytokines, and then stained for $GzmK^+$ and $GzmB^+$ expression. This graph shows the percentage of $CD8^+$ T cells in the $GzmK^+$ $GzmB^+$ quadrant gate. $CD8^+$ T cells from healthy control peripheral blood (HC PB), and HC PB in the presence of TCR stimulation are shown for reference.

To further delineate the timeline, we harvested cytokine stimulated cells everyday for 4 days by varying the duration of cytokine stimulation at 24-hour intervals. We tested the cytokines IL-12 + IL-15 in combination over 4 different time points: days 1, 2, 3, and 4. Following treatment with the cytokines IL-12 + IL-15, we saw a stable population of GzmK and GzmB expressing $CD8^+$ T cells compared to the starting (day 0) frequency of this population (Figure 9). As expected, we did not see an increase in $GzmK^+$ $GzmB^+$ $CD8^+$ T cells after TCR stimulation. Instead, the TCR stimulated cells became $GzmB^+$.

Expression of activation markers:

Four additional markers of $CD8^+$ T-cell activation were examined as part of my project: CD69, HLA DR, Nur77 and Ki67 expression. Immune cells enriched at sites of inflammation become activated not only through direct antigenic stimulation, but also via

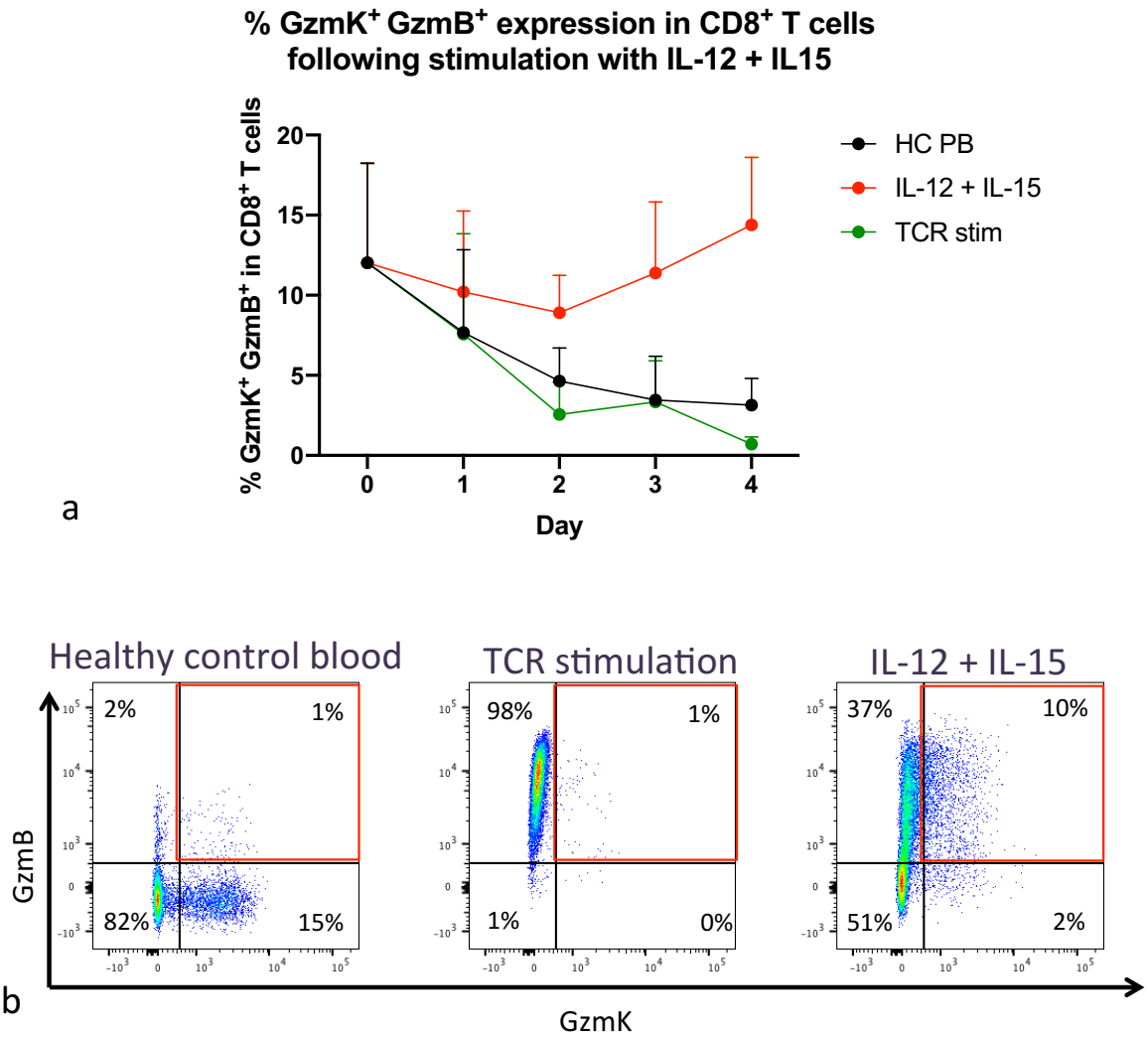


Figure 9: Cytokine stimulation of CD8$^+$ T cells with IL-12 + IL-15 led to the maintenance of GzmK$^+$ GzmB$^+$ expression. a) Cells were cultured for 1, 2, 3 or 4 days in the presence of IL-12 and IL-15, and then stained for GzmK$^+$ and GzmB$^+$ expression. This graph shows the percentage of CD8$^+$ T cells in the GzmK$^+$ GzmB$^+$ quadrant gate (outlined in red in section b). CD8$^+$ T cells from healthy control peripheral blood (HC PB), and HC PB in the presence of TCR stimulation (TCR stim) are shown for reference. b) Flow cytometry data representing day 4 expression of GzmK and GzmB in HC PB, TCR stim and CD8$^+$ T cells stimulated with IL-12 + IL-15.

other inflammatory pathways. Distinguishing antigen stimulated T cells from those activated by other factors within an inflammatory environment is challenging. However, the identification of antigen-specific T cell clones has important implications in understanding early events in human diseases, and surface and intracellular markers that serve to identify antigen specific lymphocytes in autoimmunity, infection, and cancer are critical.

Nur77 expression has previously been suggested as being specific to TCR signaling. In a study by Ashouri et al., the authors tested the effects of two cytokines: IFNα and IL-4, to conclude that Nur77 is an immediate, early gene that is rapidly induced after antigen receptor signaling in human T cells.[47] They also saw an up-regulation of Nur77 close to 4 hours after TCR stimulation, following which there was a decline in its expression. However, the authors only tested the effects of two cytokines, and limited their study to a 40-hour time point.

We decided to take advantage of our planned series of cytokine co-culture experiments to test whether other cytokines might affect Nur77 expression. Interestingly, our results show that elevated levels of Nur77 were also expressed following stimulation of human blood $CD8^+$ T cells with IL-12 in combination with IL-15 or IFNβ. This suggests that Nur77 expression can represent antigen-independent signaling by cytokines (Figure 10). We also found that elevated levels of Nur77 were expressed following stimulation of human blood $CD8^+$ T cells with IL-12 in combination with IL-15 at the day 3-4 time point (Figure 11). However, we saw conflicting results in a later experiment (Figure 12) and plan to repeat this study.

These two combinations of cytokines also have unique effects on other activation markers including CD69 and HLA DR, and on the proliferation marker Ki67, by mimicking the effect of TCR stimulation on $CD8^+$ T cells (Figure 10). CD69 (early marker) and HLA DR (late marker) are known to be up regulated by TCR independent signaling such as cytokines. We found that CD69 is up regulated on days 1 and 2, and that HLA DR is modestly up regulated on day 4. Furthermore, CD69 expression shows an increase that is transient following IL-12 + IFNβ stimulation, but persistent in both TCR and IL-12 + IL-15 stimulated $CD8^+$ T cells (Figure 11).

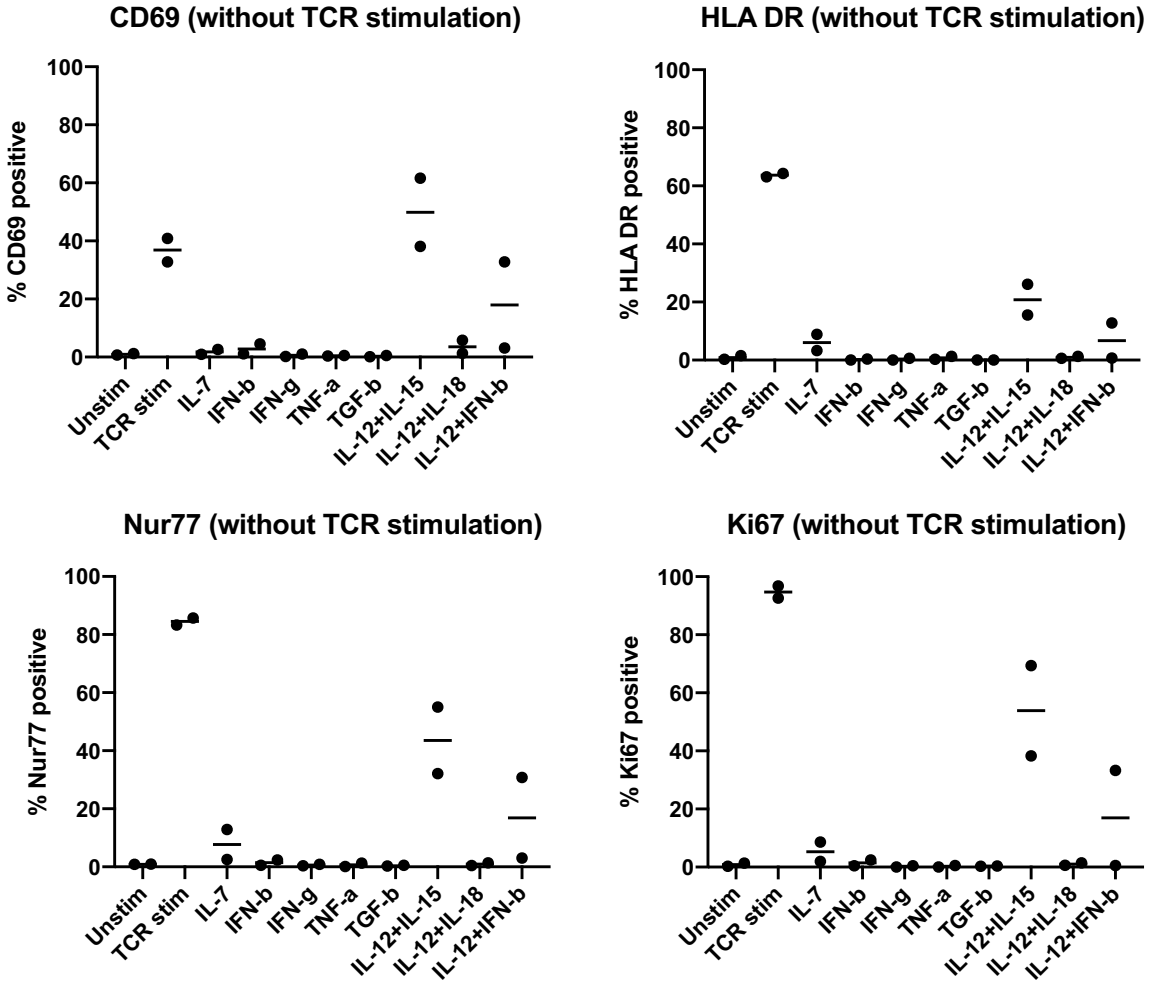


Figure 10: **Stimulation of CD8$^+$ T cells with IL-12 + IL-15 and IL-12 + IFNβ led to the up regulation of CD69, HLA DR, Nur77, and Ki67.** Cells were cultured for 4 days in the presence of the indicated cytokines, and then stained for CD69, HLA DR, Nur77 and Ki67 expression. This graph shows the percentage of CD8$^+$ T cells that stained positive for CD69, HLA DR, Nur77 and Ki67 expression. CD8$^+$ T cells from healthy control peripheral blood (HC PB), and HC PB in the presence of TCR stimulation (TCR stim) are shown for reference.

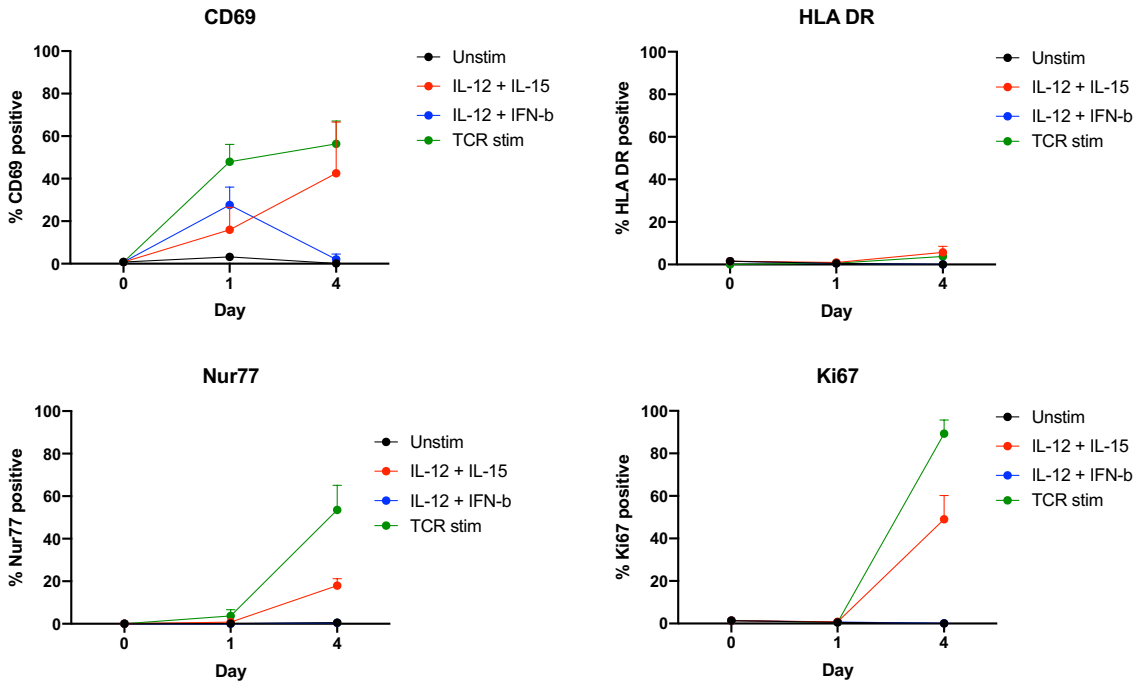


Figure 11: **Stimulation of CD8$^+$ T cells with IL-12 + IL-15 led to the up regulation of CD69, HLA DR, Nur77, and Ki67.** Cells were cultured for 1 day or 4 days in the presence of the indicated cytokines, and then stained for CD69, HLA DR, Nur77 and Ki67 expression. This graph shows the percentage of CD8$^+$ T cells that stained positive for CD69, HLA DR, Nur77 and Ki67 expression. Unstimulated CD8$^+$ T cells from healthy control blood (unstim) and CD8$^+$ T cells from healthy control blood stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies (TCR stim) are shown for reference.

a

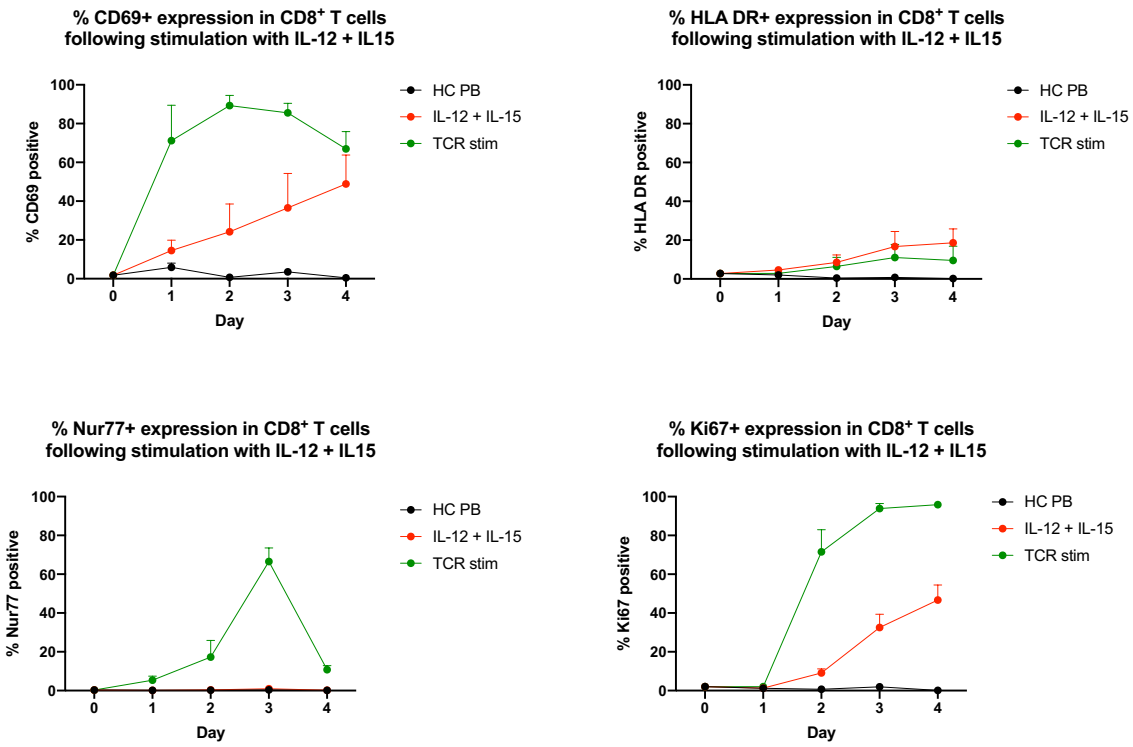
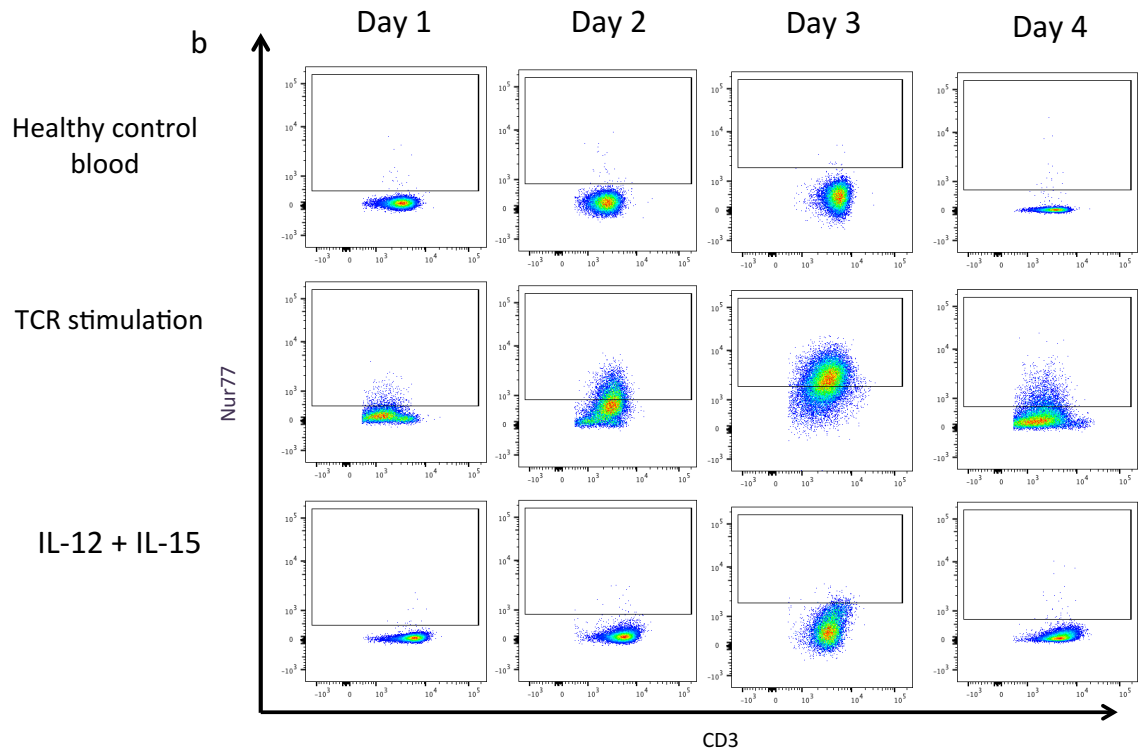


Figure 12: **Stimulation of CD8$^+$ T cells with IL-12 + IL-15 led to the up-regulation of CD69, HLA DR, and Ki67.** a) Cells were cultured for 1, 2, 3 or 4 days in the presence of IL-12 and IL-15, and then stained for CD69, HLA DR, Nur77 and Ki67 expression. This graph shows the percentage of CD8$^+$ T cells that stained positive for CD69, HLA DR, Nur77 and Ki67 expression. Unstimulated CD8$^+$ T cells from healthy control blood (HC PB) and CD8$^+$ T cells from healthy control blood stimulated with plate bound anti-CD3 and soluble anti-CD28 antibodies are shown for reference (TCR stim). b) Flow cytometry data representing day 1, 2, 3, and 4 expression of Nur77 in HC PB, TCR stim and CD8$^+$ T cells stimulated with IL-12 + IL-15. The day-to-day gates have been adjusted to allow for appropriate representation of the populations.



Discussion:

Rheumatoid arthritis is a complex, multifactorial disease in which many cell types interact to produce synovial inflammation. Furthermore, numerous cytokines have been detected in RA synovium and many effective therapies used to treat RA involve cytokine inhibitors. To date, most studies have focused on the role of $CD4^+$ T cells in RA. However, a recent study from our lab showed that half of all T cells in RA are $CD8^+$ T cells. $CD8^+$ T cells producing TNF and IFNγ are enriched in synovium and likely contribute to the inflammatory phenotype seen in RA. Interestingly, synovial $CD8^+$ T cells express IFNγ at a higher frequency, and TNF at an equivalent frequency compared to $CD4^+$ T cells.[32]

Unbiased studies of T cell subsets in RA synovium using single cell RNA sequencing revealed that the majority of $CD8^+$ T cells expressed GzmK, either alone or in combination with GzmB.[32] In this project, we studied whether cytokines regulated GzmK and GzmB expression. GzmK has pro-inflammatory effects on synovial fibroblasts, inducing them to

produce IL-6, CCL2, and ROS, all of which are up regulated in RA synovium.[22,23] $GzmK^+CD8^+$ T cells in blood also express low levels of perforin, and high levels of chemokine receptors CCR2, CCR5, and CXCR3 (unpublished data courtesy of Dr. Anna Helena Jonsson, MD PhD). In contrast, cytotoxic $CD8^+$ T cells expressing GzmB alone express CX3CR1 and high levels of perforin.[13–18]

We studied the effects of TCR and cytokine stimulation on $CD8^+$ T cells. Stimulating $CD8^+$ T cells via their TCR led to increased GzmB expression (Figure 3). $CD8^+$ T cells showed no increase in GzmK and GzmB co-expression following individual cytokine stimulation. Treatment with combination cytokines, specifically IL-12 + IL-18, and TNF + IL-17 also produced no effect. However, stimulation with IL-12 in combination with IL-15 (in the absence of TCR stimulation) led to an up-regulation of GzmK, GzmB and activation marker expression in peripheral blood $CD8^+$ T cells (Figures 8-12).

IL-15 belongs to a family of cytokines that utilize the IL-2 cytokine family receptor gamma chain (CD132; common gamma chain; γc) for signal transduction.[48] It is mainly produced by macrophages, but also by dendritic cells. IL-15 contributes to the survival and proliferation of $CD8^+$ T cells, as well as NK cells. It functions both as a homeostatic cytokine (active during steady-state in non-inflammatory conditions)[49] as well as an inflammatory cytokine. High circulating levels of IL-15 have been recorded following infections or inflammation, and one study found that levels of IL-15 in RA serum are raised at 102.4 pg/ml.[49–53] IL-15 expression has also been observed following TLR signaling, stimulation by bacterial LPS or Poly I:C, and downstream of type I interferon signaling following viral infections.[51–53]

IL-15 is known to play a role in the maintenance of $CD8^+$ T cells in a process known as homeostatic proliferation.[54] On days 2, 3, and 4 of stimulation of $CD8^+$ T cells with IL-12 + IL-15, we noted clustering of the cells in the wells (data not shown), which mimicked the

wells that were treated with plate bound anti-CD3 and soluble anti-CD28 antibodies. This observation corresponds with studies that reveal that IL-15 leads to a low level of basal proliferation that occurs in vivo, independent of any new antigen encounter, in a process known as homeostatic proliferation. Furthermore, in IL-15$^{-/-}$ and IL-15Rα$^{-/-}$ mice, homeostatic proliferation of memory CD8^{+} T cells is significantly reduced and the overall number gradually diminishes over time, suggesting that IL-15 is critical for maintaining the long-term survival of memory CD8^{+} T cells.[54,55]

Since IL-12 + IL-15 stimulation leads to some similar downstream effects as TCR stimulation, including increased expression of GzmB (Figure 9b), and activation markers CD69, HLA DR, and Ki67 (Figures 10-12), we considered whether they might have overlapping signaling mechanisms. However, according to the literature, that is not the case. IL-15 signals through a heterotrimeric receptor that comprises CD122, γc (CD132) and IL-15Rα (CD215).[48] IL-15 and its receptor, IL-15Rα form an intracellular complex within the ER which traffics to the cell surface and is delivered to CD122 and γc expressing cells (memory CD8^{+} T cell, NK cells) often via transpresentation. [51,56–58] The CD122 and γc chain then associates with JAK1 and JAK3 respectively, leading to STAT5 phosphorylation, dimer formation, and translocation to the nucleus. Studies have shown that IL-15 signaling also activates MAPK and PI3K/AKT/mTOR.[59,60] Together, these pathways induce a transcriptional program that favors the proliferation, activation, and survival of memory CD8^{+} T cells.

In contrast, when a CD8^{+} T cell is activated through its antigen receptor, the TCR associates with the CD3 complex. Following that, the immunoreceptor tyrosine-based activation motif (ITAM) is phosphorylated. Phosphorylated ITAMs provide binding sites for ZAP70, which activates multiple downstream signaling proteins such as PLCγ. Activated PLCγ can cleave phosphoinositide (PIP2) into diacylglycerol (DAG) and inositol-

triphosphate (IP3). Binding of IP3 to IP3R on the ER releases $Ca2^+$ from the ER, which increases the intracellular $Ca2^+$ and activates STIM1, which in turn activates a membrane-bound $Ca2^+$ ion channel (CRAC). This allows the flow of $Ca2^+$ into the cytoplasm. $Ca2^+$-bound calmodulin binds to calcineurin, and activated calcineurin removes the phosphate group from NFAT, which enters the nucleus and regulates transcription.[61,62]

IL-12 and IL-15 co-stimulation did not fully reproduce the GzmK and GzmB expression profile seen in RA synovium (Figure 2a), indicating that there are other factors at play. Specifically, these cytokines led to the maintenance, not accumulation of cells with a $GzmK^+$ $GzmB^+$ phenotype. Multiple components of the immune system interact and influence each other within the RA joint. We used an in-vitro system wherein $CD8^+$ T cells were cultured alone. The cell-to-cell crosstalk between $CD8^+$ T cells and other cells within the RA joint could lead to the production of additional factors that promote GzmK expression within cells. For example, previous studies have shown that IL-15 from RA synovial fibroblasts is able to stimulate freshly isolated T cells. This then generated a positive feedback loop wherein IL-15 induced and maintained T cell synthesis of TNF, IFNγ, and IL-17, which can further activate fibroblasts to produce IL-15 and also IL-6 and IL-8.[63] We did not replicate these conditions in vitro. Furthermore, the literature revealed that the levels of cytokines in the synovium of RA patients were at a lower concentration than we used in our experiments. We were guided instead by studies that used higher levels of cytokines to elicit specific effects, such as cytokine production, from $CD8^+$ T cells in vitro.[46]

Understanding the role of $CD8^+$ T cells, and their differentiation and activation pathways in RA, will open up new treatment avenues. Future work should include investigating the roles of other cell surface receptors, such as PD-1, CD27, ICOS, and other co-stimulatory or inhibitory receptors, in modulating GzmK and GzmB expression. We also plan to perform promoter region analysis to identify potential transcription factors involved

in regulating GzmK expression. Appreciating the different $CD8^+$ T cell phenotypes that may mediate destruction of the joint can help direct treatments towards inhibiting the factors, such as cytokines, that may mediate that cell state. Furthermore, these findings can be applied more broadly in autoimmunity, infection and cancer.

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