TCRγδ+ T cell Levels in Patients with Juvenile Idiopathic Arthritis Found to Be Normal

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Abstract

Because adult Still’s disease and Systemic Juvenile Idiopathic Arthritis (SoJIA) share many similarities, including several symptoms such as Macrophage Activation Syndrome and arthritis in one or more joints, the etiologies of both diseases are hypothesized to be similar. Basing our work on Hoshino et al. (1996), we tested this hypothesis by studying the levels of γδ T cells in both patients with SoJIA and control patients. This was done by taking the findings of Hoshino et al., that adult Still’s disease patients have higher levels of γδ T cells than control patients do, and applying a similar procedure to blood samples from SoJIA patients. The results revealed that SoJIA blood samples do not have higher levels of γδ T cells than control blood samples, therefore implying that adult Still’s disease is not as closely related to SoJIA as was previously speculated.

Introduction

Juvenile Idiopathic Arthritis (JIA) is a systemic inflammatory disorder that affects thousands of children each year. Systemic JIA (SoJIA) may be the most harmful and severe type of the disease. Children who are diagnosed with SoJIA not only suffer from arthritis in multiple joints, but also must endure fevers that average 102.2 degrees Fahrenheit and may last up to two weeks (Ravelli and Martini, 2007). During these periods, children’s fevers may also increase suddenly, plummet to extremely low body temperatures, then either return to normal or rise

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again to extremely high levels. These rapid fluctuations can cause organ failure. Other symptoms include rashes, lymphadenopathy (disease or swelling of the lymph nodes), and seroitis (inflammation of tissues lining the lungs, heart, and inner lining of the stomach). Commonly, fifty percent of patients remit after one year, and twenty-five percent suffer from severe joint damage. General growth abnormalities are common, as is Macrophage Activation Syndrome (uncontrolled activation and proliferation of macrophages) (Goldmuntz et al., 2006). Despite the severity of the disease, scientists do not know its cause. Thus, treatment is limited to doses of non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen (such as Advil or Motrin) and corticosteroids (anti-inflammatory hormones). By determining the biological nature of the inflammatory disease, scientists may be able to develop better treatments.

Due to the unknown etiology of the disease, information from other similar diseases can be used to provide a starting point for further research on JIA. One such disease, adult Still’s disease, shares similar symptoms to JIA such as extreme arthritis in relatively young joints. A recent study has revealed a potential link between adult Still’s disease and a group of cells called γδ T cells (Hoshino et al., 1996).

The immune system is comprised of its adaptive and innate branches. T cells and B cells make up the adaptive portion, which allows us to learn to respond to previously unencountered threats. More than 95% of T cells that make up the adaptive immune system are known as αβ T cells, so named because they express the αβ form of the T cell receptor (the molecule that lets the T cell sense its environment and determines cell specificity). The wide variability of αβ T cells is created by a process called VDJ recombination, in which the T cell’s DNA is changed dramatically to form novel versions of the αβ T cell receptor. γδ T cells, which make up less than 5% of the T cell population, are part of the adaptive branch of the immune system, yet also have been found to possess characteristics of the innate branch (Born et al., 2006). This implies that γδ T cells have specificity that is hardwired, as opposed to the highly variant αβ T cells that undergo VDJ. Even though γδ T cells are a small proportion of the overall population of T cells in the body,
they are still an essential part of the immune system, as animals lacking γδ T cells are found to be immunocompromised. γδ T cells are a proposed link to diseases like JIA and adult Still’s disease because they are related to the process of inflammation. Born et al. (2006) inferred that γδ T cells seemed to be provided with specific predetermined responses to particular threats (with ligands for mycoplasma, mycobacterium, some viruses, and many other unknown ligands) while αβ T cells were provided variably to adapt to different, very diverse, threats. Scientists (Koingshofer et al., 2006) found that a host organism has γδ T cells capable of recognizing self antigens like CD1 or MIC-A as well as some mitochondrial antigens. Since these molecules are typically associated with cellular distress, it is inferred that γδ T cells may use those signals to perceive signs of danger, such as viral infections or extensive cellular damage.

Hoshino et al. (1996) found a correlation between high levels of γδ T cells and patients with adult Still’s disease. Hoshino et al.’s study set out to answer the question, “What types of lymphocytes are involved in Still’s disease?” Initially, the study tested all types of lymphocytes: CD3, CD4, CD8, TCRαβ, and TCRγδ T cell subsets. The study found that only TCR γδ T cells were irregular in number when comparing patients infected with active Still’s disease to those who were healthy. Interestingly, the percentage of γδ T cells was three times as high as the percentage of γδ T cells found in healthy patients.

Case studies were also observed by Hoshino et al. Specifically, three case studies were observed in the greatest detail. In addition to these three female patients’ (age ranging from 20 to 41 years) γδ T cell counts, their ferritin levels, serum C-reactive protein levels (CPR) and body temperatures were also observed. These three additional assays serve as tools to measure inflammation. Ferritin and CPR levels, when higher than usual, suggest inflammation. These patients were observed over a span of one to three years. In case one, her γδ T cell percentage jumped exponentially when she was having an arthritic outbreak. This suggests that γδ T cells may have a link to Still’s disease. However, cases two and three were not in accord with the first case. While γδ T cells have not been shown to cause adult Still’s disease, there does appear to be a correlation between the disease and the raised levels.
Given the correlation that has been found between γδ T cells and adult Still's disease, and the similarity between adult Still's disease and JIA, we hypothesized that the etiology of SoJIA might also involve the overpopulation of γδ T cells. Thus we set out to test whether patients suffering from SoJIA had higher percentages of γδ T cells compared to healthy patients.

**Results**

In order to examine whether γδ T cell percentages were increased in JIA patients and thus determine if γδ T cells might be involved in the disease, we used Fluorescence Activated Cell Sorting (FACS) to quantify the percentage of γδ T cells in serum samples of SoJIA patients. FACS, or flow cytometry, is a method for examining the characteristics of single cells in a greater population. In addition to size and density, the fluorescence level of each cell can be analyzed. γδ and αβ T cells can also be distinguished by FACS based on their unique T cell receptor (TCR) forms (αβ and γδ). Specific antibodies that recognize αβ and γδ TCRs can be used to attach fluorescent probes to individual cells, allowing αβ and γδ T cells to be quantified by FACS.

All samples were stained in triplicate, according to protocol for IgG, αβ, and γδ staining. Briefly, each replicate of the sample was stained with a mixture of antibodies recognizing CD3, CD14, CD19. In addition, each replicate was stained with one of three antibodies conjugated to PE: IgG, αβ TCR, or γδ TCR. CD3 is present on all T cells and so was used to identify the T cell population in our samples. CD14 is a marker of monocytes, while CD19 is a marker of B cells. The antibodies recognizing CD14 and CD19 were therefore used as controls to confirm the purity of the T cell population. The purpose of staining with γδ TCR was to determine the percentages of γδ T cells in the T cell population, while the purpose of staining with αβ TCR was to determine the percentages of αβ T cells in the T cell population. Any increases in the γδ T cell population should correspond with the decrease of the αβ T cell population. Finally, the IgG marker was used as a negative control: no T cells should be stained with the antibody recognizing IgG.

Results were collected from a single procedure during the process of this study. Often, two to four samples were analyzed at a given time. The
samples from SoJIA cases and control cases were examined blind. After the sample cells had been stained and fixed, they were run through a four-color FACS machine, then quantified and recorded into a computer. Because the wavelengths of the fluorophores we used were so close in range, they appeared to bleed over into each other’s fluorescent ranges. This caused cells to appear to be stained with multiple markers, even if only one was present. The first two samples, 160A and 11AB, a control and SoJIA, respectively, were used to develop parameters that would be used in each experiment to follow in order to correct the overlapping wavelengths of the antibody fluorophores. FloJo, a software program used for quantifying FACS data, was used to compensate the data.

γδ Staining and Results

Hoshino et al. found a strong correlation between increased γδ T cell populations and the onset of adult Still’s disease. Control patients had often at least 6-10% fewer γδ T cells in their peripheral blood than patients with adult Still’s disease. After analysis of six SoJIA patients, we determined that SoJIA patients do not suffer from lower γδ T cell populations compared to control patients. Figure 1 shows representative populations of each control and SoJIA samples stained with PE conjugated anti-human IgG, PE conjugated anti-human γδ TCR, or PE conjugated anti-human αβ TCR. γδ T cell populations in quadrant I were recorded based on percentages of γδ T cells out of entire T cell populations. Once percentages for each sample were recorded, data was compared in a dot graph. Figure 2 is an image of this dot graph, with six points of control data on the left and six points of SoJIA data on the right. The solid line through each data column is the mean, showing where the average of the six points lies.

Two populations of γδ T cells were noticed in every sample but one, 174A. The appearance of these two populations was significant because γδ T cell populations have not been observed to behave in such a manner in previous studies. Two populations were often viewed one on top of the other with lower γδ TCR PE fluorescence levels and higher CD3 FITC fluorescence levels. The lower population, conversely, characteristically had lower CD3 FITC fluorescence levels and higher γδ TCR PE fluorescence. Figure 3 shows graphs of three SoJIA patients, each
with double populations of PE conjugated anti-human γδ TCR stained γδ populations.

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**Figure 1. Representative Populations.** Typical representative FACS graphs of TCR versus CD3 FITC reveal very little difference between SoJIA and control samples. IgG samples are both in quadrants II and III, as no TCRs should be marked by the IgG antibody. αβ populations are large and in quadrant I. γδ populations are also in quadrant I, but are split into two sub-populations.
Figure 2. The percentages of CD3 positive cells that express γδ TCR in SoJIA and control samples are very similar. The final results are shown as percentages of γδ T cells out of the total population of T cells in the given sample. The data points set off to the side are for legibility. Note how there is very little difference between percentages of γδ T cells in controls versus SoJIA patients.

Figure 3. Three examples of SoJIA samples reveal the two populations of γδ T cells. Graphs are TCR versus CD3 FITC, with γδ populations in quadrant I, as indicated by the darker boxes in the upper right of each example. Although no correlation was found between the populations of γδ and JIA, these populations were never before seen in studies. While patients 11AB and 2E each have a lower γδ T cell population around 0.8%, their upper population is more than 5% more in 2E than it is in 11AB. 3R appears to have relatively even distribution of γδ T cells in the populations, but does not relate to the other two samples.
αβ Staining and Results

The αβ populations of six SoJIA patients and six controls were analyzed. The data was analyzed similarly to the data gathered on the γδ T cell populations. Quadrant I values were recorded for each sample’s αβ staining and inserted in a dot graph shown in Figure 4. The left column of this dot graph shows six control patients, and the right column shows six SoJIA patients. The solid black line represents the average of each data set. As is revealed in Figure 5, there was a large spread of SoJIA percentages of αβ populations, from 36% to 73%. The control sample group did not have as wide a range of data; however, it was still much more spread out than the results for the γδ data.

Figure 4. The percentages of CD3 positive cells expressing αβ in SoJIA and Control Samples are very similar. The final results are shown as percentages of αβ T cells out of the total population of T cells in the given sample. The uppermost SoJIA data point is off to the side for legibility. Note the large range of αβ T cell percentages compared to the relatively close ranged data from the γδ results in Figure 3.
Discussion

In this study, we examined patients with SoJIA in order to understand the correlation, if any, between JIA and increased γδ T cell populations. Our results showed that there were no significant differences between levels of γδ T cells in JIA patients and controls.

Adult Still’s disease and JIA are both systemic inflammatory disorders that have very similar symptoms, including unexplained arthritis in otherwise healthy joints. γδ T cells are known to be involved in inflammatory processes, and Hoshino et al. discovered strong correlation between higher than average levels of γδ T cells and adult Still’s disease. Thus, it is interesting that no such correlation appears to exist between SoJIA and γδ T cell levels. It is entirely possible that the higher levels of γδ T cells are not related to the etiology of adult Still’s disease. However, if γδ T cells are linked to the etiology of adult Still’s disease, then the mechanisms that cause JIA may be completely different from those that cause adult Still’s disease.

Some limitations were presented during the experiment. Perhaps the largest of these was the source of blood. While JIA samples were from children with SoJIA, these children were in remission from severe JIA symptoms. Children with very severe JIA are typically unavailable for sample donation. Perhaps children in remission from JIA do not suffer from abnormal levels of γδ T cell percentages. If this were true, then the results gathered in this study would not be definitive. Samples from SoJIA patients not in remission should be tested; further studies would preferably be executed with samples from SoJIA patients not in remission. The study by Hoshino et al. suggests that this might indeed be the case because they saw differences only in patients with adult Still’s disease who were suffering from acute symptoms at the time of the donation. Additionally, sample sizes were limited in number due to the short period of time during which the study was performed, so the sample sizes might not have been large enough for small but significant differences between groups to be observed.

As portrayed through the data regarding αβ T cells, the percentage of αβ T cells in the SoJIA and control samples were extremely varied. This
was not so with the γδ T cell results. This may suggest some inconsistency with the results. Ideally, the αβ T cell populations would have percentages that were closer together in number. The fact that they are not suggests that the sample cells may have included many unhealthy cells, either from freezing or remaining frozen for long periods of time.

There are limitations that cause us not to be able to conclusively determine that γδ T cells are not involved in the etiology of SoJIA. However, our results reveal SoJIA and control samples to be very similar, which suggests that large differences between levels of γδ T cell populations in control and SoJIA do not cause the disease.

Experimental Procedures

Thawing Sample Cells

Affected and unaffected samples were obtained through Dr. Mellins’s lab and the Pediatrics unit of Stanford Hospital. Two to four samples were analyzed at a given time. Data was analyzed in a blinded manner. Once acquired, the samples were thawed in the following fashion: 1-1.5 mL of sample was almost completely thawed so that only a small crystal remained. This was done because Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich), while necessary for proper cell storage at low temperatures, is toxic to cells at high temperatures. Diluting the cells before they are fully thawed avoids potential toxicity of sample cells. 1 mL of 5% AB sera Heat Inactivated (pool of sera from individuals with AB blood group) (Invitrogen) in Roswell Park Memorial Institute medium (Mediatech, Inc.) (5% ABHI in RPMI) at 37 degrees Celsius was added to fully thaw and simultaneously dilute the sample. The thawed cells and 1 mL of ABHI in RPMI solution were immediately added to another 9 mL of 5% ABHI in RPMI. This was done for each sample individually. Each sample was then centrifuged for 5 minutes in a 25 degree Celsius environment at 1500 rotations per minute in order to collect a pellet of cells. The supernatant was removed, and 5 mL of 5% ABHI in RPMI were added to the pellet. The pellet was resuspended into solution and centrifuged again for 5 minutes at the same conditions as above. Again, the supernatant was removed, except for 90 μL. The cell pellet was resuspended in this remaining supernatant.
Staining Sample Cells

Each cell sample was stained in triplicate with either an antibody recognizing αβ TCR, an antibody recognizing γδ TCR, or an irrelevant antibody as a negative control. The cell suspension solution was diluted at 3:10 dilution for the purpose of staining in Phosphate Based Saline (PBS). Phycoerythrin (PE) conjugated anti-human IgG (IgG PE) (BD Pharmigen) was used at a dilution of 1:100, PE mouse anti-human γδ TCR (γδ PE) (BD Pharmigen) was used at 1:20 and PE mouse anti-human αβ TCR (αβ PE) (BD Pharmigen) was also used at 1:20.

Additionally, each replicate was stained with a cocktail of Fluorescein isothiocyanate (FITC) conjugated anti-human CD3 (CD3 FITC) (BD Pharmigen), Allophyocyanin (APC) conjugated anti-human CD19 (CD19 APC) (BD Pharmigen), and Peridinin chlorophyll protein (Percp) conjugated anti-human CD14 (CD14 Percp) (BD Pharmigen), each at a dilution of 1:10. CD3 FITC, a T cell marker, was used to select only for T cells, while CD19 APC, which recognizes only B cells, and CD14 Percp, which recognizes only monocytes, were used to confirm that the T cell population analyzed was pure.

The cells were incubated for thirty minutes to allow for antibody binding, washed in 1 mL of FACS Buffer, and centrifuged for 5 minutes. The supernatant was removed, and 200 μL of 1% Paraformaldehyde (PFA) in FACS Buffer were added. Cells were fixed in this solution until used in FACS machine for 24 to 48 hours at 4 degrees Celsius and then analyzed by flow cytometry.

Use of Four-Color FACS Machine

When using the FACS machine, it was essential to first note the levels of sheath fluid (used to take up the cells and bring them through the machine to the laser) and waste fluid. The pressure then had to be turned on so the sample could move through the machine, and then Cell Quest, a computer program, was used to quantify and record the data. Each sample was then saved onto the computer according to its stain, e.g. 14A IgG for a sample 14A that was stained with IgG PE.
Analyzing the data using FloJo

After all samples were obtained, FloJo software (Tree Star, Inc.) was used to correct what appeared to be a double staining of the sample. This was performed by the computer using equations that compensated for the overlapping wavelengths. While PE and FITC had fluorophores with the most overlapping wavelengths, all of the fluorophore wavelengths overlapped. By indicating the two populations of monocytes in the sample, the computer could then adjust the wavelengths to give comprehensible data. With the compensated data, each sample’s IgG stain was used to establish axes which would determine negatives and minimize experimental error. Using these axes, both αβ and γδ stains were analyzed.

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References


