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Secondary Immunologic Consequences in Chromosome 22q11.2 Deletion Syndrome (DiGeorge Syndrome/Velocardiofacial Syndrome)

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Abstract

Clinical evidence suggests that patients with Chromosome 22q11.2 deletion (Ch22q11.2D) have an increased prevalence of atopic and autoimmune disease and this has been without explanation. We hypothesized that the increase in atopy was due to homeostatic proliferation of T cells leading to a Th2 skew. We performed intracellular cytokine staining to define Th1/Th2 phenotypes in toddlers (early homeostatic proliferation) and adults (post homeostatic proliferation) with this syndrome. To attempt to understand the predisposition to autoimmunity we performed immunophenotyping analyses to define Th17 cells and B cell subsets. Adult Ch22q11.2D patients had a higher percentage of IL-4+CD4+ T cells than controls. Th17 cells were no different in patients and controls. In addition, adult Ch22q11.2D syndrome patients had significantly lower switched memory B cells, suggesting a dysregulated B cell compartment. These studies demonstrate that the decrement in T cell production has secondary consequences in the immune system, which could mold the patients' clinical picture.

Keywords

DiGeorge syndrome; Th1; Th2; allergy; Th17; B cells; autoimmunity; antibody

Introduction

Chromosome 22q11.2 deletion (Ch22q11.2D) syndrome, commonly referred to as DiGeorge syndrome or velocardiofacial syndrome, is due largely to haplosufficiency of the transcription factor TBX1 [1;2;3;4;5;6]. As a consequence, organs related to the third and

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fourth branchial arches have impaired development [7;8;9;10]. The most common phenotypic features are cardiac anomalies, hypoplastic parathyroid glands, palatal dysfunction, and a hypoplastic thymus. Speech delay, renal anomalies, and skeletal anomalies are also seen with some frequency [11;12;13;14;15;16]. The main manifestation of the thymic hypoplasia is diminished peripheral blood T cell numbers [17;18;19;20;21;22;23]. The T cell lymphopenia is seen much more in infancy than in adulthood and there is evidence to support the hypothesis that the T cell compartment undergoes homeostatic expansion to compensate for the diminished thymic output [24;25;26]. Ch22q11.2D syndrome patients have evidence of accelerated conversion of naïve to memory cells, more extensive replicative history of naïve CD4 cells compared to controls, and less diverse T cell repertoire as demonstrated by having both more oligoclonal peaks and V β dropouts than controls on TCR V β family analysis [26]. The lymphocyte proliferation in response to mitogen and recall antigens is similar between Ch22q11.2D patients and normal subjects, but qualitative studies of T cells have not been performed [17].

Homeostatic mechanisms are integral in the development, survival and maintenance of T cells [27]. Naïve T cells rely upon interaction with self peptide and MHC molecules and IL-7 for their homeostatic survival [27;28;29;30;31;32;33]. When T cell counts are reduced, the combination of self peptide and MHC leads to homeostatic expansion of the T cells with restoration of near normal levels. In this circumstance, the naïve cells acquire phenotypic and functional features of memory cells, even in the absence of response to foreign antigens, although commensal bacteria appear to facilitate the process [27;34;35]. Additional features of homeostatic proliferation in murine models include a limited T cell repertoire and Th2 skewing [27;35;36;37;38;39;40]. Autoimmune disease can be seen in this setting [41;42;43]. Homeostatic proliferation of memory T cells is more dependent on a combination of IL-15 and IL-7 rather than contact with self peptide and MHC [27;28].

A human example of extensive homeostatic proliferation occurs in Omenn syndrome. Omenn syndrome is seen in patients with severe combined immune deficiency where escape clones proliferate and infiltrate tissues [44]. Clinically it presents in early infancy with viral or fungal pneumonitis, chronic diarrhea, and failure to thrive and the Th2-mediated features of severe erythroderma, increased IgE levels, and eosinophilia [45]. The peripheral population of T cells is oligoclonal and the T cells are nearly uniformly of a Th2 phenotype [46;47;48;49;50].

Omenn syndrome represents an extreme example of homeostatic expansion and self-reactivity in humans. We hypothesized that patients with limited T cell production, such as is seen in Ch22q11.2D syndrome, might experience atopic features or autoimmunity as a downstream consequence of homeostatic expansion or secondary limitations of the T cell compartment. Our group has previously documented increased allergic features clinically in this patient group and there are multiple studies confirming an increased prevalence of autoimmunity [17;51;52;53;54;55]. Eczema and asthma are increased in frequency and the autoimmune diseases seen in this syndrome encompass a wide range of organ-specific disorders such as thyroiditis, autoimmune cytopenias and arthritis [17;51;52;53;54;55]. To begin to understand the mechanisms and relate the clinical findings to the known immunologic pathways, we investigated qualitative features of B cells and T cells in toddlers (early homeostatic expansion) and adults (post-homeostatic expansion). We performed intracellular cytokine staining on peripheral blood CD4 T cell subsets. In addition, although the immunodeficiency found in Ch22q11.2D syndrome is traditionally thought of as primarily T cell-mediated, there is evidence of humoral immune deficiency in some patients [17;23;56;57;58;59;60;61;62;63]. Therefore, to assess secondary effect on B cell subsets, we characterized peripheral blood B cells subsets in toddlers and adults with this syndrome.

Materials and Methods

Subjects

Patients with Ch22q11.2D syndrome and controls were recruited for this study. Eighteen adults with Ch22q11.2D syndrome, 46 adult controls, 22 children with Ch22q11.2D syndrome, and 34 pediatric controls were recruited. The demographic features are listed in Table 1. In some analyses, a subset of samples were used due to insufficient blood. This is indicated in the Figure legend and the age matching was preserved in those cases. Inclusion criteria for patients included genetic confirmation of DNA deletion in chromosome 22q11.2. Patients and controls were excluded if acutely ill. This study was approved by the IRB at The Children's Hospital of Philadelphia.

T cell preparation and analysis

Blood was diluted with phosphate buffered saline (PBS) and peripheral blood mononuclear cells (PBMC) were isolated via density gradient medium centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ). PBMCs were diluted in RPMI media (10% cosmic calf serum (HyClone, Logan, UT), 1% Pen-Strep (Invitrogen, Carlsbad, CA), and L-glutamine 2 mMol (Invitrogen). For CFSE responses, the cells were stimulated with phytohemagglutinin at 3µg/ml for five days. Cells were gated on CD3+ blasts for analysis. For intracellular cytokine analysis, the cells were incubated for five hours at 37° C after stimulation with either phorbol 12-myristate acetate (PMA) 5 ng/ml, ionomycin 500 ng/ml, and brefeldin-A (GolgiPlug, BD Biosciences, San Jose, CA) 1 µl/ml for IL-4 and IFN-γ staining; PMA 50 ng/ml, ionomycin 1 µg/ml and monensin (GolgiStop, BD Biosciences) 1.5 µl/ml for IL-17 staining; or brefeldin 1 µl/ml for control staining. PBMCs were stained with surface antibodies against CD3 (BD Bioscience) and CD4 (BD Bioscience) and intracellular antibodies against IL-4 (BD Bioscience), IFN-γ (BD Bioscience), and IL-17 (eBioscience, San Diego, CA) using the manufacturer's protocol and analyzed by flow cytometry using BD FACSCalibur and FlowJo software (Tree Star, Inc., Ashland, OR). T cell spectratyping was performed according to standard protocols. Interpretation of the data was also according to standard protocols [26;64;65].

B cell preparation and analysis

Fresh (less than 24-hour old) peripheral whole blood anti-coagulated with EDTA was prepared and stained with antibodies (all from BD Pharmingen, San Diego, CA), as described previously [66]. B cells were defined as CD19+ lymphocytes. The absolute B cell count was obtained by multiplying the absolute lymphocyte count (obtained from the complete blood count) by the CD19+ lymphocyte fraction. CD19+ lymphocytes were analyzed for CD27, CD38, IgM, IgD, CD24, CD5, CD20 and lambda expression. Flow cytometry was performed on a FACSCalibur instrument (BD Biosciences, San Jose, CA) and data were analyzed with CellQuest software (Version 5.2.1, BD Biosciences).

Analyses

Statistical analysis was performed using the Mann-Whitney U test in Prism 4 software (GraphPad, San Diego, CA). Two tailed p values are reported.

Results

T cell function

Homeostatic expansion would be expected to lead to diminished proliferative ability in adults with CH22q11.2D compared to young children, who have undergone less homeostatic expansion. A previous study documented shortened telomeres and advanced maturation of

the T cell compartment, consistent with a slow homeostatic proliferation process [26]. To test another aspect related to homeostatic expansion, we compared proliferative ability using CFSE dye dilution and phytohemagglutinin (PHA) as a stimulus. This method allows a distinction between decreased rounds of proliferation after a stimulus and fewer cells entering the cell cycle after a stimulus (Figure 1). Adults with Ch22q11.2D also had fewer T cells dividing than adult controls. The proliferation index, a measure of the rounds of division amongst dividing cells, was no different between patients and controls. The division index, a measure of average rounds of division across the entire CD3+ population, was also no different between patients and controls. Therefore, compromised proliferative ability is seen in adults with Ch22q11.2D syndrome, consistent with a prior history of homeostatic expansion. To further support the concept that homeostatic proliferation occurred, we defined the intactness of the T cell repertoire in patients and controls (Table 2). We used T cell spectratyping and defined oligoclonal and absent V β families. The increased aberrant V β families in the patients is consistent with a mechanism of homeostatic proliferation.

T cell cytokine production

To examine the relative distribution of Th1 and Th2 cells in patients, we used intracellular cytokine staining. In toddler-aged (1–3.5 years) Ch22q11.2D syndrome patients (Figure 2), there was a significantly elevated percentage of CD3+CD4+IL-4-IFN- γ + lymphocytes (Th1 cells) compared to controls ($p<0.01$). There was also a trend towards an elevated percentage of CD3+CD4+IL-4+IFN- γ -lymphocytes (Th2 cells) compared to controls ($p=0.068$). There was no significant difference in the percentage of CD3+CD4+IL-17+ lymphocytes (Th17 cells) in Ch22q11.2D patients compared to controls ($p=0.24$). The ratio of CD3+CD4+IL-4+IFN- γ -:CD3+CD4+IL-4-IFN- γ + (Th2:Th1) was significantly higher in controls than 22q patients ($p<0.01$). The intensity of staining (Figure 2) for IFN- γ and IL-4 positive cells was not significantly different between pediatric Ch22q11.2D patients and controls ($p=0.53$ and $p=0.22$). These data demonstrate that the T cell population in young Ch22q11.2D children is not Th2 skewed.

In adult Ch22q11.2D patients (Figure 3), there was a significantly elevated percentage of CD3+CD4+IL-4+IFN- γ -lymphocytes (Th2 cells) in Ch22q11.2D patients compared to controls ($p<0.05$). There was no significant difference in the percentages of CD3+CD4+IL-4-IFN- γ + or CD3+CD4+IL-17+ lymphocytes (Th1 and Th17 cells) in Ch22q11.2D patients compared to controls ($p=0.33$ and $p=0.69$). There was trend towards an increased ratio CD3+CD4+IL-4+IFN- γ -:CD3+CD4+IL-4-IFN- γ + (Th2:Th1) compared to controls ($p=0.097$), in contrast to the pediatric data which showed the inverse. The intensity of staining (Figure 3) for IL-4 positive cells was significantly higher for adult Ch22q11.2D patients compared to controls ($p=0.030$) and there was a trend towards increased geometric mean intensity of staining for IFN- γ for adults with Ch22q11.2D ($p=0.054$). Therefore, adults with Ch22q11.2D have a skewing towards Th2.

Circulating B cell subsets

A potential mechanism underlying the increased predisposition to autoimmune disease could be the dysregulated T cell help for B cell differentiation and maturation. In adults with Ch22q11.2D compared to controls (Figure 4), there was a significantly lower number of switched memory (CD19+CD27+IgM-) B cells. There were no significant differences in total B cell, naïve B cell, or unswitched memory B cell counts in adults. In children, naïve B cell counts and unswitched memory B cell counts were lower in patients than controls. In adults with Ch22q11.2D compared to toddlers with Ch22q11.2D there were significantly lower numbers of B cells, naïve B cells, transitional B cells, total CD27+ memory B cells, unswitched memory B cells, switched memory B cells ($p<0.05$ for all), a pattern also seen in

controls. These data demonstrate that Ch22q11.2D leads to a dysregulated B cell compartment.

Discussion

Our results indicate that young children with Ch22q11.2D syndrome initially have increased Th1 cytokine production compared to controls. This could reflect a high infection frequency or a higher level of memory T cells [17]. Over time, the Th1 skewing evolves towards a Th2 cytokine profile phenotype in adults with Ch22q11.2D syndrome compared with controls, a change that parallels the homeostatic expansion [26]. This supports our original hypothesis that homeostatic proliferation of T cells in patients with Ch22q11.2D could lead to a Th2 bias in adults with Ch22q11.2D syndrome and is consistent with our clinical observation of increased atopy in this population [55]. The evolution of allergies is believed to arise over time with aberrant responses developing after multiple exposures [67]. Although we did not examine intermediate ages, our data is consistent with increasing Th2 cells throughout childhood.

Previous studies have supported the concept that homeostatic proliferation leads to a limited T cell repertoire in Ch22q11.2D syndrome. Accelerated conversion of naïve to memory cells and evidence of increased replicative history has been shown [24;26;68]. The limited T cell repertoire has been confirmed via TCR V β family spectratyping [26;68;69;70;71]. The role of lymphopenia and restricted T cell repertoires leading to a Th2 bias had been previously shown in mice where homeostatic proliferation was shown to be associated with Th2 CD4 T cells and Th2-mediated eosinophilic disease with elevated levels of serum IgE [35]. This experimental model represents an extreme example of the consequences of homeostatic expansion but informs on our patient population. Compared to matched sibling controls, there was a significant association of Ch22q11.2D syndrome with both eczema and asthma [55] and the current study has identified a potential mechanism through the identification of progressive Th2 skewing of the T cell compartment.

Two other studies have examined this clinical question. Real-time PCR was used to measure PBMC cytokine levels in Ch22q11.2D syndrome [24]. Using IFN- γ to represent a Th1 cytokine, IL-10 and TGF- β as Th2 and/or regulatory cytokines, and CTLA4 and Foxp3 as Treg cell-associated molecules, they found no difference in expression levels between pediatric control and patients, and no difference in Th1/Th2 shift by comparison of the IFN- γ :IL-10 or IFN- γ :TGF- β ratios [24]. The differences between that study and ours could relate to the technology, the study populations, or the ages, as this study examined only young children. A study using intracellular cytokine staining found increased IFN γ expressing cells in Ch22q11.2D children, as was seen in the current study [70].

The etiopathogenesis of autoimmunity is complex and poorly understood. Our examination of the increased predilection for autoimmunity in this study focused on two specific mechanisms. We have previously demonstrated decreased Tregs and the homeostatic expansion seen in this syndrome could contribute to autoimmunity by selecting for T cells with self-reactive receptors [26;72]. To examine two other potential downstream consequences of T cell lymphopenia, we identified Th17 cells and effects on the B cell compartment. Th17 cell counts were no different in patients and controls. The B cell compartment exhibited characteristics which suggest delayed maturation: lower naïve and unswitched memory B cell counts in childhood and low switched memory B cells in adult patients compared to controls. This study did not investigate mechanism, but these changes could be explained by a deficiency in T cells in Ch22q11.2D patients leading to insufficient T cell-dependent activation and differentiation of B cells [73;74]. Previous studies of children with Ch22q11.2D syndrome showed that levels of peripheral CD27 $^{+}$ B cells were

reduced in patients as compared with age-matched healthy controls with the patients having significantly decreased unswitched memory B-cells (IgM+IgD+CD27+) [59;75].

The trend towards the increased transitional B cells and decreased memory B cells found in Ch22q11.2D patients is consistent with other diseases with impaired humoral immunity including common variable immunodeficiency, X-linked lymphoproliferative disease, and recent hematopoietic stem cell transplantation [76;77;78;79;80]. These diseases are each associated with an increased risk of autoimmune disease. Our study does not identify a mechanism to explain the decrement in switched memory B cells but does suggest that the B cell compartment has been dysregulated as a result of alterations to the T cell compartment.

In summary, although Ch22q11.2D syndrome is primarily thought of as a deficiency of T cell production, the immunologic aberrations are more complex. The initial decrement of T cells leads to peripheral expansion via homeostatic mechanisms that appears to lead to skewing of the T cell subsets and increased Th2-mediated disease. In addition, the restricted repertoire of T cells, could lead to abnormal T cell activation and differentiation of B cells, with a decrease in the population of memory B cells and an increase in transitional B cells. This could explain the decreased production of immunoglobulins and abnormal specific antibodies that are sometimes observed in Ch22q11.2D syndrome patients and may contribute to the autoimmunity [17;23;56;57;58;59;60;61;62;63].

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Figure 1.

CFSE was used to define T cell proliferative responses to PHA. The Proliferation Index is the average number of cell divisions that a cell in the original population has undergone. The Percent Divided is equal to the precursor frequency. Adult patients n=16, Pediatric patients n=12, Adult controls n=46. Pediatric controls n=34.

Figure 2.

Toddlers with Ch22q11.2D have increased Th1 cytokine production. Flow cytometry and intracellular cytokine staining was performed for the indicated markers and cytokines. The gating strategy included physical parameters, CD3, and CD4. The Mean Fluorescent Intensity (MFI) was calculated from the single cytokine positive cells. Pediatric patients n=9, Pediatric controls n=14. Asterisks indicate statistical significance: IFN- γ + cells in pediatric patients vs. age-matched pediatric controls p=0.0098. The ratio of IFN- γ to IL-4 positive cells in pediatric patients vs. age-matched pediatric controls p=0.0074.

Figure 3.

Adults with Ch22q11.2D have increased Th2 cytokine production. Flow cytometry was performed as above. The Mean Fluorescent Intensity (MFI) was calculated from the single cytokine positive cells. Adult patients n=10, Adult controls n=16. Asterisks indicate statistical significance: IL-4+ cells in adult patients vs. adult controls p=0.014. The IL-4 geometric mean intensity in adult patients vs. adult controls p=0.030.

Figure 4.

B cell subsets in patients with Ch22q11.2D. Flow cytometry was performed using the indicated markers. The B cell subsets are all gated on CD19+ cells and are defined as follows: Naïve (CD27–CD38+), Transitional (CD27–CD38++), Unswitched Memory (IgM +CD27–), and Switched Memory (IgM–CD27+). Adult patients n=15, Adult controls n=24, Pediatric patients n=22, Pediatric controls n=16.

Table 1

Demographic characteristics of patients and controls

Characteristic	Patients	Controls
Toddlers		
Number	22	34
Age	2.7 years	4 years
Adults		
Number	18	46
Age	21 years	24 years

Table 2Abnormal V β families

	Adult Controls n=10	Adult Ch22 n=18	Pediatric Ch22 n=16	P value adult control vs adult Ch22
Number of oligoclonal families	1.55	8.6	5.4	0.0001
Number of drop-out families	1.44	7.1	6.9	0.0009