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Scleritis and multiple systemic autoimmune manifestations in chronic natural killer cell lymphocytosis associated with elevated TCR alpha/beta+ CD3+CD4-CD8- double-negative T cells

Steven Yeh¹, Zhuqing Li¹, H. Nida Sen¹, Wee-Kiak Lim¹, Fred Gill², Katie Perkins³, V. Koneti Rao³, and Robert B. Nussenblatt¹

¹Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, MD

²Clinical Center, National Institutes of Health, Bethesda, MD

³National Institute for Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

Abstract

Background/Aims—Chronic natural killer lymphocytosis (CNKL) has been associated with systemic autoimmunity, but its association with scleritis or ocular autoimmunity has not been characterized. We evaluated the natural kill cell (NK) function and immunophenotype of a patient with CNKL who developed bilateral scleritis and multiple systemic autoimmune findings.

Methods—The ophthalmic records of a patient with CNKL and scleritis were reviewed over a 6-year period. Flow cytometry was performed to evaluate T-cell, NK, and B-cell populations. NK cellular functions (i.e. NK cytotoxicity and cytokine/chemokine production following IL-2 stimulation) were evaluated.

Results—A 56-year-old female with vitiligo, psoriatic arthritis, thyroiditis, erythema nodosum, bilateral anterior scleritis and Sjogren syndrome was managed with multiple immunosuppressive medications including prednisone, mycophenolate mofetil and methotrexate. Flow cytometry showed a persistent elevation of CD56⁺CD3⁺ NK cells greater than 40%, which was consistent with CNKL. NK cell cytotoxicity assay identified a deficiency of K562 cell lysis in the patient (1.46 mean-fold greater in control vs. patient). NK cytokine/chemokine production following IL-2 stimulation was also deficient (2.5–32.5 fold greater in control). Cytokines/chemokines assessed included pro-inflammatory (IFN- γ , TNF- α , IL-1, MCP-1) and immunoregulatory cytokines (IL-4, IL-5 and IL-10). An abnormal elevation of TCR α / β ⁺ CD3⁺CD4⁺CD8⁺ T-cells suggestive of autoimmune lymphoproliferative syndrome was observed but apoptosis dysfunction was not found.

Conclusion—The association of increased but dysfunctional NK cells in the context of multiple systemic and ocular manifestations suggests a role of NK cells in the pathogenesis of our patient's disease. Further studies regarding NK cell dysfunction and ocular autoimmunity are needed.

Corresponding Author: Robert B. Nussenblatt, MD, MPH, Chief, Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bldg 10, 10N112, 10 Center Dr., Bethesda, MD 20892, Phone No: 301-496-3123, Fax No: 301-480-1122.

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Keywords

Scleritis; thyroiditis; vitiligo; natural killer cell lymphocytosis; episcleritis; keratoconjunctivitis sicca; double-negative T-cells; autoimmune lymphoproliferative syndrome

Introduction

Scleritis is an autoimmune condition involving inflammation of the sclera, scleral vessels, and neighboring tissues. Over 40% of patients with scleritis have an associated autoimmune disease; however, a significant proportion of patients have no identifiable systemic cause for scleritis.[1] The pathogenesis of scleritis is thought to involve an infiltration of immune cells consisting of neutrophils, macrophages, T- and B-cells, with local pro-inflammatory cytokine secretion including TNF- α and IL-1. Inflammatory cell recruitment and subsequent matrix metalloproteinase secretion result in tissue destruction and eventually, severe visual loss if the inflammation is not promptly treated.[2]

Although the inflammatory processes involved in scleritis have been studied extensively, the role of natural killer cells, which also play a role in mediating and possibly regulating autoimmunity, has not been fully characterized. Chronic natural killer cell lymphocytosis (CNKL) is a relatively rare hematologic disorder characterized by the persistent expansion of mature NK cells in the peripheral blood and has been associated with autoimmune manifestations including rheumatoid arthritis, skin vasculitis, arthralgias and cytopenias that may respond to immunosuppressive therapy.[3, 4] NK cells normally comprise 15% of the mononuclear cells in the peripheral blood; a previous report thus classified a proportion exceeding the mean value by 2 standard deviations (40%) as the cutoff value to define excess NK cell populations.[3]

We report a patient with CNKL who presented to our service with scleritis in addition to a number of other systemic autoimmune manifestations. We review the 6-year longitudinal follow-up of our patient's disease ophthalmic course, CNKL immunophenotyping data, and functional analysis of her NK cells, which may have contributed to the pathogenesis of her condition. We also report herein an abnormal elevation of TCR $\alpha\beta^+$ CD4 $^-$ CD8 $^-$ double-negative T cells (DNTs), which has been previously associated with autoimmunity.

Methods

The patient was evaluated from 2002 to 2008 with serial ophthalmic and laboratory evaluation with informed consent and approval of an Institutional Review Board of the National Eye Institute, National Institutes of Health (Bethesda, MD). All research conformed to the ARVO statement on human research and the tenets of the Declaration of Helsinki.

Ophthalmic and laboratory evaluation

Serial ophthalmic evaluation included measurement of visual acuity, slit lamp biomicroscopy with and without the assistance of 10% phenylephrine to distinguish episcleral from scleral vascular injection, intraocular pressure by applanation tonometry, and dilated fundusoscopic examination. The patient was treated with immunosuppressive medications as deemed appropriate by the treating physician. Peripheral blood draws were performed for flow cytometry evaluation, NK cell-sorting, NK cytotoxicity assay, cytokine and chemokine production. Peripheral blood from normal donor controls was obtained via the NIH Blood Bank under IRB-approved protocol.

Flow cytometry analysis

The patient underwent peripheral blood isolation for lymphocyte immunophenotyping using previously described methods.[5] The following antibodies for cell surface staining were utilized -PE-Cy7 labeled CD3 (clone SK7), APC labeled anti-human CD8 (clone RPA-T8) or CD56, and PE-labeled anti-human CD4 (clone RPA T4). All of these were obtained from BD Pharmingen (San Diego, CA).

Flow cytometry data were analyzed by FlowJo software (Treestar, San Jose, CA, USA). Briefly, lymphocytes were gated based on cell optic characteristics (FSC vs. SSC). CD3⁺, CD4⁺, CD3⁻, CD4⁻, CD8⁺, CD8⁻, CD56⁺, CD56⁻ were gated based on antibody staining.

Cell separation

Peripheral blood mononuclear cells were isolated by density gradient centrifugation as previously described.[6] Briefly, fresh blood samples were diluted 1:2 with sterile PBS and overlaid with 2 volumes of Ficoll-Paque® (Ge Health Bio-Science AB, Sweden). The samples were centrifuged at room temperature and the interphase was collected, washed with PBS and the red blood cells were lysed using ACK lysis buffer (Quality Biologicals Inc, MD). The purified PBMCs were counted and ready for downstream analysis.

For purification of CD56⁺ CD3⁻ NK cells, isolated PBMCs were labeled with anti-CD3 and anti-CD56 monoclonal antibodies (BD Biosciences, CA) and sorted by a FACSARIA sorter (BD Biosciences, CA) based on CD3⁻ and CD56⁺ staining. The purity of the sorted cells was greater than 95%. The sorted cells were then used for further functional analysis.

NK cytotoxicity assay

CD56⁺CD3⁻ cells were isolated via magnetic cell-sorting. CD56⁺ NK cells were co-cultured with K562, a previously established cell line for assessing NK cell cytotoxicity. The cytosolic enzyme typically released upon cell lysis, lactate dehydrogenase (LDH), was measured via a 30-minute enzymatic reaction, in which LDH converts a tetrazolium salt (INT) into a red formazan product. The formazan product was measured by optical density (OD) utilizing the 490 nm wavelength and the LDH concentration calculated using a standard curve.

NK cytokine production assay

FACSARIA-sorted CD56⁺CD3⁻ NK cells were cultured in standard complete medium (RPMI 1640 with 10% fetal bovine serum, glutamine and penicillin and streptomycin) and stimulated with recombinant human IL-2 at a concentration of 50 ng/ml. After 48 hours stimulation, the supernatants were harvested and the concentrations of cytokines and chemokines (IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-7, IL-10, IL-12p40, IL-12p70, IL-15, IFN- α , IFN- γ , TNF- α , MCP-1, MIP-1 α , MIP-1 β .) were measured by multiplex ELISA assay (Aushon SearchLight, Boston).

Results

Case Report

A 56 year-old Caucasian female of German-French ancestry presented to the National Eye Institute in 2002 with episcleritis and scleritis and a history of multiple autoimmune conditions including autoimmune thyroiditis with associated subglottic edema, psoriatic arthritis, vitiligo, lichen sclerosis, erythema nodosum, peripheral neuropathy, and Sjogren

syndrome (Table 1). The patient's past medical history was also significant for recurrent urinary tract infections. Family history was significant for a daughter with vitiligo.

The patient's episcleritis was originally diagnosed in 1996, six years prior to presentation to the Uveitis and Ocular Immunology service. She reported approximately 3–4 episcleritis exacerbations per year, which were managed with topical Voltaren or 1% prednisolone acetate. From 1999–2000, the patient's ocular disease evolved into a recurrent, bilateral scleritis requiring oral prednisone in addition to topical 1% prednisolone acetate. In January 2001, the patient was placed on methotrexate (15 mg/week) but this was discontinued, as the prednisone could not be tapered to less than 20 mg/day. Azathioprine was later tried as a corticosteroid-sparing agent but was discontinued due to significant nausea, vomiting, and diarrhea.

Ophthalmic evaluation in 2002 revealed visual acuities of 20/20 OU. The external exam was remarkable for 360 degrees of perilimbal bluish coloration OU suggestive of mild scleromalacia. Slit lamp examination revealed bilateral, diffuse, 2+ episcleral vessel injection and prominent scleral vascular injection involving the inferotemporal quadrant of the left eye following instillation of 10% phenylephrine. The findings were consistent with diffuse, anterior non-necrotizing scleritis. Anterior chamber examination was quiet OD and showed trace cells OS. The irides were normal OU. Examination of the lenses showed a mild posterior subcapsular cataract OD. Dilated fundusoscopic examination was normal OU. Schirmer tear production testing without anesthesia was reduced with 1 mm of wetting OD and 0 mm OS. Schirmer testing with anesthesia revealed 1 mm of wetting OD and 2 mm of wetting OS. Tear break-up time to evaluate the patient's ocular surface for signs of keratoconjunctivitis sicca was 3 seconds OD and 2 seconds OS (Normal tear break-up time > 10 seconds).

Laboratory evaluation included ANA, anti-dsDNA antibody, RF, ACE, ESR, C-reactive protein, HIV, Lyme antibody, hepatitis panel, anti-cardiolipin IgM and IgG antibodies, serum immunoglobulins (IgG, IgA, IgM, IgE), CXR, and PPD, which were normal. Laboratory testing was positive for anti-thyroglobulin antibodies.

Because of the patient's active scleritis, methotrexate was re-initiated at a dose of 15 mg/week. Attempts to taper the prednisone from 20 mg were then initiated. During 2002–2003, intermittent scleritis exacerbations eventually required an increase of methotrexate to 25 mg/week (Figure 1). Subconjunctival corticosteroid injections (triamcinolone 40 mg/ml $\times$ 0.2 cc) were utilized to curb scleritis exacerbations.

During 2003–2006, methotrexate was maintained at 25 mg/week but prednisone could not be tapered past 17 mg/day without mild flare-ups of the scleritis. CSA was initiated but subsequently discontinued due to HTN symptoms. In 2006, mycophenolate mofetil at a dose of 1000 mg bid was started and prednisone was tapered to 13 mg/day. Methotrexate was later reduced to 15 mg/week. Attempts to taper the prednisone below 13 mg/day in December 2007 resulted in a scleritis exacerbation and she currently remains stable on mycophenolate mofetil 1000 mg/bid, methotrexate 15 mg/week, and prednisone 13 mg/day with a stable visual acuity of 20/25 in both eyes.

Immunophenotypic analysis

In May 2004, lymphocyte immunophenotyping revealed that 46.5% of lymphocytes were CD56⁺. Repeat immunophenotyping in September 2004 showed that 51.6% of lymphocytes were CD56⁺ and in June 2005, 43.7% of cells were CD56⁺ (Figure 1). A broader panel of immunophenotypic markers was evaluated in July 2004 to evaluate the possibility of large granular leukemia. At that time, lymphocyte immunophenotyping revealed that NK cells

comprised 46.0% of total lymphocytes and the absolute number was 3.35×10^5 cells/ml (Supplemental Table 1).

In January 2008, lymphocyte immunophenotyping revealed an elevated percentage of NK cells at 32.5% and an absolute NK cell count of 358. An elevation of double-negative $CD3^+CD8^-CD4^-$ T-cell population was observed at 14.9% with an absolute count of 164 DNT cells (Supplemental Table 1).

NK cytotoxicity assay

NK cytotoxicity assay revealed that the patient's NK cells were unable to lyse cells as efficiently as NK cells harvested from a normal donor control, suggesting a defect in cytotoxicity (Figure 2). This deficiency in killing target cells was seen in the effector vs. target cell ratios (E/T ratio) and was not due to differences of cell viability (NK cellular viability > 95% for all investigations). Specifically, for the 5:1, 2.5:1, 1.25:1, 0.625:1, 0.31:1, and 0.16:1 E/T ratios, NK cells from the normal donor control demonstrated a 1.46 mean fold increase in percentage cytotoxicity when compared to the NK cells from the patient.

NK cytokine production assay

NK cells were sorted via antibody staining-based FACS sorting. The functional differences of NK cells from the patient vs. a normal control was also tested for their ability to secrete cytokine/chemokines in response to stimulation as described in the Materials and Methods. All cytokine and chemokines assessed in the supernatant from the patient's cultured NK cells were dramatically decreased when compared to the NK cells from a normal control patient. 2.5-fold to 32.5-fold lower levels of cytokine and chemokine production were observed when comparing IL-2-stimulated NK cells from the patient compared to those of a normal donor (Table 2). This included proinflammatory cytokines, such as $IFN-\gamma$, $TNF-\alpha$, IL-1, IL-12, MCP-1 and MIP-1s, as well as immunoregulatory cytokines, such as IL-4, IL-5 and IL-10.

Conclusion

Scleritis is a destructive inflammatory eye condition, which is mediated by an influx of T-cells, B-cells and macrophages.[7, 8] Immune complex deposition vasculitis is also thought to be a primary process in the pathogenesis of this condition.[9] However, the putative antigen leading to antigen-antibody complex deposition has not been identified. Activated inflammatory cells secrete pro-inflammatory cytokines that contribute to matrix metalloproteinase elaboration and progressive scleral tissue destruction.[2] While the role of T- and B- lymphocytes and macrophages in scleritis has been described, the role of NK cells has not been characterized.[8]

Our patient presented with bilateral scleritis and a number of other systemic autoimmune findings and was diagnosed with CNKL. Clinical and laboratory heterogeneity has been observed in CNKL with autoimmune manifestations that include cytopenias, arthritis/arthralgias, immediate allergic reactions to drugs and environmental substances, and cutaneous vasculitis syndromes.[3, 10] Sjogren's syndrome-associated keratoconjunctivitis has been described with CNKL.[11] Increased immunoglobulin production has also been reported in association with NK proliferative disorders,[11] as well as elevations of ANA, RF, and anti-neutrophil antibody.[3]

The NK cell functions of our patient were assessed using a cytotoxicity assay and cytokine production assay following IL-2 stimulation of NK cells. Interestingly, NK cells derived from our patient showed significantly depressed cytotoxic function when compared to a normal donor. The production of proinflammatory cytokines ($IFN-\gamma$, IL-1 α , IL-1 β), $TNF-\alpha$),

Th2-associated cytokines, and the immunoregulatory cytokines (IL-4, IL-5 and IL-10) upon stimulation of sorted CD56⁺CD3⁻ NK cells by IL-2 was globally depressed when compared to a normal control donor. Chemokine expression of monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , MIP-1 β were also reduced following IL-2 stimulation of the patient's NK cells.

NK cells are thought to play a primary role in the innate immune system, conferring protection against viral, bacterial and parasitic infections, as well as their well-described role in tumor surveillance. However, recent work has supported the role of NK cells as regulators of adaptive immunity, providing stimulatory as well as suppressive signals for components of the immune system including dendritic cells and T cells.[6, 12] A prior study demonstrated that NK cells appear critical to the maintenance of clinical remission in multiple sclerosis patients by suppressing and/or deleting IFN- γ secreting, myelin basic protein-autoreactive CD4⁺ T cells.[13]

Reductions in NK cell populations have been reported in other autoimmune conditions including MS[14] and psoriasis.[15] It has been postulated that NK cells secrete protective Th2 cytokines; when Th2 cytokines are reduced, the predominance of Th1-associated cytokines may result in autoimmune manifestations.[16] Interestingly, defective or reduced NK cell function has also been observed in autoimmune conditions including MS[17], Sjogren's syndrome[18, 19], and psoriasis.[20]

Whether the changes in NK population and function in our patient directly contributed to the autoimmune findings in our patient remains to be determined. However, this is the first observation that NK cell dysfunction may be associated with non-infectious scleritis in association with other systemic autoimmune manifestations. IL-2 and IL-15 signaling comprise the two major pathways that lead to NK cell proliferation, survival and cytolytic activity.[21] We did not assess the NK cell response to IL-15, and it is possible that defective IL-2 signaling could have played a role in the global failure of NK cell function.

The other question that arose from immunophenotyping data was the relevance of the TCR $\alpha\beta$ ⁺ DNTs to the patient's condition. ALPS is an inherited disorder characterized by lymphadenopathy, hypersplenism, hypergammaglobulinemia, and autoimmune cytopenias, which may manifest in childhood;[22] an elevation of TCR $\alpha\beta$ ⁺ DNTs is characteristic of ALPS [23], as well as Fas-mediated apoptosis in some patients.[24] Our patient was evaluated for the ALPS, but clinical characteristics of ALPS were not found. In addition, laboratory investigations showed no evidence of defective lymphocyte apoptosis.

In summary, we have described a patient with bilateral, non-infectious scleritis and multiple systemic autoimmune findings associated with CNKL. Investigation of the patient's NK cells showed NK cell dysfunction including defective cytotoxicity and reduced cytokine/chemokine expression in response to IL-2 stimulation. The relationship of the defective NK cell populations to our patient's autoimmune manifestations requires further study. The significance of the elevated DNT cells is not clear, and although no clinical characteristics of ALPS were identified in our patient, the relevance of the abnormal DNT cell population in non-ALPS autoimmune disease also warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Slit lamp photograph of right eye and serial flow cytometry of patient
 Slit lamp photograph shows diffuse episcleral and scleral injection superiorly (top panel).
 Flow cytometry in May 2004 (lower left) demonstrated that natural killer (NK) cells
 comprised 46.5% of total lymphocytes, 51.6% in September 2004 (lower middle), and
 43.7% in June 2005 (lower right).

Figure legend/Abbreviations
 ▲ NK cytotoxicity curve of NK cells from normal donor
 ■ NK cytotoxicity curve of NK cells from patient
 E/T Ratio Effector/Target Ratio

Figure 2. Natural killer cell cytotoxicity assay

Natural killer cytotoxicity curves demonstrate that at Effector/Target (E/T) ratios varying from 5:1 to 0.155:1, percentage cytotoxicity of natural killer cells isolated from the peripheral blood of the normal control donor (▲) was greater than those isolated from the patient (■). The percentage cytotoxicity was 1.46 mean fold greater in the normal control donor than the patient.

Table 1

Clinical ocular and systemic autoimmune features

Autoimmune conditions	Age of onset
Lichen sclerosis et atrophicus	25
Vitiligo	36
Erythema nodosum	43
Psoriatic arthritis	46
Autoimmune thyroiditis	50
Bilateral episcleritis *	51
Peripheral neuropathy	54
Bilateral, non-necrotizing scleritis *	57
Sjogren syndrome keratoconjunctivitis sicca *	57

Family history was significant for a daughter with vitiligo.

* Ocular autoimmune features

Table 2

Serum cytokine or chemokine profile in response to IL-2 stimulation of NK cells

	Patient (pg/mL)	Normal donor (pg/mL)	Fold-difference: Normal vs. Patient
IL-1 α	18.3	45.0	2.5x
IL-1 β	5.7	49.4	8.7x
IL-4	6.6	42.5	6.4x
IL-5	< 0.8	2.4	> 3.0x
IL-7	3.2	10.4	3.3x
IL-10	4.6	23.6	5.1x
IL-12p40	7.6	31.6	4.2x
IL-12p70	1.8	7.6	4.2x
IL-15	3.0	16.8	5.6x
IFN- α	0.9	29.3	32.6x
IFN- γ	3.7	40.1	10.8x
TNF- α	51.3	343.0	6.7x
MCP-1	351.6	1490.5	4.2x
MIP-1 α	362.9	3400.0	9.4x
MIP-1 β	349.8	2256.9	6.5x