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Original Article
Syndrome of selective IgM deficiency with severe T cell deficiency associated with disseminated cutaneous mycobacterium avium intracellulare infection

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Abstract: Cutaneous non-disseminated, non-tuberculous mycobacterial infections have been reported in both immunocompetent and immunocompromised subjects. Systemic Mycobacterium avium intracellulare (MAI) have been reported in non-HIV patients with Idiopathic CD4 lymphocytopenia. We report a comprehensive immunological analysis in syndrome of selective IgM deficiency and T lymphocytopenia (both CD4+ and CD8+) with disseminated cutaneous MAI infection. Naive (T_N) and Central memory (T_CM) subsets of both CD4+ and CD8+ T cells were decreased, whereas terminally differentiated effector memory (T_EMRA) subset of CD4+ and CD8+ T cells were markedly increased. IFN-γ producing T cells were markedly decreased. Although CD14highCD16- proinflammatory monocytes were modestly increased, IFN-γR+ monocytes were markedly decreased. The expression of TLR3, TLR5, TLR7, and TLR9 on monocytes was decreased. Germinal center B cells (CD19+IgD-CD38+CD27lo) and B1 cells (CD20+CD27+CD43+CD70-) were markedly decreased. A role of immune alterations, including B cells and antibodies in disseminated cutaneous MAI infection is discussed.

Keywords: TLR, memory T cells, B1 cells, germinal center B cells, IFN-γ

Introduction
Non-tuberculous mycobacteria (NTM) were considered saprophytes until acquired immunodeficiency disease was discovered when Mycobacterium avium complex (MAC) species emerged as a major opportunistic infection in patients with HIV infection. The first case of Mycobacterium avium intracellulare (MAI) infection of the lung in a non-HIV patient with CD4 lymphocytopenia was described in 1992 [1]. Later in the year, the Center for Disease Control and Disease Prevention coined the termed Idiopathic CD4+ lymphocytopenia (ICL) and defined as CD4+ depletion of < 300/ul or < 20% of the total lymphocytes on two separate times with a minimum of six weeks of time without any secondary causes of immunodeficiency or immunosuppression [2]. Since then, several reviews on ICL have been published [3-7]. We described a syndrome of T cell lymphocytopenia (shared by both CD4+ and CD8+ T cells) and selective IgM deficiency associated with systemic MAI infection [8]. This syndrome is different from ICL and selective IgM deficiency; ICL is not associated with selective IgM deficiency, and selective IgM deficiency is not associated with T cell lymphocytopenia or T cell defect functional defect [9, 10]. Cutaneous NTM infections have been reported in both immunocompetent and immunocompromized hosts [11-15]. Although systemic MAI infections have been reported in patients with ICL, and in the syndrome of T cell lymphocytopenia and selective IgM deficiency, disseminated cutaneous MAI infection has not been reported in either conditions. The host immune responses to M. tuberculosis have been studied in detail; however, host immune responses to NTM are not completely understood. A role of macrophages and T cells in immune response to mycobacteria has recently been evaluated [16-18]. Here we present a comprehensive analysis of host immune responses in a patient with a syndrome of T cell lymphocytopenia and selective IgM deficiency with disseminated cutaneous MAI
Disseminated cutaneous MAI

<table>
<thead>
<tr>
<th>Table 1. Immunological analysis of the Patient</th>
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<tbody>
<tr>
<td><strong>Test</strong></td>
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<tr>
<td>Lymphocyte counts (/(^3)mm)</td>
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<tr>
<td>Percentage</td>
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<tr>
<td>Absolute counts</td>
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<tr>
<td>Serum Immunoglobulins (mg/dl)</td>
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<td>IgM</td>
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<td>IgE (IU/ml)</td>
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<tr>
<td>Lymphocyte subsets % (#)**</td>
</tr>
<tr>
<td>CD3+</td>
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<tr>
<td>CD3+CD4+</td>
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<tr>
<td>CD3+CD8+</td>
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<td>CD4/CD8 ratio</td>
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<tr>
<td>CD19+</td>
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<tr>
<td>CD3-CD56+CD16+</td>
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<tr>
<td>Delayed Type skin Hypersensitivity</td>
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<tr>
<td>Mumps</td>
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<tr>
<td>Tetanus</td>
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<tr>
<td>PPD</td>
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<tr>
<td>Lymphocyte proliferation (counts per min)</td>
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<td>PHA</td>
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<td>ConA</td>
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<tr>
<td>PWM</td>
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<td>NK cytotoxicity</td>
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<td>Lytic unit</td>
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<tr>
<td>TB Quantiferon (IU/ml)</td>
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</table>

*In subjects exposed to Mycobacterium or BCG vaccinated; **Lymphocyte subsets were performed at least on 4 separate occasions over 2 years period and were similar.

infection. This is the first report of comprehensive B cell subset analysis in mycobacterial infection. A possible role of B cell subsets and antibodies in mycobacterial defense is discussed.

Material and methods

Patient

In October 2012, the patient, a 53 year old man was involved in a motor vehicle accident where he fractured his collarbone. At that time he appreciated a small nodule on his right upper arm that began to grow. As time progressed, more lesions appeared on the medial aspect of upper right arm. A biopsy performed by a dermatologist was nonspecific. He then was referred to us for a second opinion. An immunological analysis and two biopsies were performed. His lesions at that time were two lesions that were 1 cm × 1 cm. He had no lymphadenopathy. The results of his immunological analysis are shown in Table 1, which revealed severe T cell lymphopenia that is shared by CD4+ and CD8+ T cells, selective IgM deficiency, and low NK cell functions. Similar phenotype has been reported in three patients with systemic MAI infection [8].

He was started on treatment in February 2013 with azithromycin 500 mg 3 times weekly, ethambutol 1500 mg/day and rifampin 600 mg 3 times weekly. Initially, his lesions responded to therapy, which was discontinued after 15 months. However, his lesions began to increase in size and now all four lesions were approximately 1.0 × 2.0 cm in size. He was resumed on same anti-mycobacterial regimen. However, his lesions continue to increase in size. Another biopsy was performed with culture positive for MAI. Moxifloxacin was added to his regimen. Lesions continued to increase in size. At the National Institutes of Health, he was started on IV amikacin as well as Interferon Gamma dosed at 50 mcg/m² (1 million international units/m²) subcutaneously three times weekly. Within three months, his two forearm lesions completely resolved and his two proximal lesions markedly reduced in size. Later Amikacin was discontinued because of side effects. Patient continued to receive gamma interferon.

Antibodies and reagents

**T cell subsets**: CD4 PerCP and CD8 PerCP, CD45RA APC, CCR7 FITC, CD14 FITC, CD16 PE. IFN-γ R-PE all antibodies were from BD Phar-
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mingen (San Jose, CA), and IL-12R PE from R&D Systems (Minneapolis, MN).

**TLR Expression:** Antibodies to TLR2 Alexa647, TLR4 biotin + Streptidine PE (BD Pharmingen, San Jose, California), TLR5 Alexa488 and TLR7 FITC (R&D systems, Minneapolis, MN), TLR3 PE (e-biosciences San Diego, CA) TLR6 PE (Biolegand, San Diego, CA) were used.

**B cell subsets:** The following anti-human antibodies were used: CD19 PerCP, CD20 PerCP anti-IgM APC, CD27 FITC, CD38 FITC, anti-IgD PE, CD21 PE, CD70 PE, CD27 APC, CD38 PE, all from BD Pharmingen (San Jose, CA), and CD43 APC from Biolegand (San Diego, CA).

Peripheral blood mononuclear cells (PBMNCs) were isolated from blood of patient and healthy subjects by Ficoll-hypaque density gradient. Protocol was approved by Human Subject Committee of the Institution Review Board of the University of California, Irvine.

**Immunophenotyping**

Whole blood was diluted with phosphate buffer saline (PBS), washed × 2, and then centrifuged. Cell pellet was diluted with 1 ml of PBS and stained with a panel of antibodies for various subsets of B cells and subsets of CD4+ and CD8+ T cell subsets (see below). After staining, RBC was lysed by 1 × lysing solution (BD Pharmingen, San Jose), washed with PBS, and analyzed. Flow cytometry was performed using FACS Calibur (Becton-Dickenson, San Jose, CA) equipped with argon ion laser emitting at 488 nm (for FITC, PE and PerCP excitation) and a spatially separate diode laser emitting at 631 nm (for APC excitation). Forward and side scatterers were used to gate and exclude cellular debris. Ten thousand cells were acquired and analyzed using Flowjo software (Treestar, Ashland, OR). B cell subsets included naïve (CD19+IgM+IgD+CD27+), transitional (CD19+CD38+IgM+), mature (CD19+CD21+), marginal zone (CD19+CD27+IgD+), IgM memory (CD19+IgM+CD27+), class switched memory (CD19+CD27+IgD+), germinal center (CD19+CD38+CD27lowIgD+), B1 cells (CD20+CD27+CD43+CD70-), and plasmablasts (CD19+CD38+IgM-). Subsets of CD4+ and CD8+ T cells included naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7-), and terminally differentiated effector memory/exhausted (CD45RA+CCR7-).

**Antibody panel for 4-color B cell subsets phenotype**

<table>
<thead>
<tr>
<th>Panel</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP</th>
<th>APC</th>
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<tbody>
<tr>
<td>1</td>
<td>CD27</td>
<td>anti-IgD</td>
<td>CD19</td>
<td>anti-IgM</td>
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<tr>
<td>2</td>
<td>CD38</td>
<td>CD21</td>
<td>CD19</td>
<td>anti-IgM</td>
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<tr>
<td>3</td>
<td>CD27</td>
<td>CD70</td>
<td>CD20</td>
<td>CD43</td>
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<tr>
<td>4</td>
<td>CD38</td>
<td>IgD</td>
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**Antibody panel for subsets of CD4+ and CD8+ T cell phenotype**

<table>
<thead>
<tr>
<th>Panel</th>
<th>FITC</th>
<th>PerCP</th>
<th>APC</th>
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<tr>
<td>1</td>
<td>CCR7</td>
<td>CD4</td>
<td>CD45RA</td>
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<tr>
<td>2</td>
<td>CCR7</td>
<td>CD8</td>
<td>CD45RA</td>
</tr>
</tbody>
</table>

**Detection of intracellular cytokines**

2 × 10^6/ml peripheral blood mononuclear cells (PBMC) cells in RPMI-1640 medium were activated with 10 ng/ml Phorbol 12-myristate 13-acetate (PMA) + ionomycin 1 µg/ml and 10 µg/ml Brefeldin A (BFA) (Sigma, St. Louis, MO), and Incubate for 4 hours at 37°C in a 5% CO2 atmosphere. Cells were surface stained with CD4 PerCP for 30 min, fixed with 250 ul BD Cytofix/Cytoperm™ Buffer. Cells were washed by BD Perm/Wash™ Buffer, a permeabilization and wash buffer that maintain cellular permeability and facilitate intracellular staining. Activated and unactivated cells stained for Intracellular IFN, TNF and corresponding isotype controls. Ten thousand cells were acquired and analyzed with FACS Calibur.

**Detection of TLRs**

PBMCs were surface stained either CD14 FITC or PE and surface stained with antibodies to TLR 2, 4, 5 and 6 for 30 min, washed with PBS and acquire by FACS Calibur, TLR4 tube washed stained with additional streptidine PE for 30 min wash and acquired. TLR3, TLR7, and TLR9 tubes after CD14 surface staining were fixed and permeabized with BD Cytofix/Cytoperm™ Fixation/Permeabization Kit as per manufacturer instructions, and stained with antibodies to TLR3, TLR7, and TLR9, washed, and acquire by FACS Calibur. Corresponding isotypes were used as background. Data were analyzed by Flowjo software (Treestar, Ashland, OR).
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Statistical analysis
Statistical analysis was performed using Graph Pad Prism. Differences between control and Patient sample were tested using one tail-paired t-tests. Values of $P < 0.05$ were considered significant.

Results

Naive, central memory, and effector memory subsets of CD4+ and CD8+ T cells

CD4+ and CD8+ T cells have been classified into $T_N$ (CD54RA+CCR7+), $T_{CM}$ (CD45RA-CCR7+), $T_{EM}$ (CD45RA-CCR7-), and $T_{EMRA}$ (CD45RA+CCR7-) subsets of CD4 and CD8 T cell subsets were examined with FACSCalibur and analyzed with FlowJo. $T_N$ and $T_{CM}$ cells are markedly decreased as compared to controls. $T_{EMRA}$ of CD4 is increased, whereas majority of CD8+ T cells were $T_{EMRA}$ (95.9%).

Figure 1. Naïve and memory subsets of CD4+ and CD8+ T cells. $T_N$ (CD54RA+CCR7+), $T_{CM}$ (CD45RA-CCR7+), $T_{EM}$ (CD45RA-CCR7-), and $T_{EMRA}$ (CD45RA+CCR7-) subsets of CD4 and CD8 T cell subsets were examined with FACSCalibur and analyzed with FlowJo. $T_N$ and $T_{CM}$ cells are markedly decreased as compared to controls. $T_{EMRA}$ of CD4 is increased, whereas majority of CD8+ T cells were $T_{EMRA}$ (95.9%).

Figure 2. IFN-γ and TNF-α containing CD4+ (Th1) cells. Mononuclear cells were stimulated with PMA and ionomycin and secretion of cytokine was blocked by brefeldine. Cells were stained for surface CD4, fixed and then stained for intracellular IFN-γ and TNF-α with respective antibodies and isotype controls. Cells were gated on CD4+ T cells and then analyzed for IFN-γ+ and TNF-α+ cells by multicolor flow cytometry. IFN-γ+ cells were markedly decreased (21.7%) as compared to control (85.6%). TNF-α+ cells were comparable. Blue lines are for isotype control and red lines for specific antibodies.

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Th1 cells are decreased
Th1 (IFN-γ) cells play an important role in defense against $M. tuberculosis$ and $M. leprae$. More recently, it has been reported that IFN-γ...
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and TNF-α are also important in defense against NTM [23, 24]. Therefore, we examined CD4+ T cells with intracellular IFN-γ and TNF-α. A significant reduction in IFN-γ+ T cells was observed in the patient as compared to controls (Figure 2). In addition, serum quantiferon levels were undetectable (Table 1).

Monocytes and TLR expression

When PBMNCs were isolated from whole blood CD14+ monocytes in the patient were markedly increased (26%) as compared to control (6%). Monocyte subsets were analyzed using CD14 and CD16 antibodies. CD14+CD16− “proinflammatory” monocytes are marginally increased. CD14+CD16+ “resident” monocytes are comparable.

and their stage of maturation and differentiation [27]. Although a role of antibodies in defense against mycobacteria has not been fully explored, IgM has been shown to display specificity against PGL-A1 of M. leprae [28, 29]. IgG antibodies against glycopeptidolipid (GPL) core antigen of MAC were present in a majority of patients with pulmonary MAC [30]. Therefore, we analyzed various subsets of B cells with a group of antibodies using multicolor flow cytometry. Transitional B cells (CD19+IgD+CD27+) were increased (Figure 6), whereas germinal center B cells (CD19+CD38+CD27dimIgD-) and B1 cells (CD20+CD27+CD43+CD70-) were decreased (Figure 7).

Discussion

The incidence of NTM infection is increasing worldwide. In a very large study Hoefslooot and colleagues reported 91 different species of NTM in over 20,000 patients from 30 countries [31]. Systemic NTM infections in humans manifest as hypersensitivity pneumonitis, cavitary disease, and nodular bronchiectasis. Cutaneous NTM infections are uncommon. A solitary cutaneous MAI infection was reported in a young man who underwent allogeneic bone marrow transplantation [14]. Disseminated cutaneous MAI and TNF-α are also important in defense against NTM [23, 24]. Therefore, we examined CD4+ T cells with intracellular IFN-γ and TNF-α. A significant reduction in IFN-γ+ T cells was observed in the patient as compared to controls (Figure 2). In addition, serum quantiferon levels were undetectable (Table 1).

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Mycobacterium chelonae infections, with or without cutaneous and osseous manifestation, have been reported [32, 33]. Cutaneous MAI infections are rare and disseminated cutaneous MAI infection has not been reported. Detailed immunological analyses were not performed in any of these cases. Our case also highlights a possible role of B cells and antibodies, albeit minor, in defense against mycobacterial species. Initially, our patient responded to antimycobacterial therapy; however, later became resistant. Since our patient has markedly reduced IFN-γ producing CD4+ T cells and no quantiferon, IFN-γ was added to therapy, to which he responded. Patients with ICL and MAC infection have been successfully treated with IFN-γ and IL-2 therapy [34].

Host immune responses to M. tuberculosis and M. leprae have been studied in detail [35, 36]. The host immune responses to NTM are similar to M. tuberculosis with some differences [18]. The major protective responses to mycobacteria are Th1 CD4 response and macrophages (Th1 CD4+ T cells produce TNF-α and IFN-γ); IFN-γ activates macrophages resulting in intracellular killing of mycobacteria [35]. IFN-γ deficiency has been considered as a major factor in the pathogenesis of MAC infection [35, 36]. IL-12 plays an important role in defense against mycobacterial infection by polarizing Th0 cells to Th1 cells to produce IFN-γ, which then binds to macrophages via IFN-γR, and activating them to eliminate mycobacteria [37-39]. Our patient has a deficiency of interferon produc-

Figure 4. TLR expression on monocytes. TLR-2, -4, -5, and -6 are membrane bound, whereas TRL-3, -7, and -9 are intracellular and were analyzed following fixation with respective antibodies and isotype controls. Numbers in the parenthesis are mean fluorescence channel numbers (MFC#) as an indicator of density of molecules. TLR-4 expression is increased, whereas the expression of TLR-5, -7, and -9 is decreased.
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Figure 5. Expression of IL-12R and IFN-γR on lymphocytes and monocytes. Mononuclear cells were stained with monoclonal antibodies against IL-12R or IFN-γR and isotype controls. Cells were gated for lymphocytes and monocytes, and data were analyzed by Flowjo. Numbers represent MFC#. IFN-γR on monocytes is decreased. IL-12R on lymphocytes and monocytes, and IFN-γR on lymphocytes are comparable between patient and control.

Toll-like receptors (TLRs) are a family of pattern recognition receptors that are capable of recognizing conserved pathogen-associated molecular patterns (PAMPs), including components of bacterial cell walls such as lipoproteins and lipoglycans present in mycobacteria species, and microbial nucleic acids [18, 42-44]. M. tuberculosis has lipoprotein that interacts with TLR-2 to activate NF-κB and secrete IL-12 [43]. It also results in killing of intracellular M. tuberculosis. Other PAMPs of M. tuberculosis include mannose-capped lipoarabinomannan, which does not activate TLR2 or TLR4 and may activate other TLRs [45], and mannosylated phosphatidylinositol (PIM), a component of soluble tuberculosis factor (STF) that appears to activate TLR-2 and TLR-6. In our patient, TLR2 and TLR6 expression on monocytes was comparable to control. TLR-7 ligands induce autophagy in mycobacterial-infected macrophages [46]. Bakhrui et al. [47] have demonstrated that BCG vaccine-mediated reduction in the expression of MHC II antigen on macrophages and dendritic cells is reversed by activation of TLR7 and TLR9. In our patient, expression of TLR7, and TLR9 was markedly decreased, and might contribute to MAI pathology.

Naïve T cells upon exposure to an antigen undergo a clonal expansion of effector cells, which after clearing the antigen undergo a phase of contraction when antigen-specific T cells undergo apoptosis, and then a small number of antigen-specific T cells stabilizes and retained as memory T cells [19-22]. These memory T cells differentially express adhesion molecules and chemokine receptors, which allow them to home in peripheral blood lymphoid tissues. Based upon the expression or lack of them, memory CD4+ and CD8+ T cells migrate to lymph nodes and spleen (central memory, T_{CM}) or to extralymphoid tissue like lung and liver (effector memory; T_{EM}). A small subpopulation of T_{EM} cells re-acquires CD45RA, and is termed as T_{EMRA} or terminally differentiated memory or exhausted T effector cells. T_{EM} and T_{EMRA} T cells display poor proliferation, decreased telomere length, and are resistance to apoptosis [20], whereas T_{N} and T_{CM}
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A

B cell
Naïve/ MZ/CSM
IgM Mem
CD21
Transitional & Plasmablast

Sc
IgD
IgM
CD21

CD19
CD7
CD27
CD38

Control
Subject

% positive

0
10
20
30
40
50
60
70
80
90
100

B

B cells
Naive
Marginal Zone
Class Switch Memory
IgM Memory Cells
Mature B Cells
CD21 Low
Transition B
Plasmablast

* **
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Figure 6. B cell subsets. Total B cells, mature B cells, naïve B cells, IgM memory B cells, class-switched B cells, CD21<sup>hi</sup> B cells, marginal zone (MZ) B cells, and plasmablasts were analyzed using a variety of markers (see methods). A. Flow cytograph shows increased transitional B cells. B. Data of patient is compared with 20 healthy controls.

Figure 7. B1 and Germinal Center (GC) B cells. Both germinal center (CD19<sup>+</sup>CD38<sup>+</sup>CD27<sup>+</sup>IgD<sup>-</sup>) and B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD70<sup>-</sup>) are markedly decreased as compared to 20 healthy subjects.

cells proliferate and are antigen-dependent. Therefore, a deficiency of T<sup>N</sup> and T<sup>CM</sup> cells and an expansion of T<sup>EMRA</sup> cells in our patient may be responsible for T cell functional defects contributing to increased susceptibility to disseminated cutaneous MAI infection. A deficiency of T<sup>N</sup> and T<sup>CM</sup> cells and expansion of T<sup>EMRA</sup> cells is also observed in aged human, which contributes to T cell immunosenescence [19].

Although a role of cell-mediated immunity is well established, a role of B cells and antibodies in defense against mycobacterial infection has not been investigated in detail. However, emerging evidence supports a role of B cells and antibodies in host defense against intracellular pathogens including <i>M. tuberculosis</i> [48, 49]. Evidence suggests that [A] B cells can regulate both CD4<sup>+</sup> and CD8<sup>+</sup> T cell memory responses [50-52], [B] B cells by virtue of producing antibodies and cytokines can modulate the maturation of antigen-presenting cells; thereby regulating the adaptive immune response [53]. Natural antibodies bind to and alter the activity of co-stimulatory molecules B7 and CD40, thereby affecting antigen presentation [54]. Cytokines produce by B cells can polarize T cells [51, 55]. [C] B cells can regulate the differentiation of macrophages into subsets. B1 cells promote polarization of macrophages into M2 subset [56]; macrophages are important in anti-mycobacterial defense. B1 cells spontaneously secrete natural IgM antibodies in the absence of exogenous antigenic stimulation and B1 and B2 cell-derived IgM antibodies play a protective role in intracellular microbe influenza virus infection [57-59]. One of the characteristics of B1 cells is the enrichment of their repertoire for poly- and self-reactive specificity [60]. In our patient, in addition to selective IgM deficiency, the numbers of B1 cells were markedly decreased. [D] Accumulating evidence suggest significant role of antibodies against intracellular pathogens including <i>M. tuberculosis</i>. Monoclonal antibodies specific for a number of mycobacterial components including arabinomannan, lipoarabinomannan, heparin-binding hemagglutinin, and 16kD-crystalin have been shown to protect against <i>M. tuberculosis</i> [61-65], and passive transfer of serum with polyclonal antibodies against <i>M. tuberculosis</i> is protective in relapse of tuberculosis in SCID mice [66]. Furthermore, IVIG in a mouse model of tuberculosis has been reported to be protective [67]. A role of antibodies in defense against mycobacterial defense is also supported by <i>M. tuberculosis</i> infections in patients with X-linked agammaglobulinemia [68, 69], and <i>M. tuberculosis</i> and severe NTM infection in patients treated with Rituximab that deplete B cells [70]. [E] Finally, a role of antibodies in mycobacterial defense is supported by the presence of IgG antibodies against glycopeptidolipid (GPL) core antigen of MAC in 77% of patients with pulmonary MAC and none in pulmonary tuberculosis [29].
The granuloma in *M. tuberculosis* has aggregates of B cells, which has cellular markers of typical germinal centers [71]. Therefore, a deficiency of GC B cells may result in abnormal granulomatous reaction with exacerbated pathology. In our patient germinal center B cells were markedly decreased.

In summary, a deficiency of IFN-γ secretion, Th1 cells, and IFN-γ-R expression on monocytes, as well a deficiency of T<sub>EMRA</sub> and T<sub>CM</sub> and accumulation of T<sub>EMRA</sub> T cell subsets likely play an important role in severe T cell deficiency and disseminated cutaneous MAI infection in the present patient. A deficiency of GC B cells may result in abnormal granulomatous reaction with exacerbated pathology. In our patient germinal center B cells were markedly decreased.

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Acknowledgements

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Disclosure of conflict of interest

None.

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