Selective Autoantibody Production against CCL3 Is Associated with Human Type 1 Diabetes Mellitus and Serves As a Novel Biomarker for Its Diagnosis

Naim Shehadeh, Shirly Pollack, Gizi Wildbaum, Yaniv Zohar, Itay Shafat, Reem Makhoul, Essam Daod, Fahed Hakim, Rina Perlman and Nathan Karin

*J Immunol* 2009; 182:8104-8109; doi: 10.4049/jimmunol.0803348
http://www.jimmunol.org/content/182/12/8104

**References**
This article cites 50 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/182/12/8104.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Selective Autoantibody Production against CCL3 Is Associated with Human Type 1 Diabetes Mellitus and Serves As a Novel Biomarker for Its Diagnosis

Naim Shehadeh, Shirly Pollack, Gizi Wildbaum, Yaniv Zohar, Itay Shafat, Reem Makhoul, Essam Daod, Fahed Hakim, Rina Perlman, and Nathan Karin

We have recently demonstrated that patients suffering from chronic autoimmune diseases develop an autoantibody response against key mediators that participate in the initiation and progression of these diseases. In this paper, we show that patients with type 1 diabetes mellitus (T1DM), but not those suffering from several other inflammatory autoimmune diseases, display a selective autoantibody titer to a single CC chemokine named CCL3. From the diagnostic point we show that this response could be used as a biomarker for diagnosis of T1DM, a disease that is currently diagnosed by autoantibodies to competitive anti-insulin Abs, islet cell Abs, and glutamic acid decarboxylase Abs. We show that our currently suggested biomarker is more reliable than each of the above alone, including diagnosis of T1DM at its preclinical stage, and could therefore be used as a novel way for diagnosis of T1DM. These Abs were found to be neutralizing Abs. It is possible, though hard to prove, that these Abs participate in the natural regulation of the human disease. Hence, it has previously been shown by others that selective neutralization of CCL3 suppresses T1DM in NOD mice. Theses results together with ours suggest CCL3 as a preferential target for therapy of T1DM.

Received for publication October 6, 2008. Accepted for publication April 10, 2009.

The Journal of Immunology, 2009, 182: 8104–8109.

CD8+ T cells and macrophages are required for β-cell destruction (5). Although the critical events that trigger the autoreactive process in T1DM are not clear, destruction of insulin-producing β-cells appears to be mediated by the activation of autoreactive T cells that recognize several islet β-cells Ags, including competitive anti-insulin Abs (CIAA), islet cell Abs (ICA), glutamic acid decarboxylase (GAD) 65 and 67 isotypes Abs, heat shock protein 60, and some uncharacterized β-cells Ags (6–8). The current diagnosis of T1DM is based on measuring autoantibodies to GAD, ICA, ICA 512 (IA-2) and insulin (9–17).

Chemokines are small (~8–14 kDa), structurally related proteins that regulate cell trafficking through interactions with a subset of seven transmembrane, G protein-coupled receptors (18–21). As such they are thought to be key mediators of inflammation, including inflammatory autoimmune diseases, and therefore valid targets for therapy. Based on the position of key cysteines, chemokines are divided into four subgroups: C, CC, CXC, and CX3C. Of these subgroups, various CC chemokines, CXCL8 and CXCR3 ligands, mostly CXCL10, were strongly associated with inflammatory autoimmunity, including T1DM, in experimental models (22–28). It is difficult to directly extrapolate these observations to patients with T1DM. In an attempt to indirectly identify key chemokines that could be associated with the dynamics of human T1DM, we have explored our recent target discovery strategy (1) from human RA to human T1DM. In this paper, we show that of the various chemokines that have been associated with inflammatory autoimmunity, patients with T1DM develop a selective autoantibody response to a single CC chemokine (CCL3), emphasizing its potential role as a major target not only for experimental models of disease but for therapy as well (25). The current study focuses on its potential use as a novel and effective diagnostic tool.

Materials and Methods
Study population
The basic study population was comprised of 221 children. The study groups were divided into 87 new onset diabetic patients aged 2–<18 years, who were admitted to the pediatric ward of Meyer Children’s Hospital.
(Haifa, Israel) between January 2004 and December 2007, 54 age-matched healthy subjects, 34 long-standing diabetic children (>5 years duration) who were visiting the diabetic pediatric clinic at Meyer Children’s Hospital, 20 subjects who were in prediabetic state (first-degree relatives of a T1DM patient and positive for at least one of the known autoantibodies; GAD, ICA, or CIAA), and 14 children with juvenile rheumatoid arthritis (JRA) who were being followed up by our rheumatologic pediatric clinic. All patients were diagnosed according to the following criteria: age at onset <16 years, arthritis in one or more joints, duration of disease 6 wk or longer, exclusive of other forms of juvenile arthritis. An additional group included 12 long-term patients with RA (seven women and five men, mean age 54, and range 26–73), disease duration: range 2–28 years, mean 13.8 years.

All subjects diagnosed as RA patients were rheumatoid factor positive (seropositive), accompanied by erosive RA, and fulfilled all of the American College of Radiology’s criteria for RA.

RA and JRA patients were on various nonsteroidal anti-inflammatory drugs, low steroid therapy (maximum dose 10 mg/day), and had different disease-modifying drugs like Methotrexate, Plaquenil, Imuran, Solganal (gold injections), and anti TNF α.

Twenty cystic fibrosis (CF) patients (nine females) also participated in this study; these patients were being followed up at the CF Center and the pediatric pulmonary unit within the Meyers Children’s Hospital. Ten patients are pancreatic insufficient and the other ten are pancreatic sufficient. Their average age is 16.75 years (range 1–37 years) and all patients are on traditional CF medications, none of these patients require systemic steroid treatment.

All studies were conducted according our protocol, which was approved by our local Helsinki committee at the Rambam Medical Center. All subjects provided informed consent under this protocol.

The clinical service laboratory of endocrinology at Rambam Medical Center conducted an additional comparative study (Fig. 3). This center is qualified to provide this service (ISO 9001: 2000, registration no. IL-30578). Samples were taken from sera of the type 1 diabetes Abs program, cycle V (January 2009) with RCPA quality assurance.

**Determination of anti-chemokines Ab titer**

A direct ELISA has been used to determine the chemokine autoantibody titer in human subjects. Commercially available human purified recombinant chemokines (R&D Systems) were coated onto 96-well ELISA plates (Nunc) at concentrations of 20 ng/well. Anti sera, in serial dilutions from 1:4 to 1:2 (29) were added to ELISA plates. Donkey anti-human IgG HRP-conjugated Ab (Jackson Immuno Research Laboratories) was used as detection Ab. Abs titer was determined by comparative analysis of sequential dilutions of sera (cut off Log2 Ab titer >9). Results are shown as log2 Ab titer ± SE.

**Fluid phase radioassay**

Serum levels of anti-CCL3 were determined by the method of Vardi et al. (17) as follows: 20 μl of serum was pipet to a tube followed by the 50 μl of assay buffer with or without recombinant human 125I-labeled CCL3 (~200,000 cpm/ml; PerkinElmer). The mixture was incubated for 2 h at room temperature. Then followed the addition of 50 μl of protein and a Sepharose incubation for 1 h at room temperature. The precipitate, containing bound ligand, was separated by centrifugation at 1500 g for 30 min at 4°C. After aspiration of the supernatant, the precipitate was washed with 1.5 ml of ice-cold buffer solution, centrifuged again, and the radioactivity counted.

**Migration assay**

THP-1 cells (106) were loaded in the upper chamber of a 6.5-mm diameter, 5-μm-pore polycarbonate Transwell culture inserts (Costar). The lower chamber contained 10 ng/ml CCL3 or CCL2 (R&D Systems), supplemented with 20 μg IgG purified from a pool of sera from type I diabetic patients or control subjects (protein G coulomb purification). Cells were allowed to migrate for 2 h at 37°C in 7.5% CO2 and cells that migrated were collected and counted using a FACSCalibur (BD Biosciences). The percentage of cell migration was calculated as the number of cells that migrated to the lower chamber divided by the number of cells originally plated in the upper chamber.

**Determination of autoantibody titer to CIAA, ICA, and GAD**

Production of autoantibody titer to CIAA, ICA, and GAD was determined using the following commercially available diagnostic kits: Anti-GAD and anti-insulin autoantibodies were tested by radioimmunoassay using a kit purchased from CIS Bio International.

ICA autoantibodies were determined by a standard immunofluorescence method using sections of frozen human group O pancreas (16, 29). Endpoint dilution titers were examined for the positive samples, and the results were expressed in Juvenile Diabetes Foundation units. Titers were converted to Juvenile Diabetes Foundation units as recommended by the third International Workshop of Standardization (16, 29).

**Statistical analysis**

Statistical analysis was done as according to Ref. 31. Average CCL3 specific autoantibody titer (Log2) was analyzed as mean ± SD and significance was determined by Student’s t test. Receiver operating characteristic (ROC) curve for CCL3 autoantibodies (Figs. 2 and 3) is based on the binomial distribution to obtain upper and lower bounds for 95% confidence intervals surrounding estimates of test sensitivity and specificity. When study results indicated perfect (100%) sensitivity or perfect (100%) specificity in a subgroup of interest, we report the lower 95% confidence bound. Otherwise, we report a symmetrical 95% confidence intervals.

**Results**

**Newly diagnosed patient with T1DM developed a selective autoantibody titer to CCL3 (MIP-1α)**

In the first part of our research, we evaluated the presence of a selective autoantibody response to various chemokines that have been implicated with inflammatory autoimmunity, including MIP-1α (CCL3), MCP-1 (CCL2), MIP-1β (CCL4), IL-8...
CXCL8), and IP-10 (CXCL10). Fig. 1A shows that 9 of 10 newly diagnosed patients with T1DM mount a selective autoantibody titer to CCL3, but not to each of the other chemokines ($p < 0.0001$).

It has previously been shown that neutralization of CCL3 effectively suppresses spontaneously developed T1DM in NOD mice (25). Our working hypothesis is that selective autoimmunity to CCL3 might participate in the regulation of disease in T1DM patients. In an attempt to further explore this hypothesis we have checked whether autoantibodies produced in these patients selectively neutralizes CCL3. Thus, purified IgG from a pool of sera from these patients and from the control subjects were analyzed for their ability to inhibit CCL3- and CCL2-induced migration of human THP1 cells in a Transwell system. Fig. 1B shows that sera from T1DM patients, but not from control subjects, could significantly inhibit CCL3-induced migration (Fig. 3, D compared with B and C, $p < 0.0001$) but not CCL2-induced migration (Fig. 3, H compared with G and F).

The results of our initial screening (Fig. 1A) motivated us to enlarge our study and determine whether anti-CCL3 Ab production could be used as a biomarker to distinguish newly diagnosed patients with T1DM. We therefore compared anti-CCL3 Ab titer in sera of 87 newly diagnosed patients with T1DM with 54 age-matched healthy subjects. Fig. 2A shows that mean Log$_2$Ab titer to CCL3 in 87 newly diagnosed T1DM subjects reached the level of 12.24 ± 0.14, which significantly differed ($p < 0.0001$) from those recorded in control subjects (7.75 ± 0.25). Based on these observations, we decided to set up the cut-off titer of Log$_2$Ab titer >9 as positive and analyzed our data accordingly, either with no age limitation (Fig. 2C) or with age limitation of up to 18 years of age, including all the newly diagnosed subjects in the study (Fig. 2D). According to a cut-off >9, ROC curve shows 87.4% sensitivity and 94.4% specificity for age-independent analysis (Fig. 2C) and 91.82% sensitivity with 100% specificity when limiting the study to patients up to 18 years of age.

An additional set of experiments included analysis of a blinded series of samples conducted independently by the clinical service laboratory of endocrinology at Rambam Medical Center that is qualified to provide this service (ISO 9001: 2000, registration no. IL-30578). Samples were taken from sera of the type 1 diabetes Abs program, cycle V (January 2009) with RCPA quality assurance. All analyses were conducted by this service center independently. In these experiments, sera samples from 30 newly diagnosed type 1 diabetes patients and age-matched healthy controls were analyzed for the appearance of anti-CCL3 Abs using three different methods: measuring of Log$_2$Ab titer following serial sera dilutions (Fig. 3, A and F), determining the actual OD at three different fixed dilutions (1:500, 1:1000, and 1:2000, Fig. 3, B and G, C and H, and D and I, respectively), and fluid phase radioassay (Fig. 3, E and J). All tests showed a highly significant difference ($p < 0.0001$) in the sensitivity of anti-CCL3 Ab production between T1DM subjects and healthy controls (Fig. 3).

**Autoantibody production to CCL3 in T1DM is disease specific**

We have compared the development of autoantibodies to CCL3 between 34 subjects suffering from prolonged T1DM (>5 years), 87 patients diagnosed as new onset T1DM, 12 subjects suffering from RA and 14 JRA patients, all in comparison with 54 control subjects (Fig. 4A). Possible development of autoantibody titer to TNF-1 was also recorded (Fig. 4B). Fig. 4A shows that anti-CCL3 Ab production is diseases specific, thus while the vast...
majority of newly onset T1DM patients and subjects suffering from prolonged T1DM (87.4% and 70.6%, respectively) displayed a log2 titer to CCL3, only 14.3% and 16.7% of JRA and RA patients, respectively, were anti-CCL3 positive (p < 0.01). Interestingly, T1DM patients did not display any notable Ab titer to TNF-α, which was a hallmark for the diagnosis of RA (1), as well as JRA (Fig. 4B), implicating, once again, the selectivity in the breakdown of tolerance during autoimmunity.

As a further control, we looked for a disease in which pancreatic β-cell function is also altered at an early age. Thus, CF with pancreatic insufficiency (32), in which pancreatic β-cell function is altered, leading to glucose intolerance and diabetes (33), was selected as a comparative disease for T1DM. We have compared autoantibody production to CCL3 in sera of 10 CF patients with pancreatic insufficiency and impaired glucose tolerance, which were all negative for autoantibody production to either ICA or GAD (Fig. 4C). Nine of them displayed low autoantibody production to CCL3, which was comparable to control age-matched healthy subjects, and only one patient displayed a significant titer to CCL3 (log2 11). Statistical analysis of both groups showed no significant differences (Log2 SE of 8.1 compared with 7.6). It should be noticed that two other patients with CF and pancreatic insufficiency were excluded from the study because they were found to be positive to ICA (one also to GAD). Both displayed a significant titer to CCL3 (log2 11). Of 10 patients that were identified as CF without pancreatic insufficiency and were negative to ICA or GAD, one patient displayed a significant titer to CCL3 (log2 10) (not shown). Thus, alteration of the β-cell function resulting in glucose intolerance is not sufficient for the generation of anti-CCL3 autoantibody production.

Frequency of anti-CCL3 Ab production in T1DM subjects is higher than in other known targets currently serving as biomarkers

The current diagnosis of T1DM is based on measuring autoantibodies to CIAA, ICA, and GAD (15–17). We have compared autoantibody production to CIAA, ICA, GAD, and CCL3 in 87 newly diagnosed T1DM (Fig. 5A). Although 87.4% of these subjects were positive (cut-off >9) for anti-CCL3, only 63% (54/86),
ICA, and CIAA) would cover analysis shows that a combined test of all three biomarkers (GAD, comparison of anti-CCL3 to each of the others), respectively. Our data analysis shows that a combined test of all three biomarkers (GAD, ICA, and CIAA) would cover ~85% of patients.

**Anti-CCL3 Abs as a biomarker for diabetes in prediabetic subjects**

We have examined whether anti-CCL3 also appears in prediabetic subjects (first-degree relatives of patients with T1DM with positive autoantibodies of at least one of the following: anti-Gad (85%), ICA (70%), and anti-insulin Abs (90%). At the prediabetic stage, 19 of these 20 subjects (95%) were anti-CCL3 positive.

Finally, 18 first-degree relatives of patients with T1DM, who were autoantibody negative to CIAA, ICA, or GAD, were examined for autoantibody production to CCL3 (measured by OD at a dilution of 1:500). Of these subjects, only two (11%) were anti-CCL3 positive. We are monitoring these children for possible future development of autoantibody response to CIAA, ICA, or GAD, as well as for clinical signs of T1DM.

**Discussion**

We have previously shown that targeted DNA vaccines encoding inflammatory cytokines and chemokines could be used to rapidly suppress ongoing inflammatory autoimmunity, and that disease suppression is due to an accelerated autoantibody production to the gene products of each vaccine (3, 34–37). We have then showed that the rapid effect of this way of therapy is due to amplification of an ongoing beneficial response that participates in the natural regulation of disease. It is possible, though hard to be proven, that this response is also helpful for human subjects suffering from RA, which makes it a possible target for beneficial amplification (1). Based on this study, we have established a target discovery platform aimed at identifying key mediators of different inflammatory autoimmune disease in humans.

Of the 50 known chemokines, the CC chemokines, mostly MIP-1α (CCL3), MCP-1 (CCL2), and MIP-1β (CCL4), as well as the CXC chemokines IL-8 (CXCL8) and IP-10 (CXCL10), were mostly defined as inducers of the inflammatory process in various inflammatory autoimmune diseases (38–43). The CC chemokines RANTES (CCL5) was also associated with RA (44–46). Of these chemokines, the vast majority of T1DM subjects produce neutralizing Abs exclusively to CCL3 (Fig. 1) but not others, particularly CCL2, which has been well implicated with several autoimmune diseases like multiple sclerosis, RA, myocarditis, and their experimental models (47–49). One possibility is that the inflammatory process in T1DM is regulated differently than other T cell-mediated autoimmune diseases, like RA, multiple sclerosis, and myocarditis. Indeed, Cameron et al. have shown that from the CC chemokines selective neutralization of CCL3 suppresses type 1 diabetes in NOD mice (25). Subsequently, it has been shown that the relatives at risk of developing T1DM are associated with up-regulation of CCL3 (MIP-1α) and CCL4 (MIP-1β), accompanied by down-regulation of CCL2 (MCP1). These findings suggest opposed functions of these chemokines in the disease process (50). It should be noted that vast majority of our RA and JRA patients are subjected to a low level of steroid therapy, which does not terminate their ability to spontaneously generate anti-TNF-α Ab titer (Fig. 4B).

Interestingly, T1DM subjects do not mount any autoantibody titer to this inflammatory cytokine (Fig. 4B).

It has previously been shown that neutralization of CCL3 effectively suppresses spontaneously developed T1DM in NOD mice (25). In this paper, we show that autoantibodies produced in T1DM subjects are neutralizing Abs, and speculate that they might participate in the natural regulation of disease. It is tempting to hypothesize that in an attempt to restrain the destructive immune process, the immune system would produce an autoantibody against one of the key mediators involved in the disease process and that CCL3 would be one of the strongest candidates, though the exact mechanism by which the immune system does so is still elusive. From the therapeutic perspective, this may suggest CCL3 as a favorable target for therapy. From the diagnostically oriented perspective, according to our data, anti-CCL3 Abs were positive in ~87% of patients, whereas only 63% (54/86), 60% (50/83), and 48% (39/82) were positive for anti-GAD, ICA and CIAA, respectively. Our data analysis shows that a combined test of all three biomarkers (GAD, ICA, and CIAA) would cover ~85% of patients. Therefore an additional anti-CCL3 test will increase the accuracy and reliability of T1DM diagnosis. Moreover, anti-CCL3 is positive in prediabetic patients and continues to be positive several years after the diagnosis of diabetes.

Finally, as emerging therapies for T1DM are coming, an early diagnostic of disease, even during its preclinical stage, would be critical for successful therapy. We show that 19 of 20 subjects (95%) who were at prediabetic stage displayed an autoantibody response to CCL3. We therefore believe that our study is mostly important for an early diagnosis of disease and its therapeutic implications.

**Disclosures**

N.K., N.S., and G.W. hold a patent on detection of T1DM by anti-CCL3 Abs that has been licensed out to Micro Medic (Israel).

**References**


