**ORIGINAL ARTICLE**

# Investigating the role of Natural Killer T-cells in Gram negative infections of patients with type 2 diabetes mellitus

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**Abstract**

**Background:** Invariant Natural Killer T (iNKT) cells belong to innate immunity and combine T-cell receptor specificity with Natural Killer surface markers. They can produce cytokines immediately after stimulation and direct immunity to either Th1 or Th2 cytokine production. iNKT cells participate in a variety of immune responses, such as microbial infections, autoimmunity, and cancer. Type 2 Diabetes Mellitus (T2DM) has been associated with activated innate immunity and certain cytokine profile during microbial infections. This study aimed to evaluate whether iNKT cells have a role in the immune response of T2DM patients during infections with gram-negative bacteria.

**Method:** The T2DM group consisted of patients (n =11) who had a diagnosis of T2DM for at least six months and febrile illness for three days, while the control group consisted of patients (n =11) who had not T2DM, but were febrile for three days. All patients were infected by gram-negative bacteria. Physical examination was performed, and peripheral blood was drawn on days three and six of febrile illness.

Flow cytometry was utilized for iNKT cell identification with monoclonal antibodies Phycoerythrin (PE) - Cyanin (CY) 5 anti-Human CD3, Fluorescein isothiocyanate (FITC) anti-Human CD4, PE anti-human invariant NKT T-Cell Receptor. For intracellular staining, we used Alexa Fluor anti-Human interferon-γ (IFN-γ) and Allophycocyanin (APC) anti-human interleukin-4 (IL-4).

The variables processed were: CD3+IL-4+iNKT+ , CD4+IL-4+iNKT+, CD3+IFNγ+iNKT+, CD4+IFNγ+iNKT+, CD3+iNKT+, CD4+iNKT+ ,CD3+IL4+, CD4+IL-4+, CD3+IFNγ+, CD4+IFNγ+ on days three and six of febrile illness (CD3+, CD4+: T lymphocyte surface markers, iNKT+: invariant Natural Killer T- Cell Receptor, IL4+: interleukin 4, IFNγ+: interferon γ).

**Results:** Comparisons between T2DM patients and controls revealed no statistically significant difference in any of the study's variable. Regarding within T2DM patients comparisons CD4+IL4+iNKT+, CD3+IL4+iNKT+, CD4+IFN+iNKT+, CD3+IFN+iNKT+, and CD3+iNKT+ decreased, whereas CD3+IL4+ was increased at day six compared to day three. Within control group CD4+IL4+iNKT+, CD3+IL4+iNKT+, and CD3+iNKT+ were decreased, whereas CD4+IFN+, CD3+IFN+ were increased at day six compared to day three.

**Conclusion:** The absence of statistical difference between T2DM patients and controls implies that the role of iNKT cells is virtually the same in both groups of patients during the course of gram-negative infections and that there is no numerical variance of this cell population between the two groups. Despite the small sample size, we notice that all iNKT parameters (both IL4/IFNγ) are suppressed in the T2DM group during the later phase, but only those concerning IL4+iNKT+ in the control group, suggesting that IFNγ production remains elevated in the controls.

A compensatory anti-inflammatory type-response could provide an explanation for the prevalence of IL4 production during the later phase of infection in T2DM and the sustained production of IFNγ in controls. Hippokratia 2015; 19 (3): 231-234.

**Keywords:** Type 2 diabetes mellitus, invariant natural killer T cells, iNKT cells, gram-negative infection

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## Introduction

Invariant Natural Killer T cells belong to innate immunity combining T-cell receptor (TCR) specificity with Natural Killer surface markers (NK1.1)[1]. There are at least two major subpopulations: the most studied type I or invariant NKT (iNKT) cells that express an invariant TCRa and respond to lipid antigens presented by cluster of differentiation 1 (CD1), and the less studied type II

NKT cells that express variant TCRa and recognize lipid antigens presented by CD1 as well. There is also a group of type III NKT cells that is not responding to CD1[2,3].

It seems that iNKT cells evolved to respond specifically and very fast to certain invaders since their TCR chain (Va24/Ja18) is highly preserved in humans and their ability to produce cytokines is unique[4].

iNKT cells represent a field of interest because of their capacity to influence immunity towards Th1 or Th2 cytokine production and because they participate in a variety of immune responses, such as microbial infection, autoimmunity and cancer[5].

Reactivity to a Galactosyl Ceramide (aGalCer), a glycolipid derived from marine sponges is characteristic for iNKT cells[6]. Due to this reactivity, we were able to study iNKT cells and notice that after stimulation with aGalCer, iNKT cells disappeared in a few hours. Initially, this was attributed to cell death, but afterward it was found that those cells downregulated their TCR and could not be detected[7]. In two days, iNKT cells express their TCR and increase remarkably although many of them are NK1.1 negative. Gradually in nine days, iNKT cells return to normal numbers, as were before stimulation with aGalCer[7,8]. Their usual population has not been quantified (i.e., white blood cell count) since each individual has different levels of iNKT cells circulating in peripheral blood. Their number has not been correlated with specific immune function until now[7].

Type 2 Diabetes Mellitus (T2DM) and insulin resistance have been associated with a condition of activated innate immunity and elevated acute phase markers[9]. Certain infections are more often or severe in patients with T2DM[10], but to which extend iNKT cells participate in the immune response is not elucidated[11,12]. Gram-negative bacteria have an outer cell wall of lipid antigens (lipopolysaccharide: LPS) that can activate CD1 and CD1d restricted T-cells (iNKT cells) in different ways[13,14].

Given the limited data on the role of iNKT cells in patients with T2DM, we aimed to evaluate intracellular cytokine production by iNKT cells during infections with gram-negative bacteria in T2DM patients as compared with non-diabetic patients with gram-negative infection.

**Materials and Methods**

The T2DM group consisted of patients (n =11) who had a diagnosis of T2DM for at least six months according to the criteria of American Diabetes Association [glycated hemoglobin (HbA1c) ≥6.5% or fasting plasma glucose ≥126 mg/dL or two-hour postprandial glucose ≥200 mg/dL during an oral glucose tolerance test, using a glucose load which contained the equivalent of 75 g anhydrous glucose dissolved in water, or in patients with classic symptoms of hyperglycemia or with hyperglycemic crisis, a random plasma glucose ≥200 mg/dL][15] and febrile gram-negative bacterial infection for three days. The control group consisted of patients (n =11) who had not T2DM but had febrile gram-negative bacterial infection for three days.

Morning physical examination was performed and peripheral morning blood was drawn on the days three and six after an overnight fast. Both groups had cultures (blood, urine, tissue, pus) isolating gram-negative bacteria.

All patients provided an informed consent form. The study's protocol was approved by the local Ethics Committee (decision No 12/5-3-2009) and was in accordance with the Declaration of Helsinki.

Exclusion criteria were: duration of fever more than three days, infection other than gram-negative bacteria, type 1 DM, cancer, renal or hepatic insufficiency, autoimmune diseases, psychiatric conditions, medications such as steroids, immune modifying treatments (cyclosporine, tacrolimus).

Peripheral blood was incubated for 24 hours with Brefeldin A 25μl/ml in order to stabilize intracellular cytokines. Flow cytometry [Becton Dickinson (BD) Fluorescence-activated cell sorting (FACS) Calibur 4 Colour] was utilized for iNKT cell identification with monoclonal antibodies Phycoerythrin (PE) - Cyanin (CY) 5 anti-Human CD3, Fluorescein isothiocyanate (FITC) anti-Human CD4, PE anti-human invariant NKT TCR (BD Pharmingen , New Jersey, USA).

For intracellular staining we used Alexa Fluor anti Human IFN-γ and APC anti-human IL-4 (BD Pharmingen, New Jersey, USA)[16].

The variables processed for the purposes of this study were the markers: CD3+IL-4+iNKT+, CD4+IL-4+iNKT+, CD3+IFNγ+iNKT+, CD4+IFNγ+iNKT+, CD3+iNKT+, CD4+iNKT+ ,CD3+IL4+, CD4+IL-4+, CD3+IFNγ+, CD4+IFNγ+ (CD3+,CD4+: T lymphocyte surface markers, iNKT+: invariant Natural Killer T- Cell Receptor, IL4+: interleukin 4, IFNγ+: interferon γ).

**Statistical Analysis**

Data are presented as mean ± standard error of the mean (SEM) for continuous variables and frequencies for categorical variables. Owing to the small sample size, non-parametric tests were used for all analyzes. Mann-Whitney test was used for between group comparisons and Wilcoxon signed-rank test for within group comparisons. Categorical variables were compared with Chi-Square test. Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) version 21.0 for Macintosh (SPSS Inc., Chicago, IL). Significance was set at $p < 0.05$ (two-tailed).

**Results**

The T2DM group consisted of 7 male and 4 female patients aged 74.1 ± 1.8 years and body mass index (BMI) 30.5 ± 0.7 kg/m$^2$, whereas the control group of 6 male and 5 female ($p = 0.665$) aged 73.7 ± 3.4 years ($p = 0.576$) and BMI 28.3 ± 1.1 kg/m$^2$ ($p = 0.155$). Mean HbA1c in T2DM group was 7.4 ± 0.2 % and mean diabetes duration was 10.3 years.

Comparisons between T2DM patients and controls revealed no statistically significant difference in any of the study's variable (Table 1).

Regarding within T2DM patients comparisons CD4+IL4+iNKT+,CD3+IL4+iNKT+, CD4+IFNγ+iNKT+, CD3+IFNγ+iNKT+, and CD3+iNKT+ decreased, whereas CD3+IL4+ was increased at day six compared to day three.

Within control group CD4+IL4+iNKT+,

**Table 1:** Comparative data of days thee and six of febrile illness between and within Type 2 Diabetes and Control groups.

| Variable | Day | Controls (n =11) | T2DM patients (n =11) | p-value (between groups) |
|---|---|---|---|---|
| **CD4+IL4+** | Day 3 | 56.4 ± 12.7% | 82.1 ± 7.2% | 0.094 |
| | Day 6 | 56.8 ± 13.4% | 85.5 ± 6.7% | 0.341 |
| | p-value (within group) | 0.131 | 0.059 | |
| **CD4+IFNγ+** | Day 3 | 50.0 ± 12.2% | 79.0 ± 8.2% | 0.123 |
| | Day 6 | 62.4 ± 12.4% | 81.5 ± 8.5% | 0.278 |
| | p-value (within group) | 0.016 | 0.131 | |
| **CD4+IL4+iNKT+** | Day 3 | 4.6 ± 1.6% | 3.3 ± 0.9% | 0.895 |
| | Day 6 | 1.2 ± 0.3% | 1.4 ± 0.4% | 0.668 |
| | p-value (within group) | 0.028 | 0.017 | |
| **CD4+IFNγ+iNKT+** | Day 3 | 4.8 ± 1.9% | 2.9 ± 0.7% | 0.974 |
| | Day 6 | 1.4 ± 0.3% | 1.1 ± 0.3% | 0.469 |
| | p-value (within group) | 0.168 | 0.011 | |
| **CD4+iNKT+** | Day 3 | 1.0 ± 0.3% | 0.8 ± 0.2% | 0.894 |
| | Day 6 | 0.5 ± 0.2% | 0.3 ± 0.2% | 0.421 |
| | p-value (within group) | 0.134 | 0.168 | |
| **CD3+IL4+** | Day 3 | 54.5 ± 12.5% | 79.9 ± 7.0% | 0.122 |
| | Day 6 | 56.4 ± 13.2% | 85.0 ± 6.8% | 0.941 |
| | p-value (within group) | 0.213 | 0.033 | |
| **CD3+IFNγ+** | Day 3 | 48.5 ± 11.9% | 76.5 ± 7.9% | 0.122 |
| | Day 6 | 61.3 ± 12.3% | 80.7 ± 8.4% | 0.324 |
| | p-value (within group) | 0.026 | 0.091 | |
| **CD3+IL4+iNKT+** | Day 3 | 6.6 ± 2.4% | 4.2 ± 1.3% | 0.793 |
| | Day 6 | 1.6 ± 0.4% | 1.4 ± 0.4% | 0.921 |
| | p-value (within group) | 0.028 | 0.037 | |
| **CD3+IFNγ+iNKT+** | Day 3 | 6.0 ± 2.4% | 3.2 ± 0.8% | 0.717 |
| | Day 6 | 1.5 ± 0.5% | 1.2 ± 0.3% | 0.947 |
| | p-value (within group) | 0.109 | 0.007 | |
| **CD3+iNKT+** | Day 3 | 2.4 ± 0.7% | 2.8 ± 1.0% | 0.767 |
| | Day 6 | 1.0 ± 0.3% | 0.7 ± 0.2% | 0.354 |
| | p-value (within group) | 0.018 | 0.008 | |

Data are presented as mean ± standard error of the mean (SEM). Mann-Whitney test was used for between group comparisons and Wilcoxon signed-rank test for within groups comparisons. CD3+, CD4+: T lymphocyte surface markers, iNKT+: invariant Natural Killer T- Cell Receptor, IL4+: interleukin 4, IFNγ+: interferon γ.

CD3+IL4+iNKT+, CD3+iNKT+ were decreased, whereas CD4+IFNγ+, CD3+IFNγ+ were increased at day six compared to day three (Table 1).

**Discussion**

The absence of statistical difference in the study's variables between T2DM patients and controls implies that the role of iNKT cells is virtually the same in both groups of patients during the course of gram-negative infections and that there is no numerical variance of this cell population between the two groups. To the best of our knowledge, there are currently no data regarding IFNγ/IL4 (Th1/Th2) balance during gram-negative infections in T2DM patients.

Within T2DM group, we notice reduction at day six of all iNKT parameters (IL4+/IFNγ+) (CD4+IL4+iNKT+, CD3+IL4+iNKT+,CD4+IFNγ+iNKT+,CD3+IFNγ+iNKT+, CD3+iNKT+) and increase only in CD3+IL4+ compared to day three.

Within control group only IL4+iNKT+ (CD4+IL4+iNKT+, CD3+IL4+iNKT+, CD3+iNKT+) parameters are reduced at day six whereas production of IFNγ from cells other than iNKT (CD4+IFNγ+, CD3+IFNγ+) is increased at day six compared to day three.

All iNKT parameters (both IL4/IFNγ) are suppressed in the T2DM group during the later phase, but only those concerning IL4+iNKT+ in the control group, thereby suggesting that IFNγ production remains elevated in the controls.

The compensatory anti-inflammatory response syndrome (CARS) could provide an explanation for the prevalence of IL4 production during the later phase of infection in T2DM and the sustained production of IFNγ in controls.

In the acute phase of an infection macrophages, stimulated by the microbial LPS, produce cytokines, such as tumor necrosis factor (TNF)-α, IL-6, IFNγ and IL-12 (Th1). The acute phase is followed by a regulatory reaction characterized by Th2 cytokine (IL-4, IL-10) production from lymphocytes in an attempt to minimize the inflammatory state. If the second phase is prolonged, phenomena of immune suppression occur, including: reduced expression of major histocompatibility complex (MHC) molecules; increased lymphocyte apoptosis; suppression of TNF-α; overexpression of regulatory T cells; and reduced response of monocytes to cytokines[17]. The outcomes of such a reaction are subsequent secondary infections[18].

iNKT cells can be activated either directly by recognition of microbial lipid antigens presented by CD1d or indirectly by inflammatory cytokines or via lipid molecules produced by antigen presenting cells that recognize certain microbes. The type of activation may be responsible for a different type of cytokine production[4]. The predominance of IL4 production by cells other than iNKT cells may be part of activated innate immunity in T2DM patients.

The main limitation of the present study is its small sample size; however, this was a preliminary pilot study focused on an issue that has not yet been addressed.

**Conclusion**

No significant difference was shown between T2DM patients and controls in the iNKT cell markers in this study. Larger studies are needed to determine whether iNKT response plays any specific role in gram-negative infections in T2DM patients.

**Conflict of Interest**

Authors declare no conflict of interest associated with this manuscript.

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