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**Decreased GAD<sub>65</sub>-specific Th1/Tc1 phenotype in children with type 1 diabetes treated with GAD-alum**

**Running title:** Chemokine and chemokine receptor profile after GAD-alum treatment

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## **Abstract**

**Aim:** The balance between T helper cell subsets is an important regulator of the immune system and is often examined after immune therapies. We aimed to study the immunomodulatory effect of glutamic acid decarboxylase (GAD) 65 formulated with aluminium hydroxide (GAD-alum) in children with type 1 diabetes (T1DM), focusing on chemokines and their receptors.

**Methods:** Blood samples were collected from 70 children with T1DM included in a phase II clinical trial with GAD-alum. Expression of CC chemokine receptor 5 (CCR5) and CCR4 was analyzed on CD4+ and CD8+ lymphocytes after *in vitro* stimulation with GAD<sub>65</sub> using flow cytometry, and secretion of the chemokines CCL2, CCL3 and CCL4 was detected in peripheral blood mononuclear cell (PBMC) supernatants with Luminex.

**Results:** Expression of Th1-associated CCR5 was down-regulated following antigen-challenge, together with an increased CCR4/CCR5 ratio and CCL2 secretion in GAD-alum treated patients, but not in the placebo group.

**Conclusion:** Our results suggest that GAD-alum-treatment has induced a favourable immune modulation associated with decreased Th1/Tc1 phenotypes upon antigen re-challenge, which may be of importance for regulating GAD<sub>65</sub> immunity.

**Keywords:** Type 1 diabetes, GAD<sub>65</sub>, immunomodulation, chemokines, chemokine receptors

**Abbreviations:** GAD<sub>65</sub>, glutamic acid decarboxylase 65; GAD-alum, GAD<sub>65</sub> formulated with aluminium hydroxide; PBMC, peripheral blood mononuclear cell; Th, T helper; Tc, cytotoxic T cell

## **Introduction**

Type 1 diabetes (T1DM) is an autoimmune disease where auto-reactive immune cells attack the insulin-producing  $\beta$ -cells, eventually causing a complete insulin deficiency [1]. T cells play a major pathogenic role in islet cell infiltration and destruction, and a T helper (Th)1-dominated infiltration has been observed in patients with T1DM [2]. Chemokines and chemokine receptors are associated with many tissue-specific inflammatory events. Interplay between chemokines and their receptors is important for migration of lymphocytes between blood, lymph nodes and tissues, and during an immune response the lymphocyte recruitment and activation is dependent upon the local chemokine production and the cellular expression of the appropriate receptor. Different effector Th-cell subsets express different receptors; Th1 cells preferentially express CCR5 and CXCR3, whereas expression of CCR3 and CCR4 is characteristic of the Th2 phenotype [3]. Distinct cytotoxic T (Tc) cell subsets, similar to their Th-cell counterparts, have been established in mouse models [4-5] and in humans [6-7]. Analogous to the Th1/Th2 terminology, these subsets are termed Tc1 and Tc2, and have been shown to produce type-1 cytokines or type-2 cytokines, respectively [8]. Tc cells are less well characterized with regard to chemokine receptor expression, but the pattern and regulation of chemokine receptor expression of polarized subsets of Tc cells seem to overlap that of Th cells [9-10]. Chemokines attract various types of leukocytes to sites of infection and inflammation and act as immunoregulatory molecules in driving Th1/Th2 responses. Secretion of CCR5 ligands e.g. CCL3, CCL4 and CCL5 has been associated with Th1 inflammatory responses [11], while ligands for CCR4, including CCL17 and CCL22 [12], may function as regulators of Th2 cells together with CCL2 [13-14].

Since lymphocytes specific for glutamic acid decarboxylase (GAD) 65 are among the first to enter inflamed islets in T1DM [15], they have been considered an attractive therapeutic target for immunomodulatory strategies aiming to prevent or delay disease onset. Antigen-specific

approaches to tackle T1DM are of high interest, particularly due to the limited risk of adverse effects. Despite recent setbacks in a phase II [16] and a phase III clinical trial [17] using GAD<sub>65</sub> formulated with aluminium hydroxide (GAD-alum), we and others have previously shown preservation of residual insulin secretion by GAD-alum treatment, in phase II trials involving children with recent-onset T1DM [18] and LADA patients [19]. In addition to the clinical efficacy, an early Th2-associated immune deviation in response to GAD<sub>65</sub> was observed in treated patients [20-21]. Given this, we hypothesized that also the chemokine and chemokine receptor profile would be Th2-skewed after GAD-alum treatment. Here we aimed to study expression of the Th2-associated receptor CCR4 and the Th1-associated receptor CCR5 on CD4<sup>+</sup> (Th) and CD8<sup>+</sup> (Tc) cells together with secretion of their ligands CCL2, and CCL3 and CCL4, respectively, in children with T1DM treated with GAD-alum.

## **Patients and Methods**

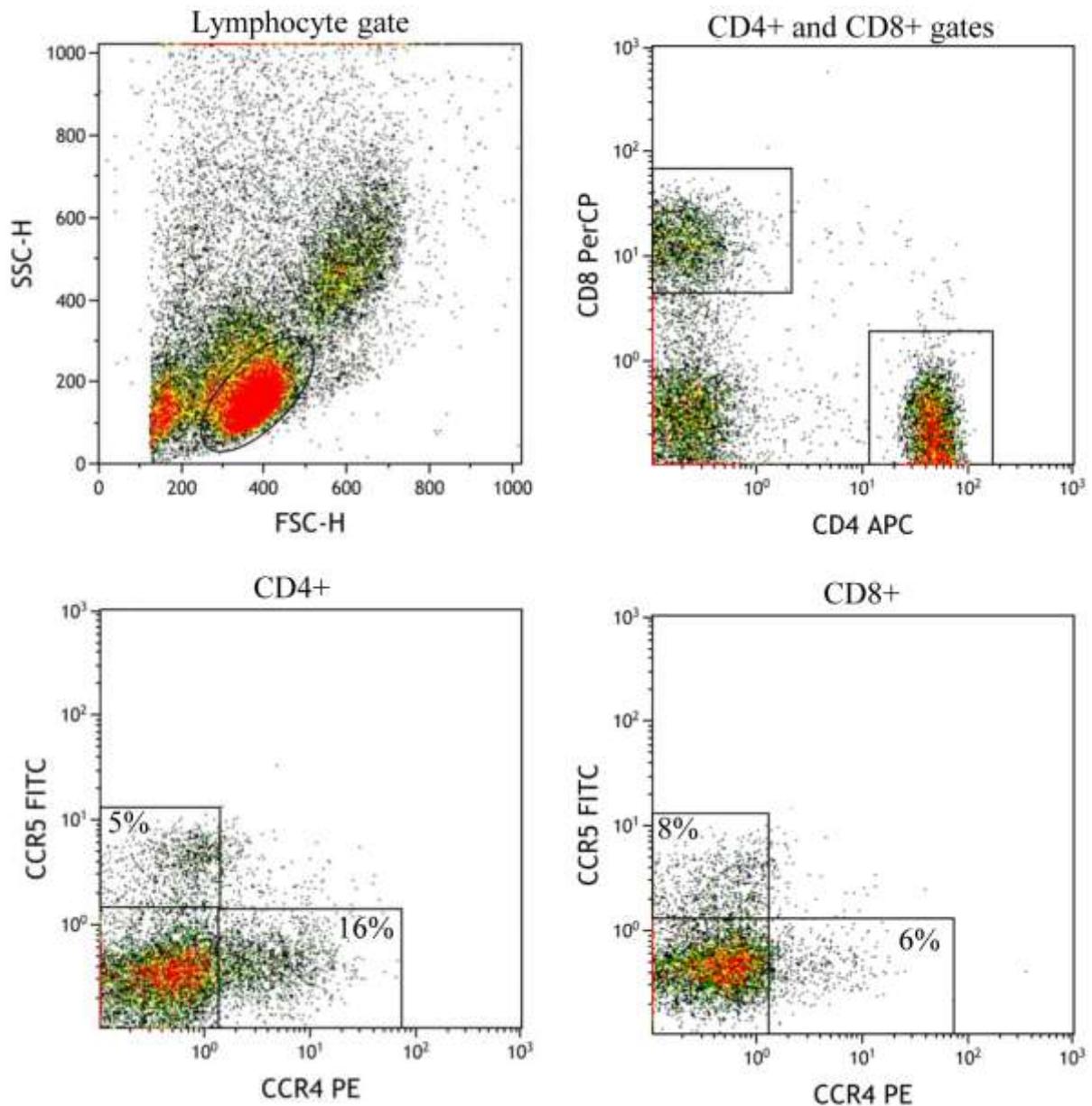
### ***Study population***

The design and characteristics of the trial have previously been described [18]. Briefly, 70 children (age 10-18 years) diagnosed with T1DM (disease duration <18 months), with fasting serum C-peptide levels above 0.1 nmol/l and presence of glutamic acid decarboxylase antibodies (GADA), were included in the trial. Patients were randomized to subcutaneous injections of 20 µg GAD-alum (Diamyd<sup>®</sup>, Diamyd Medical; n=35) or placebo (n=35) at day 0 and a booster injection 4 weeks later in a double blind setting. Based on availability, blood samples collected at baseline and at 15 and 21 months after the first injection of GAD-alum or placebo were included in the present study. All the participants received written information and consent was obtained according the Declaration of Helsinki. The study was approved by the Regional Ethics Committee for Human Research, Linköping University Hospital, Sweden.

### ***Flow cytometry analysis***

Peripheral blood mononuclear cells (PBMC) were isolated from sodium-heparinised venous fasting blood samples as described previously [20]. PBMC from baseline (GAD-alum n=21, placebo n=24) and 15 months visits (GAD-alum n=22, placebo n=20) were stained immediately after isolation as previously described [22], whereas PBMC from the 21 months visit (GAD-alum n=19, placebo n=12) were cultured over night in AIM-V medium (Invitrogen) with or without 5 µg/ml of GAD<sub>65</sub> (Diamyd Medical) prior staining.  $1 \times 10^5$  PBMC were stained with allophycocyanin (APC)-conjugated anti-CD4, peridinin chlorophyll (PerCP)-conjugated anti-CD8, fluorescein isothiocyanate (FITC)-conjugated anti-CCR5 and phycoerythrin (PE)-conjugated anti-CCR4 (BD Biosciences). Isotype controls (BD Biosciences) were included to estimate amount of non-specific binding. Four-colour flow cytometry was performed with a Becton Dickinson FACSCalibur, and data was analysed

using Kaluza version 1.1 (Beckman Coulter). Lymphocytes were gated according to forward- and side-scatter and CCR4 and CCR5 expression was analyzed on gated CD4+ and CD8+ cells, respectively (Fig. 1)



**Fig 1. Representative flow cytometry analysis.**

(A) Gating of lymphocytes was adjusted according to forward scatter (FCS) and side scatter (SSC) and (B) gating of CD4+ and CD8+ cells was determined according to positivity. Positive gates for CCR4 and CCR5 on (C) CD4+ and (D) CD8+ cells were adjusted according to isotype controls.

### ***Chemokine secretion assay***

The chemokines CCL2, CCL3 and CCL4 were measured in samples from baseline (GAD-alum n=27, placebo n=27), and at 15 (GAD-alum n=33, placebo n=29) and 21 (GAD-alum n=28, placebo n=30) months visit. One million PBMC diluted in 1 ml AIM-V medium supplemented with 20  $\mu$ M  $\beta$ -mercaptoethanol (Sigma) were cultured for 72 h with or without 5  $\mu$ g/ml of GAD<sub>65</sub> at 37°C in 5% CO<sub>2</sub>. The mitogen phytohaemagglutinin (PHA; 5  $\mu$ g/ml, Sigma) was included as a positive control. The chemokine levels were measured in cell culture supernatants using a Bio-Plex™ Human Cytokine Panel (Bio-Rad) according to the manufacturer's instructions as previously described [20]. A Luminex 100™ instrument (Luminex Corporation) was used for the identification and quantification of each specific reaction. Raw data (median fluorescence intensity) was analyzed using StarStation Software version 2.3 (Applied Cytometry Systems). The cut-offs for minimum detectable concentration were as follows: CCL2: 2.2 pg/ml, CCL3: 1.4 pg/ml, CCL4: 2.0 pg/ml. All samples had detectable levels of CCL2, CCL3 and CCL4. The specific antigen-induced secretion was calculated by subtracting the spontaneous secretion, i.e. PBMC cultured in medium alone.

### ***C-peptide***

Serum C-peptide concentrations were measured at 0, 30, 60, 90, and 120 min after a mixed liquid meal (Sustacal®) ingestion using a time-resolved fluoroimmunoassay (AutoDELFIA™ C-peptide kit, Wallac) described elsewhere [18]. Clinical effect of treatment was determined by changes in stimulated C-peptide measured as area under the curve (AUC) from baseline to 15 months (main study period). The GAD-alum treated patients were stratified in three groups; Responders (loss of C-peptide AUC < 10%), Intermediate responders (loss of C-peptide AUC between 10% and 65%) and Non-responders (loss of C-peptide AUC > 65%).

### *Statistical analysis*

As the immunological markers were not normally distributed, non-parametric tests corrected for ties were used. Unpaired analyses were performed using the Mann-Whitney U-test, and differences within groups were calculated by Wilcoxon signed rank test. A probability level of  $<0.05$  was considered statistically significant. Calculations were performed using PASW statistics version 18 for Windows (SPSS Inc).

## Results

### *Similar expression of CCR4 and CCR5 ex vivo in GAD-alum and placebo-treated patients*

Chemokine receptors CCR4 and CCR5 were analysed *ex vivo* on CD4+ and CD8+ cells at baseline and at 15 months after the first injection. Percentage CCR4 and CCR5 positive cells was similar in the two groups at both occasions (Table I).

**Table I.** Percentage (%) CC chemokine receptor 4 (CCR4) and CCR5 positive CD4+ and CD8+ cells (median and range) in GAD-alum- and placebo-treated patients. At baseline and 15 months, analyses were performed in resting cultures, and at 21 months both in resting and GAD<sub>65</sub>-stimulated cultures.

|                  |                                     | CD4+                    |                        | CD8+                   |                       |
|------------------|-------------------------------------|-------------------------|------------------------|------------------------|-----------------------|
|                  |                                     | CCR4+                   | CCR5+                  | CCR4+                  | CCR5+                 |
| <b>Baseline</b>  | <i>Resting</i>                      |                         |                        |                        |                       |
|                  | GAD-alum (n=21)                     | <b>24.0</b> (13.1-47.4) | <b>1.7</b> (0.5-3.7)   | <b>10.8</b> (6.8-41.9) | <b>6.9</b> (1.0-13.8) |
|                  | Placebo (n=24)                      | <b>26.3</b> (16.7-52.1) | <b>1.3</b> (0.1-4.6)   | <b>11.5</b> (5.1-53.6) | <b>6.3</b> (0.1-15.0) |
| <b>15 months</b> | <i>Resting</i>                      |                         |                        |                        |                       |
|                  | GAD-alum (n=22)                     | <b>23.1</b> (10.3-35.8) | <b>0.7</b> (0.2-2.1)   | <b>8.3</b> (2.1-38.0)  | <b>3.4</b> (0.4-11.9) |
|                  | Placebo (n=20)                      | <b>25.2</b> (12.1-31.2) | <b>0.8</b> (0.2-5.0)   | <b>7.3</b> (2.1-19.8)  | <b>2.7</b> (1.0-13.7) |
| <b>21 months</b> | <i>Resting</i>                      |                         |                        |                        |                       |
|                  | GAD-alum (n=19)                     | <b>20.1</b> (14.8-47.3) | <b>2.1</b> (0.5-6.5)   | <b>10.7</b> (5.0-16.8) | <b>9.1</b> (2.6-21.8) |
|                  | Placebo (n=12)                      | <b>21.1</b> (12.5-40.7) | <b>2.3</b> (0.2-5.1)   | <b>12.6</b> (1.6-30.3) | <b>9.6</b> (0.7-16.8) |
|                  | <i>GAD<sub>65</sub> stimulation</i> |                         |                        |                        |                       |
|                  | GAD-alum (n=19)                     | <b>20.1</b> (14.1-49.1) | <b>1.6</b> (0.4-5.4)   | <b>11.0</b> (6.0-25.2) | <b>7.2</b> (2.7-19.1) |
| Placebo (n=12)   | <b>22.0</b> (11.8-39.3)             | <b>1.8</b> (0.5-5.8)    | <b>12.7</b> (1.1-27.0) | <b>9.3</b> (3.4-16.4)  |                       |

GAD<sub>65</sub>, glutamic acid decarboxylase 65; GAD-alum, GAD<sub>65</sub> formulated with aluminium hydroxide

### *GAD-alum-treated patients display decreased Th1 and Tc1 cell frequencies after in vitro*

#### *GAD<sub>65</sub> stimulation*

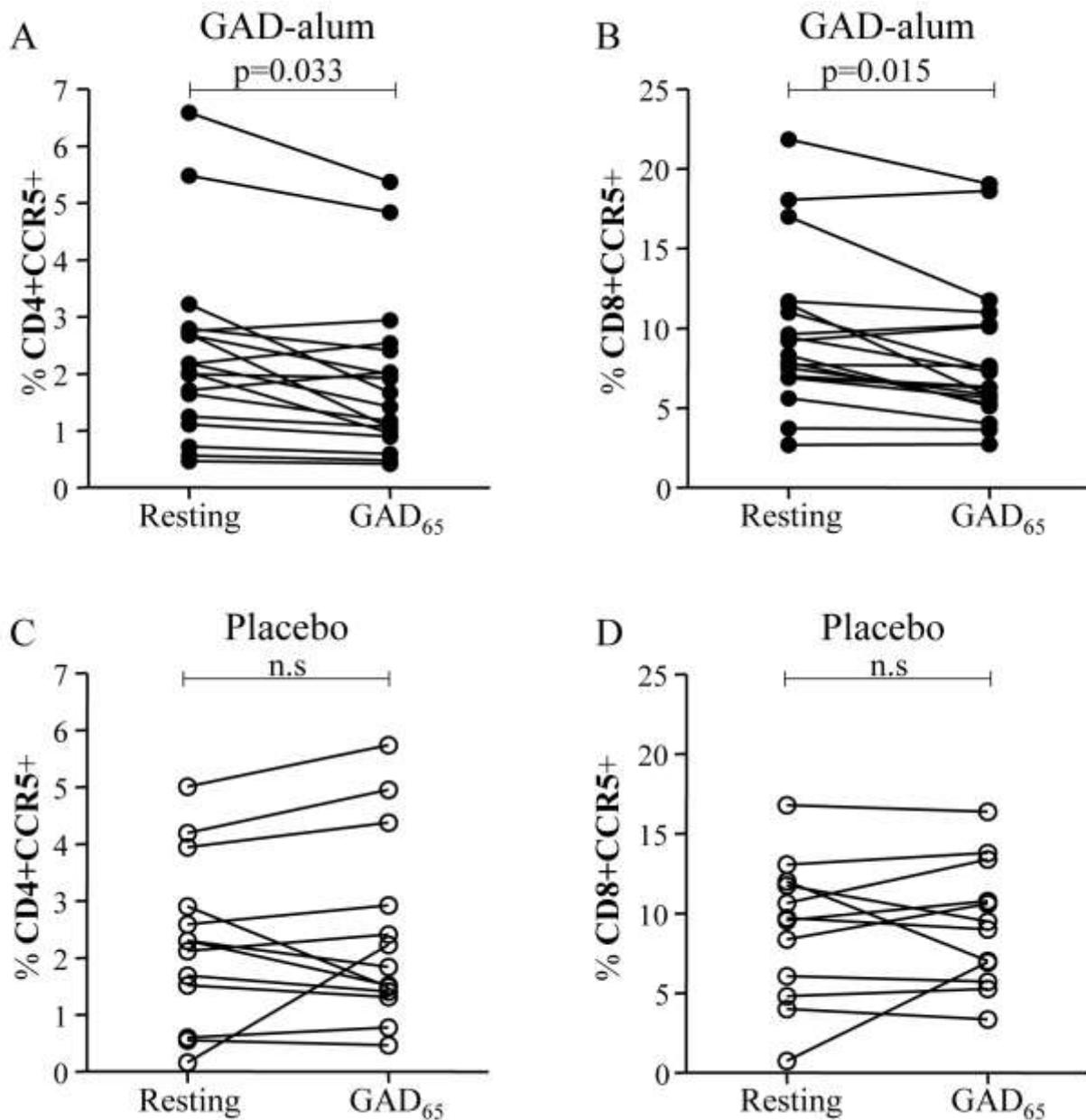
Results from the initial 15 months of the trial suggest that the immunomodulatory effect of GAD-alum treatment is antigen specific [18]. Thus, to evaluate the effect of *in vitro* antigen challenge on CCR4 and CCR5 expression, PBMC collected at 21 months were incubated over-night with and without GAD<sub>65</sub> prior staining. In line with our results *ex vivo* including baseline and 15

months samples, expression of CCR4 and CCR5 on CD4<sup>+</sup> and CD8<sup>+</sup> in resting cultures did not differ between the placebo and GAD-alum group (Table I). In contrast, when stimulated with GAD<sub>65</sub> frequencies of Th1 cells (CD4<sup>+</sup>CCR5<sup>+</sup>; p=0.033, Fig. 2A) and Tc1 cells (CD8<sup>+</sup>CCR5<sup>+</sup>; p=0.015, Fig. 2B) cells decreased in the GAD-alum treated patients, while the placebo group remained unaffected (Fig. 2C-D). In addition, the amount of CCR5 present per cell (median fluorescent intensity; MFI) was significantly decreased following GAD<sub>65</sub>-stimulation on CD4<sup>+</sup> (p=0.014) and CD8<sup>+</sup> (p=0.002) cells in the treated group (not shown). Expression of the Th2-associated receptor CCR4 on CD4<sup>+</sup> and CD8<sup>+</sup> cells remained unaffected in samples from both groups upon GAD<sub>65</sub>-stimulation. However, the CCR4/CCR5 ratio, indicating balance between Th2/Tc2 and Th1/Tc1 responses, showed a significant increase upon GAD<sub>65</sub>-stimulation in GAD-alum-treated patients both in CD4<sup>+</sup> (p=0.009, Fig. 3A) and CD8<sup>+</sup> (p=0.035, Fig. 3B) cells, whereas the CCR4/CCR5 ratio remained unaffected in the placebo group (Fig. 3C-D).

***GAD<sub>65</sub>-stimulation of PBMC induces secretion of CCL2 but not CCL3 and CCL4 in GAD-alum-treated patients***

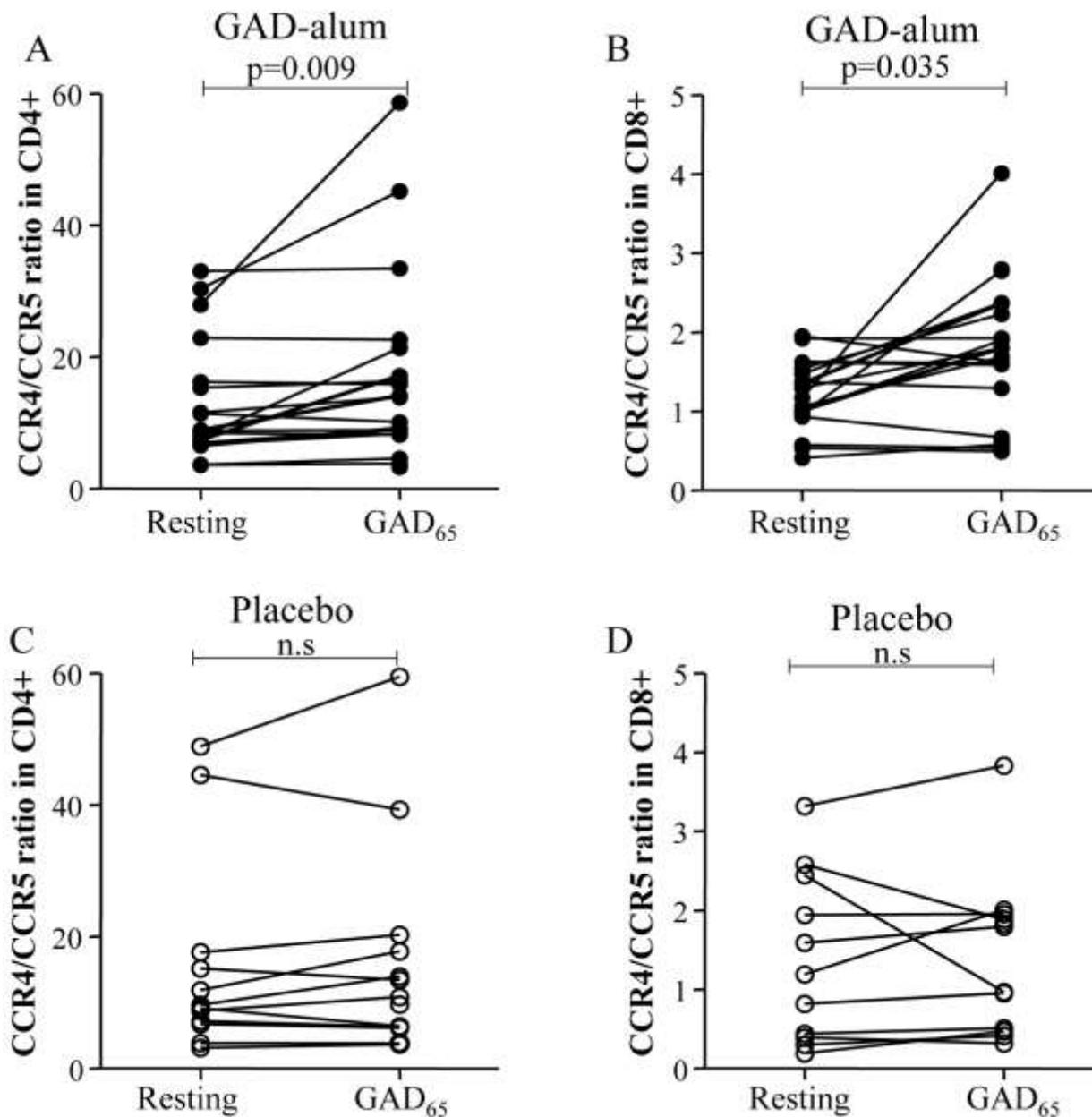
As the immunomodulatory properties of GAD-alum treatment has been characterized by an early GAD<sub>65</sub>-induced Th2 deviated cytokine response [20], we next investigated secretion of the Th2-associated chemokine CCL2, and the Th1-associated chemokines CCL3 and CCL4 in PBMC supernatants. At baseline, spontaneous and GAD<sub>65</sub>-induced CCL2, CCL3 and CCL4 secretion did not differ between the placebo- and GAD-alum-treated patients (*not shown*). Further, spontaneous chemokine secretion was also similar between the two groups 15 and 21 months after the first injection. In contrast, upon GAD<sub>65</sub>-stimulation at 15 months, secretion of CCL2 increased in GAD-alum treated patients (p=0.001; Fig 4A) but not in the placebo group, whereas secretion of CCL3 and CCL4 remained unaffected. At 21 months after treatment, secretion of CCL2, CCL3 and CCL4 was not significantly increased upon GAD<sub>65</sub>-

stimulation. Stimulation with the mitogen PHA gave significantly increased CCL2, CCL3 and CCL4 secretion compared to un-stimulated samples ( $p < 0.001$ ; *not shown*), and did not differ between the two treatment arms.



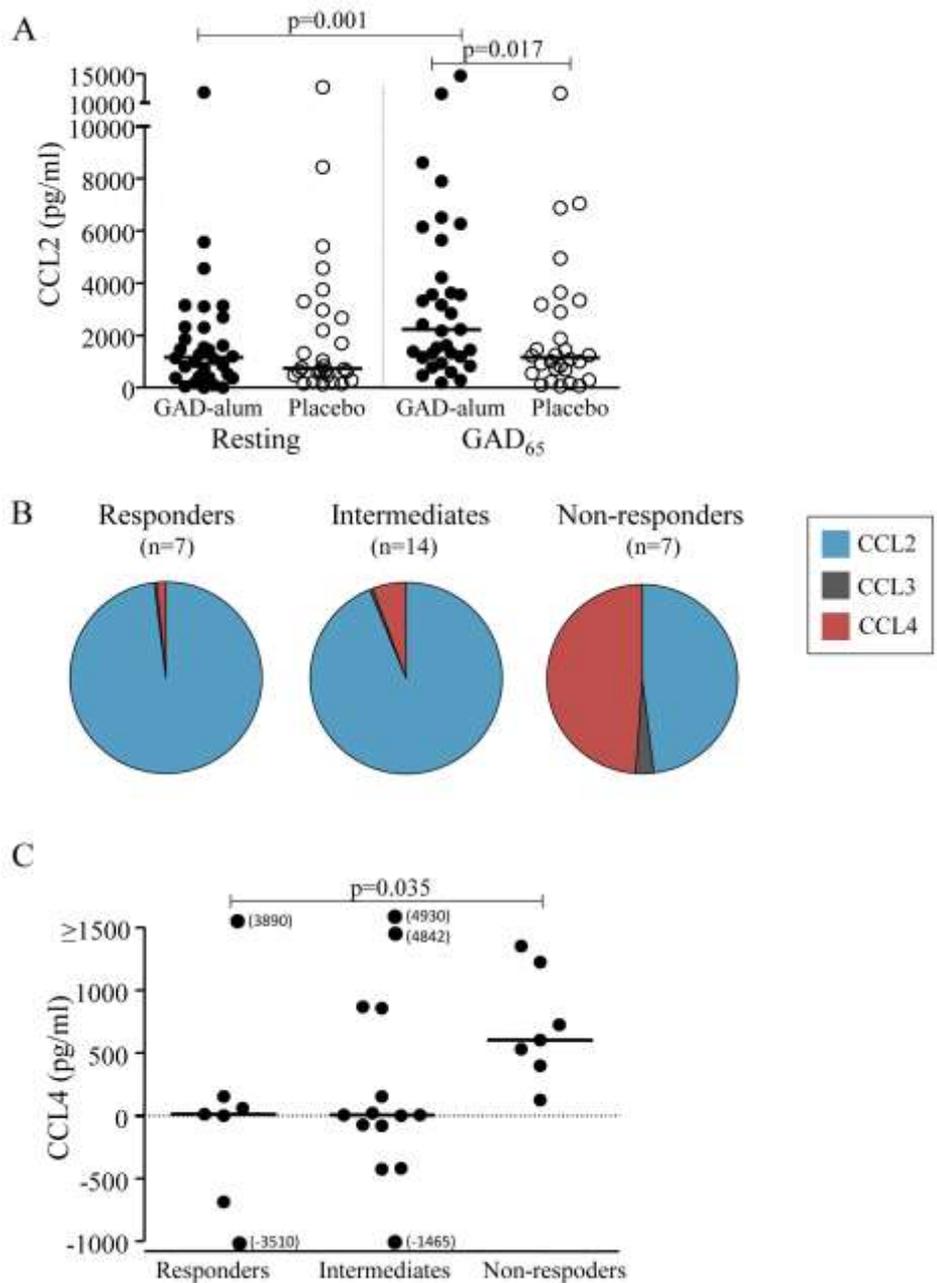
**Fig 2. Expression of CCR5 on CD4+ and CD8+ cells in GAD-alum and placebo-treated patients.**

Expression (%) of CC chemokine receptor 5 (CCR5) on resting and GAD<sub>65</sub>-stimulated CD4+ and CD8+ cells analyzed with flow cytometry at 21 months after GAD-alum (n=19; A-B) or placebo (n=12; C-D) treatment. Significant differences are indicated as p-values.



**Fig 3. The CCR4/CCR5 ratio in CD4+ and CD8+ cells in GAD-alum and placebo-treated patients.**

(A-B) The CCR4/CCR5 ratio in resting and GAD<sub>65</sub>-stimulated CD4+ cells (median in resting: 8.9 pg/ml; in GAD<sub>65</sub>-stimulated: 12.0 pg/ml) and CD8+ cells (median in resting: 1.1 pg/ml; in GAD<sub>65</sub>-stimulated: 1.8 pg/ml) at 21 months after GAD-alum treatment (n=19). (C-D) The CCR4/CCR5 ratio in resting and GAD<sub>65</sub>-stimulated CD4+ cells (median in resting: 9.4 pg/ml; in GAD<sub>65</sub>-stimulated: 10.1 pg/ml) and CD8+ cells (median in resting: 1.2 pg/ml; in GAD<sub>65</sub>-stimulated: 1.3 pg/ml) at 21 months after placebo treatment (n=12). Significant differences are indicated as p-values.



**Fig 4. Chemokine secretion upon *in vitro* PBMC stimulation**

(A) CCL2 secretion (pg/ml) detected with Luminex in resting and GAD<sub>65</sub>-stimulated PBMC supernatants in GAD-alum (n=33, black circles; median in resting: 1233 pg/ml; in GAD<sub>65</sub>-stimulated: 2225 pg/ml) and placebo-treated (n=29, white circles; median in resting: 731 pg/ml; in GAD<sub>65</sub>-stimulated: 1148 pg/ml) patients, 15 months after the first injection. Lines represent median, significant differences are indicated as p-values. (B) Pie charts illustrating the relative contribution of each of the chemokines CCL2, CCL3 and CCL4 to the GAD<sub>65</sub>-induced secretion at 21 months detected by Luminex in Responders (n=7), Intermediates (n=14) and Non-responders (n=7) to GAD-alum treatment. (C) GAD<sub>65</sub>-induced CCL4 secretion (pg/ml) in Responders (median: 11.0 pg/ml), Intermediates (median: 6.0 pg/ml) and Non-responders (median: 602 pg/ml) to GAD-alum treatment, 21 months after the first injection. Lines represent median, significant differences are indicated as p-values.

***Lower GAD<sub>65</sub>-induced CCL4 secretion in responders compared to non-responders to GAD-alum treatment***

We have previously observed a predominant GAD<sub>65</sub>-induced Th2-associated cytokine profile in clinical responders to GAD-alum treatment compared with non-responders [20]. Thus, in order to further search for possible immune surrogate markers of clinical efficacy, we analyzed the chemokine secretion profile in relation to the clinical effect of the treatment, defined by changes in stimulated C-peptide measured as AUC from baseline to 15 months (main study period). Although CCL2, CCL3 and CCL4 levels did not significantly increase upon GAD<sub>65</sub> stimulation at 21 months, the relative contribution of each chemokine to the GAD<sub>65</sub>-induced secretion shows that responders and intermediate responders to the treatment are characterized by a predominant Th2-associated profile, whereas non-responder patients display a more pronounced Th1-associated chemokine secretion (Fig 4B). Further, GAD<sub>65</sub>-induced CCL4 secretion was significantly lower in responders compared with non-responders to the treatment (p=0.035, Fig 4C). These findings were not evident at the preceding 15 months visit.

## Discussion

The balance of Th cell subsets is an important regulator of the immune system and is often examined after immune therapies in humans. The chemokine system has an essential role in optimizing T-cell response by coordinating localization and interaction of T cells and other immune cells. Modulation of chemokine receptor expression occurs upon activation, which implies changes in migratory properties of T-cell subsets [23]. Our results show a down-regulation of CCR5 on CD4<sup>+</sup> and CD8<sup>+</sup> cells following antigen-challenge, together with an increased CCR4/CCR5 ratio and enhanced secretion of CCL2 in samples from patients with T1DM treated with GAD-alum. These data suggest that immune responses tend to deviate away from a destructive Th1/Tc1 response upon GAD<sub>65</sub> re-challenge, which may be of importance for regulating the GAD<sub>65</sub> immunity.

As the microenvironment plays a crucial role in directing the T-cell responses toward type 1 or type 2 cytokine secretion, detection of cytokines and chemokines are relevant to understand the extent and direction of immune responses. The immunomodulatory properties of GAD-alum treatment has been previously characterized by an early Th2 deviated cytokine response to GAD<sub>65</sub> [20]. In this study we show that patients with T1DM treated with GAD-alum displayed an increased Th2/Tc2 associated chemokine secretion whereas the Th1/Tc1 associated secretion remained unaffected. At diabetes onset in NOD mice, CCL2 seems to correlate more closely with an early non-destructive insulinitis than with a destructive one [24-25] and primes Th2 polarization [13], whereas CCL3 secreted by antigen-specific Th1 cells invading the pancreatic islets correlated with a destructive insulinitis [24, 26]. Linkage of CCL2 to Th2 polarization has also been suggested after oral autoantigen administration in experimental autoimmune encephalomyelitis, where an increase of mucosal CCL2 resulted in enhanced Th2-associated IL-4 and reduced Th1 responses [27]. Further, it has been shown that CD8<sup>+</sup> T cells activated in the presence of IL-4 can develop into non-cytotoxic IL-4, IL-5,

and IL-10 producing cells [28]. Thus, bystander activation of CD8<sup>+</sup> T cells by cytokines could be beneficial to mount protective immune responses, and Tc2 cells could act as immunomodulatory cells producing suppressive cytokines. In line with our previous findings [20, 29], our results here support that a predominant Th2/Tc2-associated immune response to GAD<sub>65</sub> might generate an environment where auto-reactive Th1/Tc1 effector cells could be suppressed, thereby restoring immunological balance.

The recent setbacks in phase II [16] and III [17] clinical trials using GAD-alum are disappointing and indicate once more that translation from animal models to human T1DM is difficult. However, it cannot yet be excluded that GAD-alum treatment, alone or in combination therapies, might be beneficial in certain patient subgroups, and insights to the understanding of the immunomodulatory effect of antigen-specific treatment in T1DM are relevant for development of effective treatments. A clear effect of immunomodulatory therapies on disease mechanisms remains uncertain, in part because studies are limited to the analysis of the peripheral blood which may not be the major site of action of the immunomodulatory agents. Continued research to better understand how immunomodulation with autoantigen modify T- and B-cell responses and also which patients that are suitable for treatment, is crucial to optimize further intervention trials using  $\beta$ -cell antigens. We have previously demonstrated that the group of patients classified as clinical responders displayed a predominant Th2-associated cytokine secretion to GAD<sub>65</sub> compared with non-responders to GAD-alum treatment. Here we add new data suggesting that also the chemokine secretion profile in the responders group is characterized by a predominant Th2-associated secretion compared with non-responders, further supporting the hypothesis that a protective effect could be induced by a shift from Th1 to Th2 in response to GAD<sub>65</sub>.

Taken together, GAD-alum-treatment appears to deviate chemokine responses away from Th1/Tc1 which may be of importance for restoring immunological balance.

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## **Declaration of Competing Interests**

Nothing to declare.

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