Title: COMBINATION THERAPY FOR TYPE 1 DIABETES

Abstract: The present invention relates to a method of treatment of a human subject suffering from or at risk of developing type 1 diabetes comprising administering to the subject an effective amount of a) an inhibitory molecule and b) a tolerizing vaccine against a type 1 diabetes autoantigen.
Combination therapy for Type 1 diabetes

The present invention relates to a combination treatment for Type 1 diabetes. More specifically, the invention relates to a method of treatment for Type 1 diabetes based on administration of an antibody and a vaccine to a patient in need thereof.

Type 1 diabetes (T1D) is a devastating autoimmune condition in which self-reactive T cells attack pancreatic β cells that secrete insulin. Interleukin (IL)-1β is a proinflammatory cytokine produced by several cell types, including pancreatic β cells when exposed to high glucose concentrations. Since IL-1β can exert direct cytotoxic effects on β cells, one therapeutic avenue for treating T1D would be to neutralize this cytokine temporarily. This could be combined with antigen specific approaches to maintain tolerance to β cell antigens. For example, recent studies in our laboratory showed increased efficacy using combination therapies with anti-CD3 and islet autoantigens such as insulin or glutamic acid decarboxylase of 65 kd (GAD65)-expressing plasmids.

The inventors investigated whether treatment of recent-onset diabetic mice with anti-IL1β antibody alone (provided by Novartis) or in combination with a GAD85 DNA vaccine could reverse disease development. During 5 weeks, RIP-GP transgenic mice that turned diabetic upon LCMV infection (RIP-LCMV model for type 1 diabetes) were treated weekly with antibodies and at day 0, 4 and 11 with GAD65 DNA. Results show that anti-IL1p monotherapy could only protect 25% (1/4) of mice from T1D, but that combination-therapy with neutralizing IL-1β antibodies and GAD65 DNA could reverse disease in 7/9 animals (78%). Consistently with earlier studies in the lab using DNA vaccinations alone in recent-onset diabetic mice (Bresson et al. JCI, 2006) GAD65 treatment alone achieved disease reversal in 3/6 animals (50%).

Reversion of hyperglycemia was associated with preservation of insulin-producing islets and correlated in the spleen with decreased production of IFN-γ by GP33 peptide-specific ("autoreactive") CD8 effector T cells, which are required for disease in this diabetes model because they recognize the transgenically expressed GP protein on β cells. Reversion to normoglycemia was also reflected by increases in frequencies of Foxp3-expressing CD4 T regulatory cells in pancreatic lymph nodes, but no statistically significant difference was observed between GAD65 treatment with or without anti-IL-1β antibodies. In these two groups, the extent of CD4+ and CD8+ cell infiltration was also found to be similar. However, mice treated
with anti-IL1β Ab + GAD65 DNA vaccine seemed to display less severe infiltration by CD11b+ cells (presumably macrophages) compared to isotype Ab/GAD65-treated mice.

The present findings in the RIP-LCMV mouse model provide evidence that IL-1β blockade alone can only reverse diabetes to a certain extent, but that combination therapy with GAD65 DNA vaccines permits a marked improvement of clinical and immunological aspects of the disease. These data hold promise for the treatment of new-onset T1D patients with combination therapies involving blockade of IL-1β.

In one aspect the invention provides a method of treatment of a human subject suffering from or at risk of developing type 1 diabetes comprising administering to the subject an effective amount of a) an IL1β inhibitory molecule and b) a tolerizing or suppressive vaccine against a type I diabetes autoantigen.

In a further aspect the invention provides a) an IL1β inhibitory molecule and b) a tolerizing or suppressive vaccine against a type I diabetes autoantigen for use in the treatment of a human subject suffering from or at risk of developing type 1 diabetes.

In one embodiment the vaccine is directed against, tolerizes against or suppresses an immune response against one or more autoantigens selected from the group consisting of: GAD65, GAD67, insulin, proinsulin, HSP60, IA-2, IA-2β, carboxy-peptidase H, islet-cell expressed gangliosides, ZnT8 (a zinc transporter in the membrane of islet β cell secretory granules); and IGRP (islet-specific glucose-6-phosphate catalytic subunit related protein).

In one embodiment the vaccine is directed against, tolerizes against or suppresses an immune response against one or more peptide fragments of the one or more autoantigens.

In one embodiment the vaccine is a protein vaccine.

In one embodiment the vaccine is a DNA vaccine.

In one preferred embodiment the 111β inhibitory molecule is an antibody directed against IL1β.

In one embodiment the antibody is a monoclonal antibody selected from the group consisting of Canakinumab, Xoma 052, LY2189102.

In one specific embodiment, the antibody is Canakinumab and the vaccine tolerizes against or suppresses an immune response against GAD65 or peptide fragments thereof.
Brief description of the Figures

Figure 1: (A) Mean blood glucose values (BGVs) in mg/dL in all groups when treatment was started, (B) Individual BGVs for mice whose value was over 400mg/dL at treatment start and (C) Diabetes incidence in recent-onset diabetic mice. Female B6 mice expressing glycoprotein (GP) from lymphocytic choriomeningitis virus (LCMV) under the rat insulin promoter (RIP) were infected with $10^4$ PFU LCMV Armstrong intraperitoneal^a. From day 7 post-infection, BGV was assessed for all the mice and upon BGVs above 250mg/dL, mice were included in one of the following treatment groups; anti-isotype antibody (black line, n=4), anti-IL-1p antibody (red line, n=4), anti-isotype antibody plus GAD65 DNA vaccine (green line, n=6) or anti-IL-1β antibody plus GAD65 DNA vaccine (blue line, n=9). Antibodies were administered intraperitoneally (10ug per gram body weight) once a week for 5 weeks and GAD65 DNA vaccine was given intramuscularly (100ug per leg) at day 0, 4 and 11. At the end of the treatment, mice were sacrificed and spleen, pancreatic lymph nodes (PLNs) as well as pancreata were collected and processed for analysis.

Figure 2: Frequencies of IFN-γ and TNF-a producing GP33-specific CD8+ effector T cells in spleen and PLNs of treated mice. After 5-week treatment, spleens and PLNs were mashed onto 70um cell strainers to obtain single-cell suspensions. Cells were then plated in round bottom 96-well plates and left unstimulated, or stimulated for 4 to 5 hours with 5ug/mL (CD8 T-cell specific) GP33 peptide. PMA (50ng/mL) and ionomycin (1ug/mL) were used as positive controls. In all conditions, brefeldin A (10ug/mL) was used to block protein secretion. Cells were then stained in surface with fluorochrome-coupled CD8 and CD4 antibodies (Biolegend), fixed and permeabilized using FoxpS staining kit (eBioscience), and later stained with fluorochrome-coupled IFN-γ and TNF-a antibodies (Biolegend). Data were acquired on a LSRII cytometer (BD) and analyzed with FlowJo software (Tree Star). Data are expressed as % of gated CD8+ cells. Results from 2-3 pooled experiments with 2-6 mice per group are shown.

Figure 3: Proportions of Foxp3-expressing Tregs in treated mice. Spleen and PLN cells from treated mice were mashed against 70um cell strainers, resuspended, and prepared for further flow cytometry staining for CD4+CD25+Foxp3+ regulatory T cells (Tregs) using FoxpS staining kit (eBioscience) according to the manufacturer's instructions. Data were acquired on a LSRII cytometer (BD) and analyzed with FlowJo software (Tree Star). Data are expressed as % of gated CD4+ cells. Results from 2-3 pooled experiments with 2-6 mice per group are shown.

Figure 4: Preservation of insulin-producing β cells in the pancreas of treated mice. Shown is a picture representative of the size of islets counted and plotted in the lower panel. Magnification:
20X. Frozen sections (6μm) were cut from pancreata of mice treated with the different regimens and left to dry overnight. Next day, sections were fixed with acetone and blocked with Tris-buffer/NaCl solution plus protease inhibitors and goat serum. Sections were stained with guinea pig anti-insulin primary antibody (Dako), followed by staining with goat anti-guinea pig alkaline phosphatase (AP) antibody (Sigma). AP detection was performed using Vector Blue AP III (Vector) and sections were mounted with aqueous mounting solution (Lemer Lab.). Pictures were taken with a Nikon 80i fluorescence microscope.

Figure 5: CD4+, CD8+ and CD11b+ cell infiltration in mice treated with GAD65 DNA with anti-isotype or anti-IL-1β antibodies. Pancreas sections from treated mice were stained for insulin as described in Figure 4. In parallel, different stains for CD4, CD8 and CD11b were performed using biotinylated primary antibodies (1/100, BD), followed by the addition of streptavidin coupled-horseradish peroxidase (1/1 000, Vector) and developed using NovaRed Peroxidase substrate kit (Vector). The extent of CD4+, CD8+ or CD11b+ cells was counted for 16 to 111 islets from 2 to 5 mice per group. Classification was performed as follows: No infiltration; mild infiltration when cells were found around the insulin-producing islet; heavy infiltration when cells were found within the insulin-producing islet. A 2-way ANOVA test concluded that differences the two groups were statistically significant with regard to CD11b+ cell infiltration.

Experimental section

1. Anti-IL-1β treatment can reverse the course of recent-onset T1D to a modest extent when used alone, but to a much greater extent when used in combination with GAD65 DNA vaccine.

Recent-onset diabetic RIP-LCMV mice were treated with the following regimens: monotherapy with anti-IL-1β antibody alone, anti-isotype antibody alone, combination therapy with anti-IL-1β antibody and GAD65-expressing DNA plasmid, or anti-isotype antibody and GAD65 DNA. Figure 1A shows a similar average BGV when treatment was started in all groups, suggesting that randomization of mice was performed without any bias. Figure 1B demonstrates that mice whose BGV was over 400mg/dL when treatment was initiated, could not revert to normoglycemia, regardless of the tested treatment. However, when mice were treated with a starting BGV between 250 and 400mg/dL, we could observe that treatments with anti-IL-1β Ab provided a slightly protective effect in 25% (1/4) of mice while anti-isotype control Ab treatment protected none of the animals (0/4) (Figure 1C). Moreover, GAD65 DNA vaccine monotherapy resulted in a 50% (3/6) reversion to normoglycemia upon 5 weeks of treatment. Interestingly, when treating diabetic animals with anti-IL-1β Ab in combination with GAD65, 78% (7/9) of
animals reverted to normoglycemia (Figure 1C). These data suggest that an additive effect is provided by the concomitant action of IL-1β neutralization and GAD65 DNA administrations.

2. **GAD65 DNA vaccine given with either antibody leads decreased frequencies of GP33-specific IFN-γ producing CD8 cells in the spleen.**

Cells from spleen and PLNs were isolated and restimulated with LCMV GP33 peptide in order to evaluate CD8 T-cell specific cytokine production. In comparison to isotype control antibody treatment, we noticed a tendency towards diminished IFN-γ secreting cells in the spleen of anti-IL-1β-treated animals (Figure 2). The co-administration of GAD85 DNA together with either antibody correlated with decreased production of IFN-γ (and TNF-ct, to a lesser extent) by GP33-specific CD8 effector T cells in the spleen, but not PLNs.

3. **The improved diabetes incidence upon combination therapy is not reflected by higher proportions of Foxp3-expressing Tregs.**

We then assessed whether the increased success upon IL-1p blockade in conjunction with GAD65 DNA could be explained by a greater presence of Foxp3-expressing Tregs in spleen and PLNs. We found that compared to single-antibody treatment, the addition of GAD65 DNA vaccination significantly increased the frequencies of CD4+CD25+Foxp3+ Tregs locally in PLNs. Nonetheless, no statistically significant difference could be seen between GAD treatment with or without anti-IL-1β antibodies (Figure 3). Taken together, we can conclude that in comparison to GAD65 treatment alone, the improved benefit provided by IL-1β blockade cannot be attributed to an increase in Foxp3-expressing Tregs.

4. **GAD65 DNA vaccine therapy preserves insulin-producing pancreatic β cells, regardless of the antibody used in combination.**

We further investigated whether the protection, or lack thereof, seen in the different groups of mice would correlate with the number of insulin-producing β cells. To do so, 6-um pancreata sections were cut and stained for insulin. We found that anti-isotype or anti-IL-1β antibody-treated animals did not present large islets, as defined in Figure 4 (upper panel), while anti-isotype/GAD65- and anti-IL-1p/GAD65-treated mice presented averages of 3.34 and 3.71 islets per section, respectively. Moreover, anti-isotype/GAD65-treated mice had in average higher numbers of medium (4 vs 1) and small (7.67 vs 1) islets compared to anti-isotype Ab-treated mice. Likewise, anti-IL-1p/GAD65-treated mice presented in average more medium (6.71 vs 3) and small (8.57 vs 2.5) islets compared to anti-IL-1β-treated mice. Altogether, these findings are
consistent with the generally improved diabetes outcome observed in GAD65-treated mice with or without IL-1β blockade.

5. **IL-1β blockade upon diabetes onset seems to participate in the reduction of pancreatic infiltration by CD11b+ macrophages, but not by CD4+ nor CD8+ T cells,**

IL-1β can be produced by β cells, especially in conditions of hyperglycemia, but also by macrophages which can count among the different inflammatory cell infiltrates in diabetic mice and patients. Thus, we assessed by immunohistochemistry the extent of pancreatic infiltration by CD4+ and CD8+ T cells, but also CD11b+ macrophages in anti-isotype/GAD65- and anti-IL-1p/GAD65-treated mice. In these two groups, we found that overall CD4+ and CD8+ cell infiltration was similar. However, mice treated with anti-IL1p/GAD65 DNA vaccine seemed to display less severe infiltration by CD11b+ macrophages compared to isotype Ab/GAD65-treated mice.

In sum, the results suggest that the protective effect of anti-IL-1β therapy alone in recent-onset mouse model of diabetes is modest, which is consistent with the minor effect IL-1R deficiency had on diabetes development in the RIP-LCMV model (Barral et al. J. Autoimmunity 2006). When performed alone, the induction of an antigen-specific tolerance using GAD65 DNA vaccination resulted in 50% reversal from hyperglycemia, which is in line with previous studies (Bresson et al., JCI 2006). However, combining the neutralization of IL-1β with GAD65 DNA administration for a period of 5 weeks significantly improved the reversal of recent-onset diabetes in transgenic RIP-GP B6 mice. Compared to either antibody treatment, the addition of GAD65 DNA vaccine resulted in (i) diminished IFN-γ production by GP33-specific CD8 T cells in the spleen, (ii) higher proportions of Foxp3-expressing Tregs in PLNs and (iii) higher numbers of preserved insulin-producing β cells. IL-1β blockade together with GAD65 DNA vaccination resulted in a reduced infiltration of CD11b+ macrophages, but not CD4 and CD8 T cells, in the pancreas of treated mice. These data confer a potential explanation for the improved clinical outcome observed upon this combination therapy (78% reversion) in comparison to separate administrations of either IL-1β blockade (25% reversion) or GAD65 DNA vaccination (50% reversion). The perspective that the efficacy of an anti-inflammatory drug such as anti-IL-1β can be improved by combination with antigen specific approaches known to have no minimal systemic side effects is clinically promising.
Claims

1) A method of treatment of a human subject suffering from or at risk of developing type 1 diabetes comprising administering to the subject an effective amount of a) an IL1β inhibitory molecule and b) a tolerizing vaccine against a type I diabetes autoantigen.

2) The method according to claim 1, wherein the tolerizing vaccine is directed against one or more autoantigens or fragments thereof, selected from the group consisting of: GAD65, GAD67, insulin, proinsulin, HSP60, IA-2, IA-2 β, carboxy-peptidase H, ZnT8, IGRP, and islet-cell expressed gangliosides.

3) The method according to claim 1 or 2, wherein the tolerizing vaccine is a protein vaccine.

4) The method according to any of the preceding claims, wherein the tolerizing vaccine is a DNA vaccine.

5) The method according to any of the preceding claims, wherein the IL1β inhibitory molecule is a monoclonal antibody directed against IL1β.

6) The method according to any of the preceding claims, wherein the antibody is selected from the group consisting of Canakinumab, Xoma 052, LY2189102.
Figure 2
(gated on CD8+ cells)

% IFN-γ+

% TNF-α+

Spleen

PLNs

- Anti-isotype Ab
- Anti-IL1β Ab
- Anti-isotype Ab / GAD65 DNA
- Anti-IL1β Ab / GAD65 DNA
Figure 3

(gated on CD4+ cells)

% CD4^+CD25^+Foxp3^+

Spleen

- Anti-isotype Ab
- Anti-IL1β Ab
- Anti-isotype Ab / GAD65 DNA
- Anti-IL1β Ab / GAD65 DNA

PLNs
### A. CLASSIFICATION OF SUBJECT MATTER

- INV. A61K39/00
- A61K39/395
- C07K16/24
- A61P5/50
- A61P3/10

**ADD.**

According to International Patent Classification (IPC) and both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols): A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**EPO-Internal**

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

**X** Further documents are listed in the continuation of Box C.  
**X** See patent family annex.

* Special categories of cited documents:
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

**T** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

**X** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

**Y** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

**S** document member of the same patent family

**Date of the actual completion of the international search:** 25 January 2012  
**Date of mailing of the international search report:** 08/02/2012

**Name and mailing address of the ISA/**  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040,  
Fax: (+31-70) 340-3016

**Authorized officer:** Noe, Veerle
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Wo 2010/028273 AI (XOMA TECHNOLOGY LTD [US]; SCANNON PATRICK J [US]; SOLINGER ALAN M [US]) 11 March 2010 (2010-03-11) paragraphs [0018], [0019], [0043], [0044]; claim 74</td>
<td>1-6</td>
</tr>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>WO 2010028273 A1</td>
<td>11-03-2010</td>
<td>AU 2009289545 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 2009289547 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2735939 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2735940 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2012003226 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2012005127 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2010028273 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2010028275 A1</td>
</tr>
</tbody>
</table>