

# Prevention of type 1 diabetes by inducing subclinical dermatitis on a small area

Kaare Engkilde<sup>1,2\*</sup>

Jeanne Duus Johansen<sup>1</sup>

Axel Kornerup Hansen<sup>3</sup>

Torkil Menne<sup>1</sup>

Karsten Buschard<sup>2</sup>

<sup>1</sup>Department of  
Dermato-Allergology,  
National Allergy Research Centre,  
Gentofte Hospital,  
University of Copenhagen,  
Copenhagen, Denmark

<sup>2</sup>Bartholin Institute,  
Rigshospitalet,  
University of Copenhagen,  
Copenhagen, Denmark

<sup>3</sup>Department of Veterinary  
Disease Biology,  
Faculty of Life Sciences,  
University of Copenhagen,  
Copenhagen, Denmark

\*Correspondence to:  
Kaare Engkilde,  
Department of  
Dermato-Allergology,  
National Allergy Research Centre,  
Gentofte Hospital,  
University of Copenhagen,  
Copenhagen, Denmark  
E-mail: kaaeng02@geh.regionh.dk

## Abstract

**Objective** We have previously epidemiologically shown that type 1 diabetes is inversely associated with contact allergy. This finding is intriguing as type 1 diabetes and contact allergy are two completely different diseases, although T cells are involved in both diseases. The objective of this study was therefore to experimentally study the effect of contact allergens on the development of diabetes in non-obese diabetic mice.

**Methods** Non-obese diabetic mice 4 weeks of age were separated into seven groups. One group was exposed to tap water every 14th day, whereas the remaining six groups were split into sensitization groups or elicitation groups (exposure every 14th day). These groups were then treated with one of the selected contact allergens (PPD or DNCB) or vehicle (AOO). All groups received the sensitizing treatment regime, and hereafter only the elicitation groups were further treated. If the blood glucose reached 14 mM, the mice were considered diabetic and euthanized. Cardiac heart blood was drawn at euthanization, and a Luminex analysis was done on the serum.

**Results** We showed that repeated application of a low dose of PPD reduced the incidence of diabetes compared to application with water (47% versus 93%,  $p = 0.004$ ). The rest of the groups developed diabetes with a cumulative incidence rate above 80%. The Luminex cytokine analysis revealed no differences between the groups, and no elevated cytokine level suggested a systemic response. Dermatitis was not noticeable by visual inspection, a histological examination, however, revealed a slight infiltration in the ears in the elicitation groups exposed to contact allergens.

**Conclusion** This study showed that repeated topical application on the ears with a contact allergen could prevent the development of diabetes in non-obese diabetic mice. The contact allergens gave a non-visible, subclinical dermatitis on the application site. Activation of NKT cells to the ear lymph nodes seems to be involved. Copyright © 2011 John Wiley & Sons, Ltd.

**Keywords** allergic contact dermatitis; CHS; dermatitis; diabetes; hapten; incidence; NKT cells; NOD mice

**Abbreviations** A-LN – ear-draining lymph node; AOO – acetone : olive oil (4:1); CHS – contact hypersensitivity; DNCB – 2,4-dinitrochlorobenzene; NKT cell – natural killer T cell; PPD – p-phenylenediamine.

## Introduction

The incidence of type 1 diabetes appears to be increasing [1] and it is therefore of interest to investigate environmental factors which could modulate the

Received: 12 April 2011

Revised: 24 June 2011

Accepted: 25 June 2011

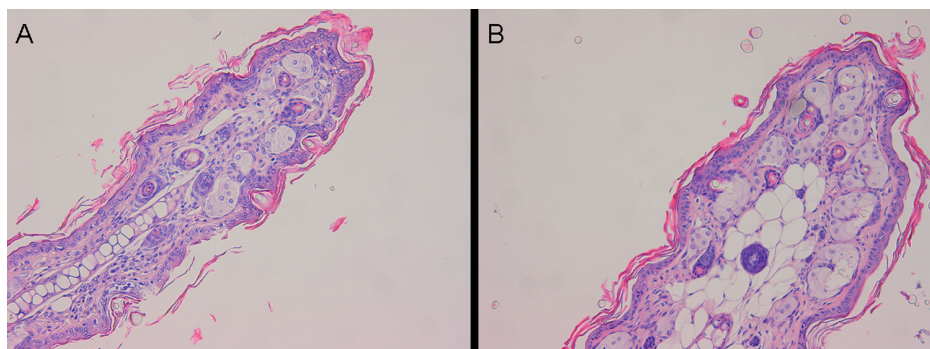


**Figure 1.** Mononuclear infiltrations in the ear after provocation with PPD at the end of this study. (A) A haematoxylin and eosin stained ear from the group of mice repeatedly exposed to PPD. (B) An ear from the handling control (water group) repeatedly exposed to water

development of disease. Allergic contact dermatitis is a skin disease, driven by skin exposures to contact allergens. These are often present in consumer products and allergic contact dermatitis can therefore be considered an environmental diseases. The allergens or haptens which due to their size and lipophilic nature can penetrate the skin and interact with proteins making the hapten–protein molecule immunogenic [2]. Recent studies using potent contact allergens have shown that CD8<sup>+</sup> T cells are the effector cells of allergic contact dermatitis in mice, whereas CD4<sup>+</sup> T cells behave as down-regulatory cells [3–6]. We have previously in an epidemiological study shown an inverse association between type 1 diabetes and allergic contact dermatitis [7]. On the background of this finding, the idea for this study was conceived in which we examine whether exposure to a contact allergen can prevent the development of diabetes in non-obese diabetic (NOD) mice, as first outlined in 2010 [8].

It has been established that stimulation of NKT cells can protect NOD mice against diabetes [9,10]. As contact allergens stimulate NKT cells [11,12], we additionally explored whether the used allergens actually did influence NKT cells.

## Materials and methods

### Diabetes incidence study

To study the incidence rate of diabetes in contact allergen exposed mice, we took receipt of 104 females, 3-week-old NOD/BomTac mice (Taconic, Ry, Denmark). The mice were allocated to seven groups. These groups were further allocated to exposure with PPD, DNCB, vehicle (AOO) or water (one group). All groups received three topical exposures a week after being delivered, i.e. their 4th week of life, equivalent to the first 3 days of the local lymph node assay protocol, previously described in Dean *et al.* [13]. Four groups were further exposed repeatedly every 2 weeks until a maximum of 32 weeks of life. These groups consisted of one group from each of the PPD,

DNCB and AOO exposure groups and the group from the water exposure group.

The exposures were topical application of 25  $\mu$ L substance on the dorsal side of each ear. For the first exposures, on the 4th week of life, the concentration for the allergens (PPD or DNCB) was 0.1% (w/v) and pure solvent for the other exposure groups (AOO and water). For the repeated exposures (four groups), the amount remained at 25  $\mu$ L, whereas the concentration of allergen was reduced to 0.01% (w/v). The vehicle (AOO) and water repeated exposure groups received only AOO or water, respectively. The dilutions were freshly made immediately before application.

The experiment ended when the mice were 32 weeks old, at which time the mice in the repeatedly exposed groups had received 14 applications of the repeated dose.

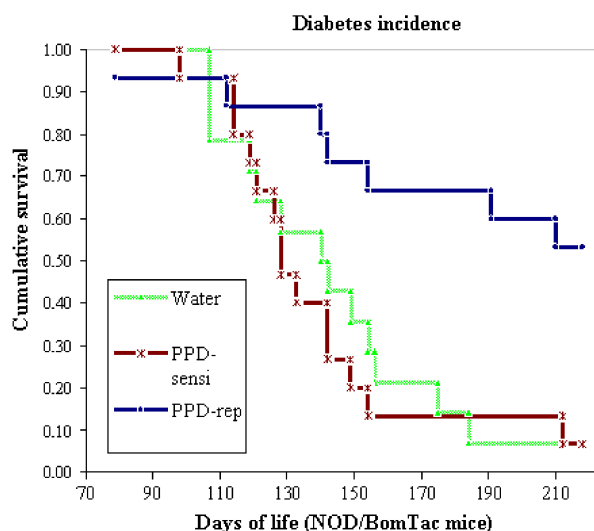


**Figure 2.** Repeated application of PPD inhibits development of diabetes in non-obese diabetic mice. It shows a Kaplan–Meier curve of the diabetes incidence for three groups of mice. PPD-rep and water refer to the groups that were repeatedly exposed to PPD or water every other week. PPD-sensi refers to the group that was only exposed to PPD in the 4th week of life. The non-obese diabetic mice exposed to PPD repeatedly, displayed a cumulative diabetes incidence of 47%, in contrast to 93% of the water-treated group ( $p = 0.004$ )

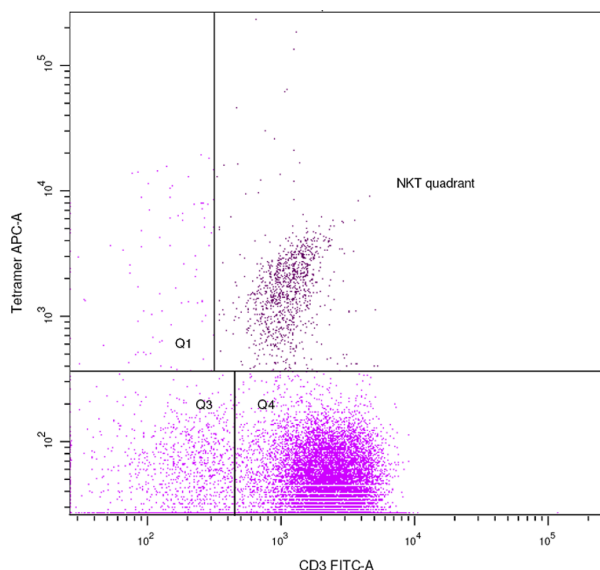


Figure 3. The flow cytometry analysis. It shows the gate containing NKT cells, from the liver of PPD-exposed mice euthanized 72 h after challenge

At 12 weeks of age, weekly measurements of tail blood glucose commenced. If the glucose level was equal to or above 14 mmol/L on two consecutive days, the mice were considered to be diabetic and were euthanized. At the end of this study, the mice still alive in the PPD repeatedly exposed group were provoked with 25  $\mu$ L, 0.1% (w/v) PPD, and euthanized 2 days later. A histological examination was made to assess cellular infiltration of the ears (Figure 1). All samples were fixed in formalin and stained in haematoxylin and eosin. The rest of the mice still alive at the end of this study were euthanized and their ears were also examined for cellular infiltration. We did a cytokine analysis of blood serum drawn by cardiac puncture at euthanization. The

analysis was done by use of the flow metric Luminex xMAP technology. The staining was done according to the manufactures guideline

The cumulative diabetes incidence was evaluated by the Kaplan–Meier estimation, and the significance was estimated by the log-rank test. The analysis was done with SPSS version 15.0 (SPSS, Chicago, IL, USA).

## NKT-cell study

We took receipt of 20 females, 10-week-old C57BL/6JBomTac mice (Taconic). One week after arrival 10 mice were sensitized with 0.5% (w/v) DNCB in AOO and 10 mice with PPD 0.5% (w/v). Two weeks after sensitization the mice were separated into 10 groups of two mice – accordingly there were 5 groups in each interval. In eight groups, the respective allergen in a concentration of 0.5% (w/v) was reapplied. At the following time points after reapplication (18, 24, 48 and 72 h), a group of mice were anaesthetized, fixated and vena portae was ligated. Vena cava was cut and the mice bled. Subsequently, the liver was perfused with phosphate buffered saline containing heparin until pale; afterwards the liver was removed along with the A-LN. The groups which did not receive a reapplication, control group, were euthanized at 48 h.

The livers and A-LNs were strained (70  $\mu$ m; BD Biosciences, Brøndby, Denmark), to gain a single cell suspensions, and the liver lymphocytes were isolated using Lympholyte-M (CEDARLANE Laboratories, Burlington, Canada). Subsequently, the cell suspensions were stained with antigen-presenting cell (APC)-conjugated CD1d tetramers (ProImmune, Oxford Science Park, UK) loaded with  $\alpha$ -galactosylceramide (Alexis Biochemicals/Axxora, San Diego, USA) according to the manufacture guidelines. Thereupon, the following cellular receptors were stained:

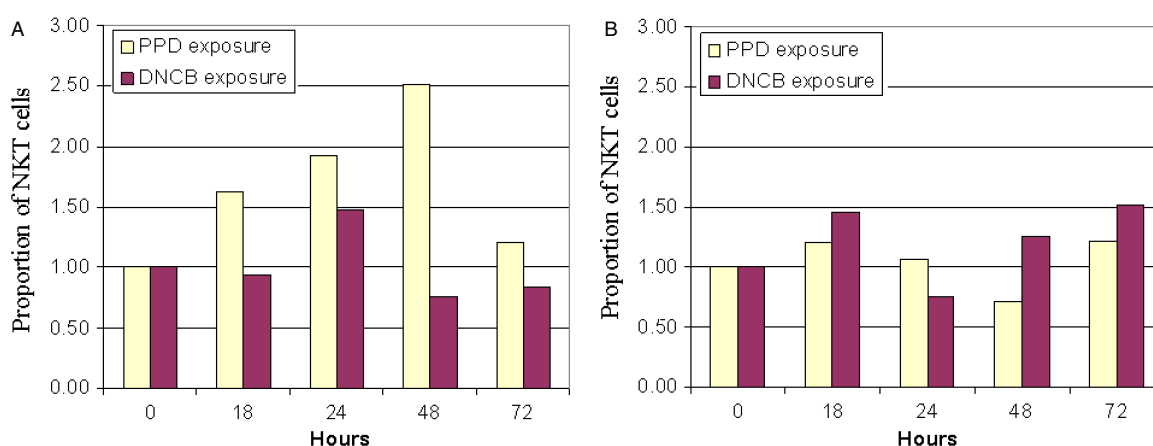


Figure 4. PPD is a strong peripheral activator of NKT cells. (A) The proportion of NKT cells in the A-LNs, for the mice exposed to either PPD or DNCB. The proportions were found, by dividing with the number of NKT from the control groups (0 h). As shown, the proportion of NKT cells appears to rise in the beginning, but at 48–72 h there is a sharp drop that probably has to do with a down-regulation of receptors. (B) The proportion of NKT cells in the liver, for mice exposed to PPD or DNCB. The proportions were found by dividing with the number of NKT cells from the control groups (0 h). Both allergens increased the number of NKT cells in the liver in a comparable manner. The NKT cells were stained for T-cell receptor (TCR)- $\beta$ , CD3 and CD1d loaded tetramers ( $\alpha$ -GalCer), CD19 positive cells were gated out

fluorescein isothiocyanate-conjugated anti-CD3 (BD Biosciences), PE-Cy5.5-conjugated anti-T-cell receptor (TCR)- $\beta$ , PE-Cy7-conjugated anti-NK1.1 (eBioscience, San Diego, USA) and APC-Cy7-conjugated CD19 (BioLegend, San Diego, USA). Afterwards the cells were analysed on a FACSCanto II (BD Biosciences). The CD19 positive cells were gated out, and TCR- $\beta$  positive cells were selected. Subsequently, cells in the quadrant containing CD3 positive and CD1d were designated as CD1d restricted NKT cells. Next, the quantity of NKT proliferation was determined by dividing with the number of NKT cells in the control groups, and a paired samples *t*-test was used to evaluate the difference in NKT-cell stimulation.

## Results

The diabetes incidence study revealed that NOD mice repeatedly exposed to PPD displayed a cumulative diabetes incidence of 47%, in contrast to 93% of the water-treated group ( $p = 0.004$ ). The group repeatedly exposed to DNCB had an incidence of 92%, and the group which only received the PPD sensitizing regime 93% (Figure 2). The three other groups developed diabetes by a rate similarly with a cumulative incidence from 80 to 86% (data not shown).

Dermatitis was not noticeable by visual inspection, however, sensitization in the group repeatedly exposed to PPD was confirmed by a typical lymphocytic infiltration in the ears following PPD provocation, and a more moderate infiltrate was also noticeable in the group repeatedly exposed to DNCB (Figure 1). The Luminex cytokine analysis revealed no differences between the groups, and no elevated cytokine level suggested a systemic response.

The NKT study demonstrated that both allergens influenced the quantity of NKT cells in an analogous manner in the liver. Figure 3 shows the gating strategy. This is in contrast to the A-LNs where PPD appears to influence the amount of NKT cells more strongly than DNCB (Figure 4), although this was not statistically significant.

## Discussion

Here we describe the interesting finding that repeated applications of PPD prevent the diabetes development in NOD mice. The repeated application of allergen gave a subclinical dermatitis.

Moreover, we studied the effect of hapten application on NKT cells in a separate study using C57BL/6 mice as NOD mice have a aberrant immunophenotype and do not

express the NK1.1 receptor. We found that both allergens modulated the number of NKT cells in the liver, which is in accord with previous published data [11], while PPD gave a more pronounced NKT-cell response in the A-LN (Figure 3). The found peripheral stimulation of NKT cells by contact allergens is in agreement with a human study [12]. The mechanism by which allergic contact dermatitis can protect against diabetes is unknown, but activation of NKT cells could be an explanation, as up-regulation of NKT cells have been shown to protect NOD mice against diabetes [9,10,14,15]. Correspondingly, alcohol has been shown to inhibit diabetes development, probably due to facilitated loading of CD1d and thereby activation of NKT cells [16]. Furthermore, hapten application has been shown to increase glycolipids levels in the liver which might be an endogenous stimulation of NKT cells [17]. Moreover, we have preliminary results indicating that repeated application of BALB/c mice with 0.5% PPD increases the proportion of NKT cells by 2.5-fold in the pancreatic lymph nodes, which further indicates a stimulation of NKT cells in peripheral lymph nodes. The difference between PPD and DNCB in the peripheral activation of NKT cells, as indicated in A-LNs could be an explanation for the selective protective effect of PPD.

The epicutaneous application of the contact allergens gave in the repeatedly treated mice a local harmless subclinical dermatitis. The application involved only the outside of the ears which is in contrast to the systemic and immunological unspecific treatments with anti-CD3 [18] and complete Freund's adjuvant [19] or the systemic treatment with Bacillus Calmette-Guérin vaccination [20]. These treatments have previously been shown to protect against diabetes development in the NOD mouse

In conclusion, we show that controlled repeated epicutaneous exposures to a contact allergen prevented the development of diabetes in the NOD mouse. The mechanism may be a peripheral up-regulation of NKT cells. This observation may lead to a new understanding of the integrated immune function and might lead to a new strategy for the prevention of type 1 diabetes.

## Acknowledgements

We are grateful for general financial support from Aage Bang's Foundation and The Capital Region's Research Foundation.

## Conflict of interest

The authors have no conflicts of interest.

## References

1. van der Werf N, Kroese FG, Rozing J, Hillebrands JL. Viral infections as potential triggers of type 1 diabetes. *Diabetes Metab Res Rev* 2007; **23**(3): 169–183.
2. Cavani A, De PO, Girolomoni G. New aspects of the molecular basis of contact allergy. *Curr Opin Allergy Clin Immunol* 2007; **7**(5): 404–408.
3. Xu H, Dilulio NA, Fairchild RL. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of

- cytokine production: interferon gamma-producing (Tc1) effector CD8+ T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulatory CD4+ T cells. *J Exp Med* 1996; **183**(3): 1001–1012.
4. Kehren J, Desvignes C, Krasteva M, *et al.* Cytotoxicity is mandatory for CD8(+) T cell-mediated contact hypersensitivity. *J Exp Med* 1999; **189**(5): 779–786.
  5. Saint-Mezard P, Berard F, Dubois B, Kaiserlian D, Nicolas JF. The role of CD4+ and CD8+ T cells in contact hypersensitivity and allergic contact dermatitis. *Eur J Dermatol* 2004; **14**(3): 131–138.
  6. Vocanson M, Hennino A, Cluzel-Tailhardat M, *et al.* CD8+ T cells are effector cells of contact dermatitis to common skin allergens in mice. *J Invest Dermatol* 2006; **126**(4): 815–820.
  7. Engkilde K, Menné T, Johansen JD. Inverse relationship between allergic contact dermatitis and type 1 diabetes mellitus: a retrospective clinic-based study. *Diabetologia* 2006; **49**(4): 644–647.
  8. Engkilde K, Buschard K, Hansen AK, Menne T, Johansen JD. Prevention of diabetes in NOD mice by repeated exposures to a contact allergen inducing a sub-clinical dermatitis. *PLoS One* 2010; **5**(5): e10591.
  9. Poulton LD, Baxter AG. Clinical application of NKT cell assays to the prediction of type 1 diabetes. *Diabetes Metab Res Rev* 2001; **17**(6): 429–435.
  10. Cardell SL. The natural killer T lymphocyte: a player in the complex regulation of autoimmune diabetes in non-obese diabetic mice. *Clin Exp Immunol* 2006; **143**(2): 194–202.
  11. Campos RA, Szczepanik M, Lisbonne M, Itakura A, Leite-De-Moraes M, Askenase PW. Invariant NKT cells rapidly activated via immunization with diverse contact antigens collaborate in vitro with B-1 cells to initiate contact sensitivity. *J Immunol* 2006; **177**(6): 3686–3694.
  12. Gober MD, Fischelevich R, Zhao Y, Unutmaz D, Gaspari AA. Human natural killer T cells infiltrate into the skin at elicitation sites of allergic contact dermatitis. *J Invest Dermatol* 2008; **128**(6): 1460–1469.
  13. Dean JH, Twerdok LE, Tice RR, Sailstad DM, Hattan DG, Stokes WS. ICCVAM evaluation of the murine local lymph node assay. Conclusions and recommendations of an independent scientific peer review panel. *Regul Toxicol Pharmacol* 2001; **34**(3): 258–273.
  14. Chen YG, Choisy-Rossi CM, Holl TM, *et al.* Activated NKT cells inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes. *J Immunol* 2005; **174**(3): 1196–1204.
  15. Cain JA, Smith JA, Ondr JK, Wang B, Katz JD. NKT cells and IFN-gamma establish the regulatory environment for the control of diabetogenic T cells in the nonobese diabetic mouse. *J Immunol* 2006; **176**(3): 1645–1654.
  16. Buschard K, Hansen AK, Jensen K, *et al.* Alcohol facilitates CD1d loading, subsequent activation of NKT cells, and reduces the incidence of diabetes in NOD mice. *PLoS One* 2011; **6**(4): e17931.
  17. Dey N, Szczepanik M, Lau K, Majewska-Szczepanik M, Askenase PW. Stimulatory lipids accumulate in the mouse liver within 30 minutes of contact sensitization to facilitate activation of naive iNKT cells in a CD1d-dependent fashion. *Scand J Immunol* 2011; **74**(1): 52–61.
  18. Kaufman A, Herold KC. Anti-CD3 mAbs for treatment of type 1 diabetes. *Diabetes Metab Res Rev* 2009; **25**(4): 302–306.
  19. Sadelain MW, Qin HY, Lauzon J, Singh B. Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. *Diabetes* 1990; **39**(5): 583–589.
  20. Gazda LS, Baxter AG, Lafferty KJ. Regulation of autoimmune diabetes: characteristics of non-islet-antigen specific therapies. *Immunol Cell Biol* 1996; **74**(5): 401–407.