Introduction
Vaccine adjuvants enhance the antigen-induced immune response by targeting the innate immune response. The discovery of several classes of pattern-recognition receptors (PRRs), mainly Toll-like receptors (TLRs) and lectin receptors (LRs), has shed light on the mechanisms responsible for the adjuvant-mediated amplification of interactions between antigen-presenting cells (APCs) and T-cells. Furthermore, particulated adjuvants, such as mineral salts and oil-in-water emulsions which are TLR and LR independent, have been shown to play a critical role in the recruitment of innate immune cells to the site of infection. Several adjuvants are of special interest. First of all, the TLR9 ligand CpG has been shown to induce a strong Th1 response in the presence of an antigen. Furthermore, the lectin receptor ligands beta-glucan and mannan from *C. albicans* have been found to predominantly induce Th17 responses. In addition to these LR- and TLR-dependent adjuvants, the widely used particulated adjuvant alum has been found to activate the NALP3 inflammasome, which is important for IL-1β and IL-18 processing. IL-1β has been shown to be important for the induction of Th17 cells and IL-18 will induce IFNγ, which is the prototypical cytokine of the Th1 response. Potential antigens that could be used for vaccination against *C. albicans* infections are mannoproteins derived from *C. albicans*. For tuberculosis, the antigens ESAT6 derived from *M. tuberculosis*, and BCG from *M. bovis* are of special interest. By combining these antigens with the specific TLR, LR or NALP3 adjuvants, we investigated which innate cytokine responses and which predominant T-helper cell response(s) will be induced, and whether this is dependent on the antigen to which the adjuvant is combined.

Hypothesis
Distinctive classes of adjuvants based on TLR, LR and NLRP3 ligands can differentially modulate antigen-induced T-helper responses.

Study results
**Adjuvants: Alum and CpG induce cytokines in human PBMCs in contrast to LR ligands.**

To determine whether adjuvants have intrinsic capacity to induce innate and T-helper cytokine production in human peripheral blood mononuclear cells, we stimulated PBMCs with Alum, CpG and the LR ligands beta-glucan and O-linked mannans derived form *Candida albicans*. None of the adjuvants induced TNF production, and only Alum induced IL-1β. CpG and Alum both induced IFNγ, whereas Alum was the only adjuvant able to induce IL-22. None of the adjuvants induced the Th1 cytokine IL-17, and the LR ligands did not induce any of the measured cytokines. In addition, IL-4, IL-23 and IL-12p70 were measured, however none of the conditions resulted in a detectable concentration of these cytokines.

**ESAT6-induced TNF is decreased or increased dependent on the adjuvant that is present.**

Innate cytokines, such as TNF and IL-1 are important for the host defense against *Candida* and *M. tuberculosis*. We investigated whether adjuvants have the capacity to alter innate cytokine responses in PBMCs stimulated with the antigens BCG, mp65 or ESAT6. TNF was not produced by any of the combinations of the adjuvants with BCG or mp65, however the adjuvants Alum and CpG had significant effects on ESAT6-induced TNF production (Figure 3). Strikingly, Alum decreased TNF production, whereas CpG increased TNF production. In response to IL-1β, CpG in combination with mp65 resulted in a trend towards higher IL-1β production, but this was not observed for BCG or ESAT6 (Figure 3). These data suggest that the production of innate cytokines, such as TNF can be influenced by adjuvants, and that dependent on the type of adjuvant this production can be increased or decreased.

**Alum can inhibit or augment Th17 responses, whereas CpG augments Th1 responses**

Th17 responses provide protection against mucosal *Candida* infections, whereas Th1 responses are important for protection against invasive *Candida* infection or tuberculosis. To determine whether TLR, LR and particulate adjuvants can shape the T helper response in a specific direction, we studied the capacity of PBMCs to produce the characteristic T-helper cytokines IL-17, IL-22, and IFNγ. BCG by itself did not induce any of the measured cytokines. The addition of LR ligands did not result in any production of IL-17, IL-22 or IFNγ. Alum together with BCG induced IL-22, but this concentration was similar to Alum alone. In contrast Alum + BCG resulted in synergism fro the production of IFNγ. CpG did not have an effect on cytokines in combination with BCG. The antigen derived from *C. albicans*, namely mp65 induced some production of IL-17, IL-22 and IFNγ by itself. Beta-glucan as an adjuvant did not have any significant effect on the mp65 induced cytokines, however the LR ligand O-mannan did result in a slightly higher production of IL-17 and IL-22, but not IFNγ. Alum however, strongly boosted the IL-17 and IL-22 production induced by mp65, but it had minimal effects on mp65 induced IFNγ. CpG did not have an effect on mp65 induced IL-22 or IL-17, but boosted the IFNγ production induced by mp65.

Similar to mp65, ESAT6 the antigen from *M. tuberculosis* induced IL-17, IL-22 and IFNγ by itself. However, adding Alum to this antigen resulted in a striking decrease of IL-17 production, while it had no effect on IL-22 production induced by ESAT6. CpG did not have an effect on ESAT6-induced IL-17 and IL-22, but boosted the production of IFNγ induced by ESAT6.

These data suggest that Alum can have different effects on the production of inflammatory T helper cytokines depending on the antigen with which Alum is present. In contrast CpG does not show different effects, but only seems to have effect on Th1 responses and when it has an effect it increases the characteristic cytokine of the Th1 response, namely IFNγ.

**Alum is able to induce master regulator genes of T helper cells**
Since we observed that Alum can have inhibiting as well as augmenting effects on T helper cytokine production induced by different antigens in contrast to the other adjuvants used in this study, we compared the capacity of Alum to modulate master genes of T helper subsets such as T-bet, RORγt, and GATA3, which are the master regulators of Th1, Th17 and Th2 respectively. Interestingly, Alum was able to induce the Th1 and Th2 gene regulators, which was not observed with beta-glucan, O-mannan, or CpG (Figure 5). Alum did not induce RORγt by itself. Only O-mannan showed an induction of RORγt, however this was only 1 donor. In all 4 donors Alum induced GATA3 expression.

Conclusions from the investigation

In the present study we demonstrate that adjuvants based on TLR ligands, such as CpG, or lectin-receptor ligands such as mannans and beta-glucans, are not potent as single ligands to induce monocyte-derived innate cytokines such as IL-1 and TNF as single ligands. In contrast the particle-based adjuvant Alum has the capacity to stimulate IL-1 by itself. The characteristic Th1 helper cytokine IL-17, was not induced by any of the adjuvants alone, while CpG and Alum can stimulate the Th1 cytokine IFNγ as single ligands. When the adjuvants were combined with the three antigens, namely BCG, mp65 and ESAT6, we observed that the innate cytokine response induced by an antigen can be augmented or inhibited dependent on the type of adjuvant used. This is underscored by the observation that ESAT6-induced TNF production is augmented by CpG, but inhibited by Alum. Furthermore, the same adjuvant can differentially modulate the T-helper response dependent on the type of antigen, since we observed that Alum augments IL-17 production induced by mp65, while Alum inhibits IL-17 production induced by ESAT6. These data provide evidence that it is possible to modulate in a rational manner the type of response, innate as well as adaptive, induced by a certain vaccine candidate by using a well-chosen ligand as adjuvant.

Whereas vaccine adjuvants have historically been selected on a trial-and-error basis, the present studies provide a proof-of-principle approach for a rational design of antigen/adjuvant vaccines. One very important question that remains unanswered to date is whether the cytokine profile induced by the adjuvant, and subsequently its polarizing capacity, is dependent on the antigen to which it is combined. Here we show that some adjuvants do not merely augment an immune response induced by an antigen but that they can modulate the immune response induced by the antigen. This is best illustrated by the adjuvant Alum. Despite the widespread use of alum in several vaccines over the past decades, its mechanism of action remained unknown until recently. Alum consists of aluminum salts that can be emulsified with the antigen and this substance has been reported to generally induce a Th2-biased response. Recently, it has been shown that that alum signals through the NLRP3 inflammasome. Thus, antigen presenting cells stimulated in vitro with alum plus a stimulus like LPS, induces IL-1β and IL-18 which is dependent on caspase-1 and NLRP3. However, the mechanisms by which alum induces Th2 responses still remain poorly understood. Moreover, this study provides new insights in the effects that alum can have on antigen induced T helper responses.

Th17 responses are crucial for anti-Candida host defense, especially for mucosal host defense. Furthermore, the Th1 response is important for preventing and controlling invasive disease caused by Candida. An optimal vaccine strategy to control Candida infections would ideally induce robust Th1 and Th17 responses. When we combined a mannanprotein from the cell wall of Candida, called mp65, with Alum we observed strongly augmented mp65-induced Th1 and Th17 responses. Thus, combining alum with an immunogenic cell wall protein from Candida could be protective. These results also provide a rationale to test the augmenting effects of alum on ESAT6, an antigen from M. tuberculosis, -induced Th1 and Th17 responses since these T helper responses are also crucial for optimal host defense against M. tuberculosis. However, the opposite was true, we observed not only that alum did not augment Th1 and Th17 responses induced by ESAT6, moreover we observed that alum inhibited significantly the Th17 response induced by ESAT6. This is in line with recent reports that have observed that alum is not effective in boosting Th1 and Th17 responses in vivo in ESAT6 based vaccines. Furthermore, we also demonstrate that alum not only inhibits Th17 responses induced by ESAT6, but also inhibits innate cytokine production induced by ESAT6, namely TNF, a crucial cytokine in the host defense against M. tuberculosis. These observations have important consequences. In shaping immune responses alum can augment or inhibit, and thus the effects of alum on antigen induced T helper responses needs to be carefully addressed before using it in vaccines in patients. Moreover, the capacity of alum to suppress completely TNF alpha production is an unexpected but highly relevant observation, since TNF blocking is detrimental in many infections, and this capacity of alum to suppress TNF production needs to be studied in more detail.

In conclusion, the present study underscores the need to study in more detail the interaction between adjuvants and antigens, since adjuvanticity has effects on both innate and adaptive immune responses and does not always augment responses, and can even inhibit optimal immune responses. This study has highlighted the unpredicted outcome of an immune response when antigen and adjuvants are combined, and has revealed that the adjuvant can have different effects dependent on the antigen to which it is combined.