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Running Title: Adenylate cyclase toxoid activates dendritic cells

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Abstract

The adenylate cyclase toxin-hemolysin (CyaA) of *Bordetella pertussis* is a bi-functional leukotoxin. It penetrates myeloid phagocytes expressing the complement receptor 3 and delivers into their cytosol its N-terminal adenylate cyclase enzyme domain (~400 residues). In parallel, the ~1300 residue-long RTX hemolysin moiety of CyaA forms cation-selective pores and permeabilizes target cell membrane for efflux of cytosolic potassium ions. The non-enzymatic CyaA-AC$^-$ toxoid, has repeatedly been successfully exploited as an antigen delivery tool for stimulation of adaptive T cell immune responses. We show that the pore-forming activity confers on the CyaA-AC$^-$ toxoid a capacity to trigger Toll like receptor and inflammasome signaling-independent maturation of CD11b-expressing dendritic cells (DC). The DC maturation-inducing potency of mutant toxoid variants *in vitro* reflected their specifically enhanced or reduced pore-forming activity and K^+ efflux. The toxoid-induced *in vitro* phenotypic maturation of DC involved the activity of mitogen activated protein kinases p38 and JNK and comprised increased expression of maturation markers, interleukin 6, chemokines KC and LIX and granulocyte-colony stimulating factor secretion, Prostaglandin E2 production and enhancement of chemotactic migration of DC. Moreover, intravenously injected toxoids induced maturation of splenic DC in function of their cell-permeabilizing capacity. Similarly, the capacity of DC to stimulate $CD8^+$ and $CD4^+$ T cell responses *in vitro* and *in vivo* was dependent on the pore-forming activity of CyaA-AC$^-$. This reveals a novel self-adjuvanting capacity of the CyaA-AC$^-$ toxoid that is currently under clinical evaluation as a tool for delivery of immunotherapeutic anti-cancer $CD8^+$ T cell vaccines into DC.

Introduction

The adenylate cyclase toxin-hemolysin (CyaA) is a 1706 residue long bi-functional leukotoxin that plays a central role in *Bordetella pertussis* virulence, particularly in the early phases of airway colonization [1, 2]. CyaA is endowed with a cell-invasive N-terminal adenylate cyclase (AC) enzyme domain (~400 residues) that is fused to a pore-forming RTX (Repeat in ToXin) 'cytolysin' (Hly) moiety of ~1300 residues. The Hly portion binds the complement receptor 3 (CR3, known as the $\alpha_M\beta_2$ integrin CD11b/CD18 or Mac-1) and penetrates the membrane of myeloid phagocytes [3, 4]. CyaA insertion into the cytoplasmic membrane of phagocytes then triggers influx of calcium ions [5] that activates calpain-mediated cleavage of talin [6]. This mobilizes the CyaA-CR3 complex for relocation into membrane microdomains (lipid rafts), from where the translocation of the AC domain across cellular membrane is completed [6, 7]. Inside cells the AC enzyme binds calmodulin and catalyzes unregulated conversion of ATP into the key signaling molecule cAMP, which ablates bactericidal activities of phagocytes [4]. In parallel, the Hly moiety of CyaA, which is functionally independent of the invasive AC domain, forms cation-selective pores and permeabilizes cellular membrane for efflux of cytosolic potassium ions from cells [7, 8]. The pore-forming activity of CyaA-AC$^-$ was then shown to synergize with Toll-like receptor (TLR) signaling in promoting NALP3 inflammasome complex assembly and IL-1β secretion in dendritic cells (DC) [9].

The cell-invasive AC domain of CyaA was shown to accommodate large polypeptide inserts without losing its capacity to penetrate cells [10, 11]. Therefore, non-enzymatic CyaA-AC$^-$ toxoids, having a variety of heterologous antigens inserted into, or in place, of the AC domain of CyaA, have been extensively exploited over the past two decades for delivery of antigens into CD11b$^+$ DC for processing and antigenic presentation to T cells. These recombinant CyaA-AC$^-$

constructs were, indeed, shown to induce potent antigen-specific cytotoxic $CD8^+$ T lymphocyte (CTL) responses against various viruses (e.g. HIV, CMV, LCMV, HPV or influenza), bacteria (*M. tuberculosis*), parasites (*P. berghei*), or tumors (melanoma or HPV-induced) [12, 13]. Building on these studies, three CyaA-derived toxoids currently reached the stage of phase I and II clinical trials in humans and are being evaluated as immunotherapeutic CTL vaccines for treatment of metastatic melanoma and papilloma virus-induced cervical cancer, respectively.

Besides inducing T cell immune responses through delivery of antigens for presentation into DC, the CyaA-AC^- toxoid was also shown to possess an inherent adjuvant capacity. CyaA-AC^- was shown to enhance antibody and T cell responses to co-administered *B. pertussis* antigens [14, 15]. Moreover, co-administration of LPS-free CyaA-AC^- with a diluted commercial pertussis vaccine was found to shift the polarization of the resulting immune response from a typical Th2 type to a mixed Th1/Th2 type of response [16]. This importantly improved the resulting protection of mice against intranasal challenge by *B. pertussis,* well beyond the contribution of anti-CyaA antibodies [16]. These observations indicated that the CyaA-AC^- toxoid might exert its adjuvant activity through targeting and activation of DC. Recently Dadaglio et al. showed that adjuvant activity of high doses of LPS-free CyaA-AC^- toxoid on DC depends on TLR4/TRIF signaling. The $CD8^+$ T lymphocyte response was, however, not completely abolished in $TLR4^{-/-}$ and $TRIF^{-/-}$ mice *in vivo* [17], suggesting that CyaA-AC^- may employ an additional adjuvant mechanism on DC to potentiate their capacity to induce T cell responses. Here we show that specifically at low toxoid concentrations, the CyaA-AC^- exerts a novel intrinsic adjuvant activity on DC *in vitro* and *in vivo*. This is independent of Toll like receptor (TLR)- and inflammasome-mediated signaling and depends on the pore-forming activity of the toxoid that causes K^+ efflux-mediated activation of p38 and JNK mitogen activated protein kinases (MAPKs).

Results

At low concentration the CyaA-AC$^-$ toxoid triggers CD80 and CD86 expression on BMDC *in vitro* by a mechanism that does not involve TLR or inflammasome-mediated signaling.

We first tested a minimal toxoid concentration required for efficient antigen delivery into DC for T cell stimulation *in vitro*, using toxoids that contained less than 0.12 EU of LPS per 1 μg of toxoid (<12 pg LPS/ 1 μg of CyaA-AC$^-$). Murine bone marrow-derived DC (BMDC) were incubated for 4 h with various concentrations (100 - 1000 ng/ml) of the CyaA-OVA-AC$^-$ toxoid carrying ovalbumin epitopes presented on MHC class I and II molecules. Cells were then washed, incubated with OVA-specific naïve CD8$^+$ (OT-I) or CD4$^+$ (OT-II) T cells and IL-2 production in supernatants of DC and T cell co-cultures was measured after 3 days. As shown in Fig. 1A, BMDC incubated with as low concentration of CyaA-OVA-AC$^-$ as 300 ng/ml displayed similar CD8$^+$ and CD4$^+$ T cell stimulatory capacity as BMDC incubated with the higher toxoid concentration of 500 or 1000 ng/ml. Given the very low endotoxin content of the toxoid preparations, this indicated that it was the activity of the toxoid itself that conferred a DC maturation-dependent T cell stimulatory capacity on the BMDC.

Since, CyaA-AC$^-$ at the highest concentration of 1000 ng/ml induced DC maturation (data not shown), we examined in detail whether as little as 300 ng/ml of CyaA-AC$^-$ would induce DC maturation and what signaling pathway(s) might be triggered by the toxoid. Towards this aim we used a set of BMDC derived from mice deficient in CD14, TLR 2, 4, 2/4 and 9, TLR adaptors TRIF (TIR-domain-containing adaptor-inducing interferon-β) and MyD88 (Myeloid differentiation primary response gene 88), or deficient in molecules participating in inflammasome and IL-1 signaling such as IL-1R, P2X7, ASC (apoptosis-associated speck-like protein containing a CARD) and NLRP3 (NOD-like receptor family, pyrin domain containing 3,

respectively. The BMDC were treated with 300 ng/ml of CyaA-AC$^-$ for 24h and analyzed by flow cytometry, using CD80 and CD86 expression as markers of maturation. When added into BMDC suspensions this CyaA-AC$^-$ dose (300 ng/ml) yielded a final endotoxin concentration of less than 0.036 EU/ml (e.g. <3.6 pg/ml of LPS). As a positive control DC maturation stimulus, a dose of 100 ng/ml of *E. coli* LPS was used (Fig. 1B). As however shown in Fig. 1C, the used LPS contained also traces of TLR2 ligands, inducing to some extent also maturation of TLR4-deficient BMDC. It, however, did not trigger maturation of BMDC deficient for both the TLR2 and TLR4 (TLR2/4) receptors (Fig. 1B and 1C). In contrast, the highly purified CyaA-AC$^-$ toxoid (300 ng/ml) triggered induction of CD80 and CD86 marker expression not only in the TLR2/4 knock-out BMDC, that were insensitive to LPS (Fig. 1B and 1C), but promoted also maturation of all other used knock-out BMDC that were deficient in the other TLR or inflammasome signaling components (Fig. 1C). This indicated that the CyaA-AC$^-$ toxoid used at 300 ng/ml possessed an intrinsic DC-activating capacity that did not depend on TLR- or inflammasome-mediated signaling pathways.

As however shown in Fig. 1D, at higher toxoid concentrations ranging from 1 to 3 μg/ml, a significantly higher expression of CD80, CD86, I-A/I-E and CD40 maturation markers was observed on DC generated from wild type mice (C57BL/6) than on DC from TLR2/4 KO mice. These results, hence, show that CyaA-AC$^-$ induces both TLR-dependent and TLR-independent DC maturation. The TLR-dependent maturation is then particularly triggered at high toxoid concentrations of ≥ 1 μg/ml, presumably via the TLR4/TRIF signaling mechanism reported previously[17].

Pore-forming activity-dependent K^+ efflux accounts for phenotypic maturation of BMDC exposed to low concentrations of the CyaA-AC$^-$ toxoid. The enzymatically inactive CyaA-AC$^-$

toxoids still possess the capacity to form pores in membranes of CD11b-expressing cells and permeabilizes cells for efflux of cytosolic potassium ions [9, 18, 19]. As K^+ efflux was shown to induce DC maturation [20], we examined whether it was the cell-permeabilizing activity of the CyaA-AC$^-$ toxoid at 300 ng/ml that induced maturation of BMDC. Towards this aim, advantage was taken of a previously characterized set of CyaA-AC$^-$ toxoids that have the specific pore-forming activity selectively enhanced (CyaA-KK-AC$^-$) or strongly reduced (CyaA-QR-AC$^-$) by specific residue substitutions in the pore-forming domain (Fig. 2A). These toxoids were purified close to homogeneity and all contained <120 EU of endotoxin/mg of toxoid. As shown in Fig. 2B, the CyaA-AC$^-$ toxoid and mutant CyaA-KK-AC$^-$, exhibiting an enhanced pore-forming activity, promoted importantly faster leakage of K^+ ions from BMDC than the CyaA-QR-AC$^-$ toxoid that exhibits a reduced pore-forming activity. While the resolution of the here used fluorescence probe-based method was insufficient for demonstrating the quantitative differences in the specific membrane-permeabilizing potencies of the CyaA-AC$^-$ and CyaA-KK-AC$^-$ toxoids at shorter time points after addition, this was previously established in planar lipid bilayer measurements and erythrocyte lysis experiments and was found to be in the order of CyaA-KK-AC$^-$ >> CyaA-AC$^-$ [18, 21]. As further shown in Fig. 2C, all purified toxoids bound BMDC *in vitro* and competed equally well for CR3 binding with the biotinylated intact CyaA toxin. As then shown in Fig. 2D, 300 ng/ml of the less pore-forming CyaA-QR-AC$^-$ toxoid did not trigger an upregulation of the maturation marker molecules on BMDC. In contrast, the intact CyaA-AC$^-$ toxoid at 300 ng/ml and the highly cell-permeabilizing CyaA-KK-AC$^-$ toxoid at 300 ng/ml, or as well as at 100 ng/ml, triggered significantly enhanced expression of the maturation molecules, such as the MHC class II molecules I-A/I-E, CD80, CD86 and CD40, respectively, which were upregulated to a similar extent then upon BMDC stimulation with 100 ng/ml of LPS (Fig. 2D). At the same time, the viability of BMDC at the end of incubation with LPS or with the CyaA-AC$^-$

and CyaA-QR-AC$^-$ toxoids at 300 ng/ml remained high (Fig.2E), while the increased cytotoxicity of the highly cell-permeabilizing CyaA-KK-AC$^-$ mutant toxoid could be mitigated by decreasing its working concentration to 100 ng/ml, as documented in Fig. 2E.

Since production of inflammatory molecules is a hallmark of the DC maturation process [22], we next used a cytokine array (Suppl. Fig. 1) to analyze inflammatory cytokine and chemokine production induced by CyaA-AC$^-$ in BMDC after 24 h of incubation. As shown in Fig. 2F, co-incubation with 300 ng/ml of CyaA-AC$^-$ enhanced several-fold the production of the IL-6 cytokine, of the chemokines KC (IL-8) and LIX, and of the granulocyte-colony stimulating factor (G-CSF), respectively. However, no enhancement of secretion of the main pro-inflammatory cytokines tumor-necrosis factor-α (TNF-α), IL-12 and IL-1β was observed. These results confirm that the used CyaA-AC$^-$ toxoid preparations were free of detectable TLR ligand contamination and that already at 300 ng/ml the CyaA-AC$^-$ toxoid induced phenotypic maturation BMDC through its pore-forming activity that causes K^+ efflux form cells.

Pore-forming activity of CyaA-AC$^-$ toxoid drives maturation marker expression in BMDC via activation of p38 and JNK MAPKs. Potassium efflux from cells can trigger MAPK activation [38-42] and expression of DC maturation markers was shown to be regulated by signaling involving MAPKs such as p38, JNK and ERK and NF-κB translocation into cell nucleus [22-26]. Therefore, 1h before addition of the CyaA-AC$^-$ toxoid (300 ng/ml), the BMDC were preincubated with 10 μM MAPK inhibitors, e.g. the MEK1/2 (ERK1/2) inhibitor PD98059 (PD), the p38 inhibitor SB203580 (SB), the JNK inhibitor SP600125 (SP) or the NF-κβ inhibitor BAY-11-7082 (BAY), respectively. After 24h of incubation with the toxoid, the expression of I-A/I-E, CD80 and CD86 molecules on CD11c$^+$Hoechst-negative cells was determined by flow cytometry. As

shown in Fig. 3A, a significant decrease of CyaA-AC$^-$-induced expression of I-A/I-E, CD80 and CD86 molecules was observed upon inhibition of p38 and JNK signaling, even if expression of I-A/I-E was not statistically different in the presence of the p38 inhibitor. The enhancement of expression of I-A/I-E, CD80 and CD86 molecules by CyaA-AC$^-$ treatment was, however, not affected in BMDC treated with MEK1/2 (ERK1/2) and NF-κβ inhibitors.

To investigate if the pore-forming activity of CyaA-AC$^-$ (K^+ efflux) accounted for the activation of p38 and JNK signaling in BMDC, cells were incubated with LPS or CyaA-AC$^-$ and its mutant variants CyaA-QR-AC$^-$ and CyaA-KK-AC$^-$, exhibiting reduced or enhanced pore-forming activity, respectively. The amounts of phosphorylated and total JNK and p38 kinases were detected by immunoblots after 30, 90 or 180 min of cell exposure to toxoids (Fig. 3B). Quantification of the signals (Fig. 3C) of phospo-MAPK-specific antibodies then revealed that both JNK and p38 phosphorylation was activated above the background levels only by the pore-forming CyaA-AC$^-$ and CyaA-KK-AC$^-$ toxoids that were capable to provoke K^+ efflux from cells, but not by the CyaA-QR-AC$^-$ toxoid. These findings suggest that the cell-permeabilizing activity and K^+ efflux induced by CyaA-AC$^-$ activated JNK and p38 MAPK signaling and this mediated expression of co-stimulatory molecules on BMDC.

Pore-forming activity of CyaA-AC$^-$ promotes chemotactic migration of BMDC through p38 and JNK MAPKs activation. Activated DC migrate *in vivo* from the periphery into the lymphoid tissue [27]. This involves enhanced expression of the CCR7 receptor of CCL19 and CCL21 chemokines [28] and enhanced production of prostaglandin E2 (PGE2) that contributes to activation of the CCR7 receptor [29]. We thus analyzed if activation by CyaA-AC$^-$ toxoid (300 ng/ml) also enhanced the migratory capacity of BMDC. As shown in Fig. 4A, enhancement of production of PGE2 was observed with BMDC exposed to the pore-forming CyaA-AC$^-$ but not

upon treatment with low-permeabilizing CyaA-QR-AC$^-$ toxoid. The same pattern was observed also for CCR7 expression (Fig. 4B). This could then be inhibited by pre-treatment of BMDC with JNK and p38 inhibitors prior to CyaA-AC$^-$ addition (Fig. 4C). Finally, as shown in Fig. 4D, activation of BMDC for 24 h by 300 ng/ml of CyaA-AC$^-$, but not by CyaA-QR-AC$^-$, enhanced significantly the capacity of BMDC to migrate from the upper chamber across the transwell plate membrane into the lower chamber medium that contained chemokines CCL19 or CCL21. As documented in Fig. 4E, the p38 and JNK inhibitors interfered with CyaA-AC$^-$ toxoid-induced migration towards both cytokines. Hence, cell permeabilization by CyaA-AC$^-$ toxoid enhanced the migratory capacity of BMDC by a mechanism involving p38 and JNK signaling.

Pore-forming capacity of CyaA-AC$^-$ toxoid induces maturation of splenic DC and expansion of antigen-specific CD8$^+$ and CD4$^+$ T cells *in vivo.* To corroborate that the CyaA-AC$^-$ toxoid can promote maturation of DC also *in vivo*, 25 μg of the CyaA-AC$^-$ or CyaA-QR-AC$^-$ toxoids were intravenously injected into mouse tail vein in PBS with 1M urea and the same buffer was used in control mice. Splenocytes were isolated 24h later and the expression of maturation markers I-A/I-E, CD80, CD86 and CD40 was assessed by flow cytometry, splitting the lymphoid-tissue resident CD11c$^+$ DC by gating into the CD8$^+$CD11b$^-$ and CD8$^-$CD11b$^+$ subpopulations [30]. As documented by representative dot plots in Fig. 5A, both toxoids similarly decreased the percentage of CD8$^-$CD11b$^+$ cells by 13-15 %, as compared to control mock-treated mice. This likely reflected the internalization of the CD11b receptor resulting from toxoid binding. However, the percentage of CD8$^+$CD11b$^-$ cells remained similar among all experimental groups. Quantification of I-A/I-E, CD80, CD86 and CD40 expression in both splenic DC subpopulations is shown in Fig. 5B. The expression of CD80, CD86 and CD40, but not of I-A/I-E molecules, was significantly enhanced on CD8$^-$CD11b$^+$ DC from mice injected with CyaA-AC$^-$

and CD86 and also to a lower extent on cells from mice that received the CyaA-QR-AC$^-$ toxoid, but not on cells from control mice that received only buffer. Moreover, a significant increase of only CD86, but not of the other markers was observed on CD8$^+$CD11b$^-$ DC lacking the CR3 receptor of CyaA. This shows that as expected, the CyaA-AC$^-$ and CyaA-QR-AC$^-$ toxoids acted predominantly on CD11c$^+$CD8$^-$CD11b$^+$ DC *in vivo*, with a potency reflecting their respective cell-permeabilizing capacities.

Therefore, we investigated if DC maturation induced by pore-forming activity of CyaA-AC$^-$ impacted on T cell stimulation *in vitro* and *in vivo*. BMDC were incubated with CyaA-OVA-AC$^-$ or the non-permeabilizing CyaA-OVA-QR-AC$^-$ toxoid for 24h. The cells were then washed and OVA-specific CD8$^+$ (OT-I) and CD4$^+$ (OT-II) T cells were added for 3 days. As shown in Fig. 5C BMDC treated with CyaA-OVA-AC$^-$ stimulated higher IL-2 production in both CD8$^+$ and CD4$^+$ T cells than BMDC incubated with low pore-forming mutant CyaA-OVA-QR-AC$^-$. Similarly, as shown in Fig. 5D, in contrast to BMDC treated with low-permeabilizing CyaA-OVA-QR-AC$^-$ toxoid, the BMDC treated *ex vivo* with CyaA-OVA-AC$^-$ for 24h and subsequently injected into mice were capable of expanding adoptively transferred CD8$^+$ T cells. These data suggest that pore-forming activity of CyaA-AC$^-$ endowed DC with a higher T cell stimulatory capacity *in vitro* and *in vivo*. Finally, we tested if this activity translated directly into a higher capacity of CyaA-OVA-AC$^-$ to induce antigen-specific CD8$^+$ and CD4$^+$ T cell responses *in vivo* at a dose of 25 μg of the toxoid. This reduced dose was used here because at the previously used higher dose of 50 μg of the same toxoids, no difference in T cell inducing potency of the two toxoids was observed in an *in vivo* killing assay, likely due to saturation of the response [20]. Here, CyaA-OVA-AC$^-$ or CyaA-OVA-QR-AC$^-$ were injected at 25 μg *i.v.* into mice 1 day after the adoptive transfer of OVA-specific CD8$^+$ or CD4$^+$ T cells and T cell expansion was detected 4

days later by flow cytometry. As shown in Fig. 5E and 5F, CyaA-OVA-AC$^-$ induced significantly higher expansion of OVA-specific CD8$^+$ and CD4$^+$ T cells.

Discussion

In this study we identified a novel intrinsic adjuvant activity of CyaA-AC$^-$. We show that non-enzymatic but pore-forming CyaA-AC$^-$ toxoid at as low concentration as only 300 ng/ml induces maturation of CD11b-expressing DC by a mechanism that is independent of TLR 2, 4 and 9, CD14, TRIF, MyD88 or inflammasome signaling. The CyaA-AC$^-$-induced DC maturation depended on the specific pore-forming capacity of the toxoid that permeabilized cells and induced K^+ efflux that triggered activation of the JNK and p38 MAPKs. We further show that the adjuvanting pore-forming activity of CyaA-AC$^-$ on DC contributes to the potency of the toxoid in induction of T cell responses both *in vitro* and *in vivo*.

We observed that as low amounts as 300 ng/ml of CyaA-AC$^-$ carrying antigenic ovalbumin T cell epitopes endowed BMDC with a capacity to effectively stimulate CD8$^+$ and CD4$^+$ T cell responses *in vitro.* At this toxoid concentration, the CyaA-AC$^-$ induced phenotypic maturation of BMDC led to production of KC (IL-8) and IL-6 cytokines, of the chemokine LIX and of the differentiation factor G-CSF, respectively. Interestingly, all of these molecules regulate predominantly the activities of neutrophils that are important mediators of innate immunity. We have not detected production of the major pro-inflammatory cytokines such as TNF-α, IL-12p70 or IL-1β by toxoid-activated BMDC, which reflects the absence of TLR signaling of CyaA-AC$^-$ at the low toxoid concentrations. This was also confirmed by the capacity of the CyaA-AC$^-$ toxoid to trigger CD80 and CD86 expression in BMDC generated from TLR 2, 4, 2/4 and 9, TRIF, MyD88 and CD14 knock-out mice. These experiments clearly showed that if traces of

TLR ligands were still present in the used toxoid preparations, their levels were too low to activate the expression of costimulatory molecules in BMDC any importantly. Similarly, the expression of CD80 and CD86 induced in BMDC generated from mice deficient in inflammasome signaling proteins, such as IL-1R, P2X7, ASC and NLRP3, was not decreased. This shows that whereas CyaA pore formation-dependent K^+ efflux activates NLRP3 complex formation and stimulates IL-1β production under infection with *Bordetella pertussis*, there is no inflammasome activation by enzymatically inactive CyaA-AC^- alone in the absence of BMDC priming through TLR signaling [9]. Similarly, there was no involvement of inflammasome signaling in the $CD8^+$ T cell stimulatory adjuvant activity of CyaA-AC^- *in vivo*, as shown in the recent study of Dadaglio et al. [17]. We identified here that K^+ efflux induced by CyaA-AC^--mediated cell permeabilization acts as a major trigger for CyaA-AC^--induced BMDC maturation and migration. This goes well with the published effects of K^+ and Ca^{2+} ion signaling on induction of DC maturation and migration towards CCL21 [20, 31]. Of note, Ca^{2+} influx, which accompanies the translocation of AC domain into the cells [5-7], must not necessarily play a significant role in CyaA-AC^--induced BMDC maturation. Indeed, the highly pore-forming toxoid CyaA-KK-AC^- triggers very little Ca^{2+} influx [5], while induced efficient BMDC maturation. Conversely, the low pore-forming mutant CyaA-QR-AC^- did not stimulate BMDC's maturation and migration, despite inducing Ca^{2+} influx into the cells to a similar extent as the wild type toxoid CyaA-AC^- [6]. Nevertheless, some cooperation between Ca^{2+} and K^+ signaling in CyaA-AC^--mediated DC maturation cannot be fully excluded at present.

The activation of p38 and JNK signaling pathways by CyaA-AC^- is in agreement with the role of stress-activated p38 and JNK MAPKs in numerous processes underlying induction of adaptive immune responses, including DC maturation and chemotactic migration [32]. Whereas *in vitro* CD80 and CD86 molecules seem to be regulated by CyaA-AC^- pore-dependent activation of

both kinases, the expression of I-A/I-E may be predominantly regulated only by the JNK kinase. This would go well with a described differential regulation of DC maturation markers [33, 34] but it would not explain the lack of I-A/I-E expression on splenic DC that is observed after *in vivo* toxoid administration. Hence, a more complex regulation of DC maturation may occur under *in vivo* conditions. In our study, p38 and JNK activation was also involved in upregulation of CCR7 and enhancement of chemotactic migratory capacity of the maturing BMDC towards CCL19 and CCL21, since inhibition of these signaling pathways abolished CyaA-AC$^-$-mediated induction of BMDC migration. This is in agreement with previous reports that p38 and JNK kinases are involved in DC migration [27, 35, 36]. p38 activity triggers synthesis and secretion of PGE2 [37] and this might explain the observed enhancement in PGE2 production induced by CyaA-AC$^-$ in BMDC. Interestingly, K^+ efflux from cells permeabilized by pore-forming toxins (PFTs) was recently shown to trigger specifically the phosphorylation of p38 [38] and JNK kinases [39]. This appears to reflect a highly conserved mechanism of cellular stress response to PFT attack on cell membrane integrity, which is found in nematodes, insects and mammals [40-42].

Over the past 20 years, various CyaA toxoids were used without any added adjuvant to successfully deliver antigens into cytosol of dendritic cells for induction of potent protective anti-viral and anti-tumor CD8$^+$ T cell-mediated immunity in mice [13]. It is, therefore, plausible to propose that it is the self-adjuvanting capacity of the CyaA-AC$^-$ toxoid, i.e. its capacity to trigger maturation and migration of DC, which accounts for its potent T cell stimulatory capacity *in vivo*. We show here that the intravenously administered CyaA-AC$^-$ toxoid (25 µg) strongly enhances CD86 expression and upregulates CD80 and CD40 molecules on splenic CD11c$^+$CD8$^-$CD11b$^+$ DC *in vivo*. Intriguingly, at a dose of 25 µg per mice, the CyaA-QR-AC$^-$ toxoid was still capable to rather efficiently trigger a lower but still significant expression of CD86 on splenic CD11c$^+$CD8$^+$CD11b$^-$ DC, despite its reduced cell-permeabilizing activity (c.f. Fig. 5). We cannot

exclude that at the high CyaA-QR-AC$^-$ concentration its residual pore-forming activity may still promote enough K^+ efflux from DC to prime CD86 expression *in vivo*.

Recently, Dadaglio et al. showed that after the intravenous application of a higher dose of 50 μg of LPS-free CyaA-Tyr toxoid the permeabilization-primed maturation of splenic DC is largely potentiated by signaling through the TLR4/TRIF pathway [17]. This signaling may result from a direct interaction/clustering of toxoid-CD11b/CD18 complexes with TLR4 receptors in the membranes of CD11b$^+$ DC. In keeping with these results, we show here that CyaA-AC$^-$ at concentrations 0.1 - < 1 μg/ml induces DC maturation solely via potassium efflux, whereas at concentrations ≥ 1 μg/ml, the CyaA-AC$^-$ induces DC maturation through both the potassium efflux-triggered and the TLR4-dependent mechanisms, presumably operating in synergy. This is most likely potentiating the induction of antigen-specific T cell responses by CyaA toxoids. Indeed, the induction of antigen-specific CD8$^+$ T cell response after administration of CyaA-AC$^-$ carrying ovalbumin epitope SIINFEKL was shown to depend only in part of the TLR4/TRIF signaling triggered by the toxoid [17]. This is explained by our present observation that the pore-forming activity of CyaA-AC$^-$ is necessary for toxoid-treated BMDC to induce T cell responses. We show here, indeed, that BMDC treated with low concentration of wild type CyaA-OVA-AC$^-$ (300 ng/ml), but not with the less pore-forming toxoid CyaA-OVA-QR-AC$^-$, were able to induce CD8$^+$ and CD4$^+$ T cell responses *in vitro* and expanded adoptively transferred CD8$^+$ T cells *in vivo*. Moreover, we observed a significantly higher expansion of adoptively transferred OVA-specific CD8$^+$ and CD4$^+$ T cells after administration of 25 μg/ml of CyaA-OVA-AC$^-$ in comparison to administration of CyaA-OVA-QR-AC$^-$. This confirms the higher stimulatory activity of the pore-forming wild type toxoid towards antigen presenting cells *in vivo*. Based on these findings we propose a concentration-dependent model of adjuvant activity of the

immunotherapeutically relevant CyaA-AC$^-$ toxoid (Fig. 6). It predicts that at low toxoid concentrations the cell permeabilizing activity provokes K^+-efflux-mediated p38 and JNK activation, triggering DC maturation, which is largely potentiated by TLR4/TRIF signaling upon high level of toxoid binding to cells.

In conclusion, the present study demonstrates that on top of the previously described ability to deliver passenger antigens into cytosol of DC for processing and subsequent presentation on surface MHC molecules to T cells, the capacity of the CyaA-AC$^-$ toxoid to permeabilize DC triggers their maturation. This is then required for efficient antigen-specific priming of T cells to induce anti-tumor or anti-viral immunity [13]. The CyaA-AC$^-$ toxoid was further shown to induce a prominently Th1-polarized type of immune responses [43, 44]. This observation is potentially relevant also for the use of the toxoid as a new antigen and adjuvant in the next generation of acellular pertussis vaccines. Indeed, shifting of the predominantly Th2-polarized immune response to currently used acellular pertussis vaccines towards induction of a more Th1/Th17-polarized responses appears to be highly desirable in view of induction of longer-lasting and more efficient protective immunity against *B. pertussis* infection [45, 46].

Methods

Production and purification of CyaA-AC$^-$ and its mutants. The CyaA-AC$^-$ toxoid is devoid of adenylate cyclase enzyme activity due to insertion of the GlySer dipeptide between residues 188 and 189 in the ATP binding site of the AC domain [47]. The toxoid variants used in this study were CyaA-E570Q-K860R-AC$^-$ (abbreviated CyaA-QR-AC$^-$), a mutant with strongly reduced pore-forming activity and CyaA-E509K-E516K-AC$^-$ (abbreviated CyaA-KK-AC$^-$) a mutant with an increased pore-forming activity [5, 18, 19, 21]. CyaA-AC$^-$ and low-permeabilizing CyaA-QR-AC$^-$

toxoids carrying MHC class I epitope from chicken egg ovalbumin SIINFEKL (CyaA-SIINFEKL-AC$^-$) inserted in position of 233 of AC domain are described in [17, 19]. In this study we constructed a new CyaA-OVA-AC$^-$ toxoid and low-permeabilizing CyaA-OVA-QR-AC$^-$ which carry a polyepitope consisting of 3 chicken egg ovalbumin epitopes: $OVA_{257-264}$ (SIINFEKL) for MHC class I and $OVA_{323-339}$ (ISQAVHAAHAEINEAGR) and $OVA_{258-276}$ (IINFEKLTEWTSSNVMEER) for MHC class II presentation (CyaA-OVA-AC$^-$), respectively, inserted at position 233 of the AC domain. CyaA-AC$^-$ derived toxoids were produced in *Escherichia coli* BL21/pMM100(*lacI*^{q}) and were purified close to homogeneity as described previously [48, 49]. The endotoxin content in all CyaA samples was determined by the Limulus amebocyte lysate assay (QCL-1000; Cambrex) according to the manufacturer's instructions and was below 120 EU/mg.

Cell binding of CyaA-AC$^-$ and its mutants. The competitive CyaA binding assay was performed as described elsewhere [50, 51]. Briefly, BMDC (5 x 10^5 per well) in 100 µl of DMEM with 1% FCS were preincubated with different concentrations of various toxoids for 30 min on ice. Then, the competitor CyaA-biotin (30 nM) was added for another 30 min. Finally, unbound proteins were removed by repeated washing, and cells were stained with PE-conjugated streptavidin (Exbio) at 1:400 dilution (50µl/well) for 30 min on ice. The amount of surface bound CyaA-biotin was then determined by flow cytometry using a FACS LSR II instrument (BD Biosciences) and FlowJo version 7.2.1 (Treestar, Inc.). Results are expressed as the percent of CyaA-biotin binding, which means: CyaA-biotin bound in the presence of the competitor/maximal CyaA-biotin binding in the absence of the competitor CyaA x 100 (%).

Potassium efflux. Fluorescence measurement of cytosolic K^+ was performed as described previously [19]. Briefly, BMDC were loaded with 9.5 μM PBFI/AM (Molecular Probes) for 30 min at 25°C in the presence of 0,05% (w/w) Pluronic F-127 (Sigma-Aldrich) in the dark. Fluorescence intensity of PBFI (excitation wavelength 340, emission wavelengths 450 and 510 nm) was recorded, ratio of these intensities are shown in the graphs.

Mice. 6-12 weeks old C57BL/6 (Ly5.2) mice were obtained from a breeding colony at the Institute of Physiology of ASCR in Prague, Czech Republic. C57BL/6 (Ly5.1) and OT-I mice were provided by Marek Kovar, Institute of Microbiology of ASCR v.v.i. Prague, Czech Republic. OT-II mice were generous gift of Pavel Otahal, Institute of Molecular Genetics of ASCR, Prague, Czech Republic. Mice deficient in P2X7 [52], ASC [53] and NLRP3 [54] were generous gift of Prof. Stefan F. Martin, University Medical Center, Freiburg, Germany. Mice deficient for TLR2 [55], TLR4, TLR2/4 [56], TLR9 [57], TRIF [58], MyD88 [59], CD14 [60], IL-1R [61], P2X7, ASC and NLRP3 [62] and their corresponding WT C57BL/6 and C57BL/10 progenitor mice were bred under specific pathogen-free conditions in the animal facilities of the Max Planck Institute of Immunobiology and Epigenetics and the University Medical Center, Freiburg, Germany where all of the experimental procedures were performed in accordance with institutional, state and federal guidelines on animal welfare and every effort was made to minimize suffering. The animal experiments were approved by the Regierungspräsidium Freiburg and supervised by the Animal Protection Representatives of the University Freiburg Medical Center or the MPI. Mice were anesthetized before sacrificing with 1% pelltobarbitalum natricum at the dose of 10 mg/kg. All animal experiments were also approved by the competent Animal Welfare Committee of the Institute of Microbiology of the ASCR v.v.i. and handling of animals was performed according to the Guidelines for the Care and Use of Laboratory Animals, the Act of the Czech National

Assembly, Collection of Laws No. 149/2004, inclusive of the amendments, on the Protection of Animals against Cruelty, and Public Notice of the Ministry of Agriculture of the Czech Republic, Collection of Laws No. 207/2004, on care and use of experimental animals.

Generation of bone marrow-derived dendritic cells. Bone marrow-derived dendritic cells (BMDC) were generated according to the method of Lutz [63]. Bones for isolation of BMDC of knock-out and corresponding wild type mice were prepared at the Max Planck Institute of Immunobiology and Epigenetics and the University of Freiburg Medical Center and shipped on ice. Briefly, bone marrow cells were flushed from femurs and tibias of mice, and cultured at 2×10^6/ml in 100-mm dishes in 10 ml of RPMI 1640 medium supplemented with 10 % FCS (Life Technologies), 0.1 mg/ml streptomycin, 1000 U/ml penicillin and 0.25 μg/ml amphotericin (Sigma-Aldrich), 50 μM 2-mercaptoethanol, 1 % non-essential amino acids (Biochrom), 1 mM sodium pyruvate, 2 mM glutamine and 200 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). Fresh medium was added on day 3 or changed on day 6. Loosely attached cells were used for experiments at days 6 to 8. 70-80 % of cultured cells expressed CD11c (APC-anti-mouse-CD11c antibody, clone N418, eBioscience) and 90 % CD11b (PE-anti-mouse-CD11b antibody, clone M1/70, BD Pharmingen) [2]. Prior to all experiments with CyaA toxoids the RPMI medium used for cultivation of BMDC was replaced by DMEM medium to avoid uncontrollable chelation of calcium ions by the phosphate ions contained in RPMI medium, as calcium is required for the activity of CyaA. BMDC were allowed to rest in DMEM at 37 °C in 5% CO_2 atmosphere for at least 1 h before performing experiments.

Antibodies and reagents. LPS from *E. coli* serotype 0111:B4, MAPK inhibitors MEK1/2 (ERK1/2) (PD98059), p38 (SB203580) or JNK (SP600125) and NF-κβ inhibitor BAY-11-7082

were obtained from Sigma Aldrich. The following antibodies were used: APC-anti-mouse CD11c (clone N418), FITC-anti-mouse-I-A/I-E (clone M5/114.15.2), FITC-anti-mouse-CD80 (clone 16-10A1), FITC-anti-mouse-CD86 (clone GL1), PE-anti-mouse CCR7 (CD197; clone 4B12), PE-anti-mouse-CD3 (clone 145-2c11), PE-Cy7-anti-mouse-CD11b (clone M1/70) and PerCP-Cy5.5-anti-mouse-CD8a (53-6.7) from eBioscience or FITC-anti-mouse-CD40 (clone 3.23) and Alexa700-anti-mouse-CD8 (53-6.7), PerCP-anti-mouse-CD4 (RM4-5) and APC-anti-mouse-Ly5.2 (CD45.2 clone 104) from BD Pharmingen. Phospho-p38 (28B10), p38, phospho-SAPK/JNK (G9) and SAPK/JNK antibodies were obtained from Cell Signaling Technology.

Detection of BMDC's viability. BMDC (1×10^6/ml) in DMEM at 37 °C in 5% CO_2 atmosphere were left untreated or were treated with LPS (100 ng/ml) or with CyaA-AC$^-$, CyaA-QR-AC$^-$ (300 ng/ml) or CyaA-KK-AC$^-$ (300 or 100 ng/ml), respectively. After 24h of incubation, the viability of BMDC was detected with Hoechst 33258 (0.5 μg/ml; Invitrogen) by flow cytometry as a percentage of CD11c$^+$Hoechst 33258-negative cells.

BMDC's maturation markers and CCR7 expression. BMDC (1×10^6/ml) were left untreated or were treated with LPS (100 ng/ml) or with CyaA-AC$^-$, CyaA-QR-AC$^-$ (300 ng/ml) or CyaA-KK-AC$^-$ (100 ng/ml) for 24h in DMEM at 37 °C in 5% CO_2 atmosphere. In some experiments BMDC were pre-treated with MAPK and NF-κβ (10 μM) inhibitors for 1 h before addition of toxoids. After 24h the expression of I-A/I-E, CD80, CD86, CD40 and CCR7 on CD11c$^+$Hoechst33258-negative cells was detected by flow cytometry. Mean fluorescence intensity (MFI) values of samples were normalized as fold change of marker expression of untreated control BMDC.

Cytokine and Prostaglandin E2 production. For inflammatory cytokine and chemokine detection BMDC (1×10^6/ml) were left untreated or were incubated with CyaA-AC$^-$ at 300 ng/ml. After 24 h the level of cytokines and chemokines in culture supernatants was assessed by Mouse inflammation antibody array kit (RayBiotech) according to manufacturer's instructions. The chemiluminescence was detected using LAS-1000 (Luminiscence Analyzing System, Fuji) and analyzed by AIDA 1000/1D Image Analyzer software, version 3.28 (Raytest Isopenmessgeraete GmbH). The final graph shows the ratio of relative production of cytokines and chemokines induced by CyaA-AC$^-$ that were normalized to the background production of untreated control BMDC. For Prostaglandin E2 (PGE2) detection, the BMDC ($1x10^6$/ml) were left untreated, or were treated with LPS (100 ng/ml) or CyaA-AC$^-$ or CyaA-QR-AC$^-$ (300 ng/ml) for 24 h. Culture supernatants were collected and the amount of PGE2 was detected using the PGE2 EIA Kit (Cayman Chemicals) according to manufacturer's instructions.

Western blot analysis of p38 and JNK MAPK activation. BMDC ($1x10^6$/ml) were left untreated, or incubated with LPS (100 ng/ml) or with CyaA-AC$^-$, CyaA-QR-AC$^-$ or CyaA-KK-AC$^-$ for 30, 90 and 180 min. The BMDC were placed on ice and lysed for 10 min with 100 µl lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 50 mM NaF, 10 nM Calyculin A and Complete Mini protease inhibitors (Roche). The samples were boiled with Laemmli buffer for 5 min and analyzed by Western blot using specific antibodies recognizing phosphorylated and total JNK and p38, respectively. The signal of secondary HRP-conjugated antibody (GE Healthcare) was detected by the West Femto Maximum Sensitivity Substrate (Pierce) on LAS-4000 (Luminiscence Analyzing System, Fuji) and quantified by AIDA 1000/1D Image Analyzer

software as the ratio of signal of phosphorylated p38/JNK to non-phosphorylated total p38/JNK, respectively.

BMDC migration. BMDC ($1x10^6$/ml) were left untreated, or were incubated with 100 ng/ml LPS or with CyaA-AC$^-$ or CyaA-QR-AC$^-$ (300 ng/ml). In some experiments BMDC were pre-treated with MAPK inhibitors (10 μM) for 1 h before addition of toxoids. After 24h cells were washed and aliquots of 1×10^5 BMDC were transferred to the upper chambers of 96-well Transwell cell culture plates with 5-μm pore size polycarbonate filters (Corning Costar). The lower chambers of the Transwell plates were filled with DMEM medium with or without 200 ng/ml of chemokine CCL19 or CCL21 (Peprotech). BMDC were allowed to migrate for 4h and transmigrated cells were subsequently stained for viability by Hoechst 33258 and counted by flow cytometry. The number of transmigrated mock-treated BMDC was set to 1 (migration index).

Splenic DC maturation *in vivo*. C57BL/6 mice were intravenously injected into tail vein with PBS buffer with 1M urea (control), or with 25 μg of CyaA-AC$^-$ or CyaA-QR-AC$^-$ toxoids diluted in PBS with 1M urea. After 24h mice were sacrificed, the spleens were removed and cut into pieces and dissociated with Colagenase D (1 mg/ml) for 30 min at 37°C. Cellular suspensions were filtered through a 70 μm cell strainer and erythrocytes were lysed using ACK Lysing buffer (Life Technologies) for 10 min. Cell suspensions were centrifuged for 5 min at 300 x g and filtered through a 30 μm strainer. The expression of maturation markers I-A/I-E, CD80, CD86 and CD40 was detected on living (Hoechst 33258-negative) CD3$^-$CD11c$^+$CD8$^-$CD11b$^+$ or CD3$^-$ CD11c$^+$CD8$^+$CD11b$^-$ subpopulations of splenic DC by flow cytometry.

T cell expansion *in vitro* and *in vivo.*

For T cell expansion *in vitro*, the BMDC ($5x10^4$/sample) were left untreated or were incubated with CyaA-OVA-AC^- or CyaA-OVA-QR-AC^- (300 ng/ml) for 24h, repeatedly washed and co-incubated for 3 days with naïve OVA-specific $CD8^+$ (OT-I) or $CD4^+$ (OT-II) T cells ($2x10^5$/sample). T cells were isolated from lymph nodes and spleens of OT-I or OT-II transgenic mice by a magnetic cell separation using $CD8^+$ or $CD4^+$ T cell Isolation kits (Miltenyi), respectively. IL-2 production was determined from cell culture supernatants by ELISA. For T cell expansion *in vivo*, purified OVA-specific $CD8^+$ or $CD4^+$ T cells (Ly5.2) were injected intravenously (*i.v.*) into C57BL/6 recipients (Ly5.1) at 1.5×10^6 cells per mouse. 24h later mice were injected *i.v.* with 1.5×10^6 BMDC that had been pretreated with 300 ng/ml of CyaA-OVA-AC^- or CyaA-OVA-QR-AC^- for 24h or with 25 μg CyaA-OVA-AC^- or CyaA-OVA-QR-AC^- toxoids. As a positive control for T cell proliferation, mice were injected intraperitoneally (*i.p.)* with or $OVA_{257-264}$ peptide (SIINFEKL) + polyI:C (75 μg) or $OVA_{323-339}$ peptide (ISQAVHAAHAEINEAGR) + polyI:C (75 μg), respectively. As a negative control mice were injected with non-treated DC or PBS + urea (2M). Four days later, spleen cells were harvested, fixed by 4 % paraformaldehyde and the expansion of adoptively transferred $CD8^+Ly5.2^+$ or $CD4^+Ly5.2^+$ T cells, respectively, was detected by flow cytometry.

Statistical analysis. The significance of differences between groups was determined by the unpaired two-tailed Student's *t* test and ANOVA. Experimental data were compared to those obtained with the corresponding controls and differences were considered statistically significant if $p < 0.05$ (*), $p < 0.005$ (**) or $p < 0.001$ (***).

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Figure legends

Fig. 1. Low concentration of CyaA-AC$^-$ toxoid triggers CD80 and CD86 expression on BMDC *in vitro* by a mechanism that does not involve TLR- or inflammasome-mediated signaling. (a) BMDC (1 x 10^6/ml) were treated with indicated concentrations of CyaA-OVA-AC$^-$ toxoid carrying ovalbumin epitopes for MHC I and MHC II for 4 h, washed and subsequently added to OVA-specific CD8$^+$ T cells (OT-I) or CD4$^+$ T cells (OT-II). IL-2 was detected by ELISA in cell culture supernatant after 3 days. Values are representative ofthree independent experiments. **(b, c)** BMDC (1 x 10^6/ml) prepared from indicated knock-out mice were incubated with LPS (100 ng/ml) or CyaA-AC$^-$ toxoid (300 ng/ml) for 24 h. The expression of CD80 and CD86 molecules on CD11c$^+$Hoechst negative cells was determined by flow cytometry. **(b)** Histograms are representative of n=3. Grey histogram represents isotype control, grey line represents non-treated BMDC, black line represents LPS or CyaA-AC$^-$ treatment. **(c)** Values represent the means ± S.E from at least three independent experiments expressed as fold change of marker expression on mock-treated BMDC according to an appropriate genetic background from C57BL/6 or C57BL/10 mice) (* $p < 0.05$). **(d)** CyaA-AC$^-$ concentration-dependent induction of maturation of DC generated from wt C57BL/6 and TLR4 KO mice. Values represent the means ± S.E expressed as fold change of marker expression on mock-treated BMDC for each mouse strain (* $p < 0.05$).

Fig. 2. Pore-forming activity-dependent K$^+$ efflux determines the capacity of CyaA-AC$^-$ toxoid to trigger phenotypic maturation of BMDC. (a) CyaA-AC$^-$ toxoids and its mutants used in this study **(b)** Fluorescence measurement of cytosolic K$^+$ concentration elicited by 3 μg/ml of

indicated toxoids. BMDC were loaded with 9.5 μM PBFI/AM for 30 min at 25°C in the presence of 0.05% (w/w) Pluronic F-127 in the dark. Fluorescence intensity of PBFI (excitation wavelength 340, emission wavelengths 450 and 510 nm) was recorded, ratio of these intensities is shown in the graph. **(c)** Binding of indicated toxoids to BMDC was assessed as a competition for the CD11b/CD18 (CR3) receptor with biotinylated intact CyaA (CyaA-AC$^-$-biotin) and detected with streptavidin-PE by flow cytometry. Graph shows a percentage of CyaA-AC$^-$-biotin bound to BMDC in the presence of non-biotinylated CyaA-AC$^-$, CyaA-QR-AC$^-$ or CyaA-KK-AC$^-$ toxoids. **(d)** BMDC (1 x 10^6/ml) were left untreated, or were incubated with LPS (100 ng/ml) or with CyaA-AC$^-$, mutant toxoid with reduced pore-forming activity CyaA-QR-AC$^-$ or toxoid with enhanced pore-forming activity CyaA-KK-AC$^-$ at indicated concentrations for 24h. The expression of I-A/I-E, CD80, CD86 and CD40 was detected in CD11c$^+$Hoechst$^-$ cells by flow cytometry. Graphs represent the means ± S.E from n = 4 expressed as fold change of marker expression on mock-treated BMDC (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$). **(e)** Cell viability was determined as % of CD11c$^+$ Hoechst$^-$ cells. **(f)** BMDC (1 x 10^6/ml) were left untreated, or incubated with CyaA-AC$^-$ (300 ng/ml) for 24 h. The inflammatory cytokines and chemokines were evaluated from cell culture supernatant using cytokine array kit with chemiluminescence detection. Membranes are representative of n=3. Graph represent fold change of chemiluminiscent signal for each cytokine obtained in mock-treated BMDC (* $p < 0.05$, ** $p < 0.005$).

Fig. 3. Pore-forming activity-dependent K^+ efflux induced by CyaA-AC$^-$ toxoid drives maturation marker expression in BMDC via p38 and JNK MAPK activation. (a) BMDC (1x10^6/ml) were pre-incubated with the MEK1/2 (ERK1/2) inhibitor PD98059 (PD) p38 inhibitor SB203580 (SB), JNK inhibitor SP600125 (SP) or NF-κβ inhibitor BAY-11-7082 (BAY) all at 10

μM for 1 h before CyaA-AC$^-$ toxoid (300 ng/ml) was added. After 24 h of incubation the expression of I-A/I-E, CD80 and CD86 molecules on CD11c$^+$Hoechst$^-$ cells was determined by flow cytometry. Values represent the means ± S.E from n = 3 expressed as fold change of marker expression on mock-treated BMDC (* $p < 0.05$). **(b)** BMDC (1x10^6/ml) were incubated with LPS (100 ng/ml) or indicated CyaA-AC$^-$ mutants (300 ng/ml) for indicated time and the amount of phosphorylated and total MAPKs were detected by specific antibodies using Western blot analysis. The immunoblots are representative of n=3. **(c)** Relative JNK and p38 activation was quantified as P-JNK and P-p38 signal intensities normalized to the respective total JNK and p38 signal. Ratio of signals in lyzates of mock-treated BMDC at time point 180 min was set as 1. The signal intensity data show means ± S.E from n = 3 (* $p < 0.05$, ** $p < 0.005$).

Fig. 4. Pore-forming activity of CyaA-AC$^-$ promotes chemotactic migration of BMDC via JNK and p38 activation. BMDC (1x10^6/ml) were incubated with LPS (100 ng/ml) or CyaA-AC$^-$ or CyaA-QR-AC$^-$ mutant (300 ng/ml) for 24 h **(a, b, d)** or pre-incubated with the p38 inhibitor SB203580 (SB; 10 μM) or JNK inhibitor SP600125 (SP; 10 μM) for 1 h before the addition of toxoids **(c, e)**. **(a)** PGE2 production in BMDC culture supernatants was determined by ELISA after 24 h. Data represent means ± S. E. of n=3. **(b, c)** The expression of CCR7 detected by flow cytometry in CD11c$^+$Hoechst$^-$ cells after 24 h. Values represent the means ± S.E from n = 4 expressed as fold change of CCR7 expression on mock-treated BMDC (* $p < 0.05$, ** $p < 0.005$). **(d, e)** After 24 h of toxoids treatments, cells were washed and allowed to migrate for 4 h across Transwell membrane towards medium alone or containing CCL19 or CCL21 (200 ng/ml) chemokines. Transmigrated cells (Hoechst$^-$) were counted by flow cytometry. Values represent the means ± S. E. of n=3. The number of transmigrated mock-treated BMDC was set as 1 (migration index) (* $p < 0.05$, ** $p < 0.005$).

Fig. 5. Pore-forming capacity of CyaA-AC$^-$ toxoid induces maturation of splenic DC and expansion of antigen-specific CD8$^+$ T cells *in vivo*. Mice received intravenously PBS with 1 M urea (control) or 25 μg of CyaA-AC$^-$ or CyaA-QR-AC$^-$ toxoid. 24 h later mice were sacrificed, total splenocytes were isolated and expression of splenic DC maturation markers was determined by flow cytometry in CD8$^-$CD11b$^+$ and CD8$^+$CD11b$^-$ subpopulations of CD3$^-$CD11c$^+$Hoechst$^-$ cells. **(a)** Dotplots and histograms are representative of n=3, 3 mice per group. **(b)** Graphs represent means ± S.E from n = 3 expressed as marker fold change of control CD8$^-$CD11b$^+$ DC or CD8$^+$CD11b$^-$ DC (* $p < 0.05$, ** $p < 0.005$). **(c)** BMDC ($5x10^4$/sample) were incubated with CyaA-OVA-AC$^-$ or CyaA-OVA-QR-AC$^-$ mutant (300 ng/ml) for 24 h prior to washing and co-cultivation with naïve OT-I CD8$^+$ T or OT-II CD4$^+$ T cells ($1x10^5$/sample). IL-2 production was determined from cell culture supernatants by ELISA after 3 days. Graph represents means ± S.E from n = 3. **(d)** Expansion of adoptively transferred CD8$^+$ T cells by BMDC pre-treated 24h with CyaA-OVA-AC$^-$ or CyaA-OVA-QR-AC$^-$ (300 ng/ml) *in vivo* was determined after 3 days by flow cytometry as a percentage of CD8$^+$Ly5.2$^+$ T cells out of 2×10^6 counted spleen cells. Dot plots are representative of n=2, 2 mice per group. Graphs represent means ± S.E. **(e, f)** Expansion of adoptively transferred OVA-specific CD8$^+$ **(e)** and CD4$^+$ **(f)** T cells by *i.v.* administered CyaA-OVA-AC$^-$, or CyaA-OVA-QR-AC$^-$ (25 μg/ml), was determined after 4 days by flow cytometry as a percentage of CD8$^+$Ly5.2$^+$ or CD4$^+$Ly5.2$^+$ T cells, respectively, using 2×10^6 counted spleen cells. Dotplots are representative of n=2, 3 mice per group. Graphs represent means ± S.E.

Fig. 6. Proposed mechanism of adjuvanticity of CyaA-AC$^-$.

A model of CyaA-AC$^-$-mediated enhancement of T cell stimulatory functions of DC which depends on pore-forming-dependent K^+ efflux at low toxoid concentrations and it is potentiated

by TLR4/TRIF signaling [17] at higher toxoid concentrations. Grey untested hypotheses in this study.

Suppl. Fig. 1

Layout of cytokines in cytokine array kit.

Fig. 1

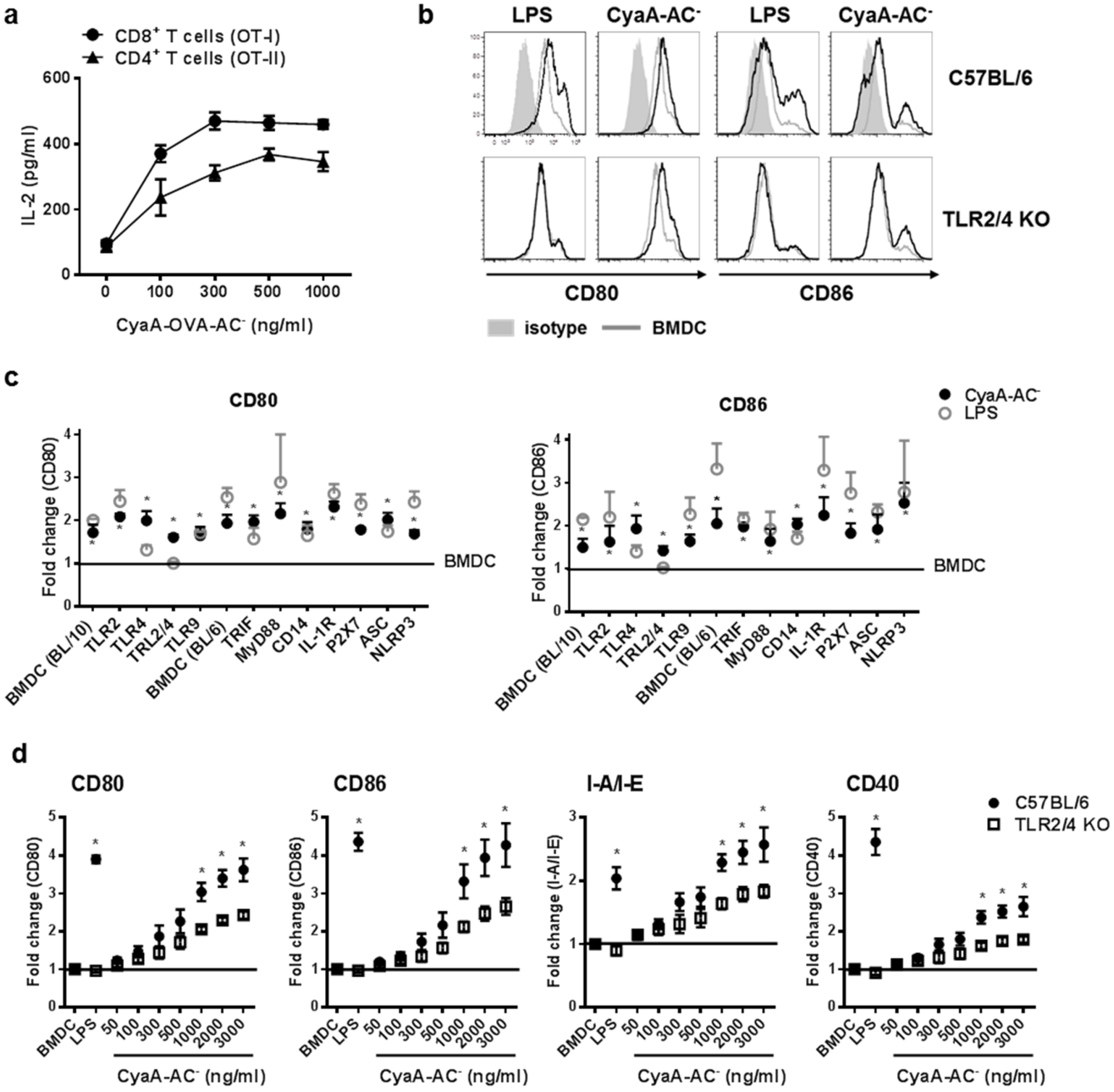
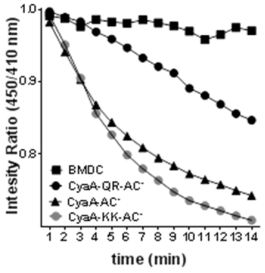
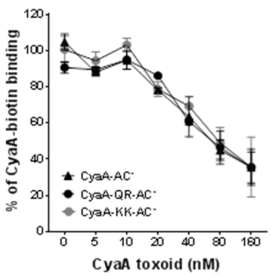

a
CD8+ T cells (OT-I)
CD4+ T cells (OT-II)
IL-2 (pg/ml)
CyaA-OVA-AC- (ng/ml)
b
LPS
CyaA-AC-
C57BL/6
TLR2/4 KO
CD80
CD86
isotype
BMDC
c
CD80
CD86
CyaA-AC-
LPS
Fold change (CD80)
Fold change (CD86)
BMDC
BMDC (BL/10)
TLR2
TLR4
TRL2/4
TLR9
BMDC (BL/6)
TRIF
MyD88
CD14
IL-1R
P2X7
ASC
NLRP3
d
CD80
CD86
I-A/I-E
CD40
C57BL/6
TLR2/4 KO
Fold change (CD80)
Fold change (CD86)
Fold change (I-A/I-E)
Fold change (CD40)
BMDC
LPS
CyaA-AC- (ng/ml)

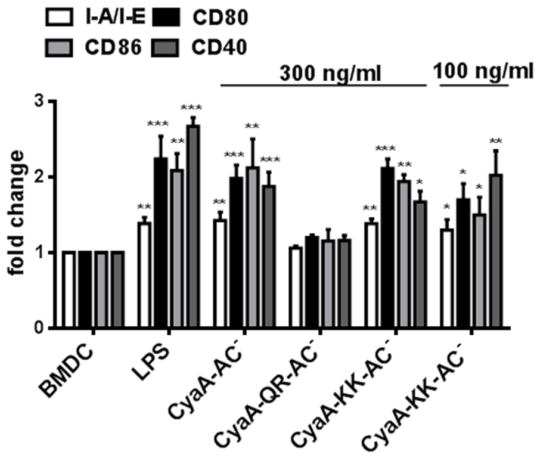
Fig. 2

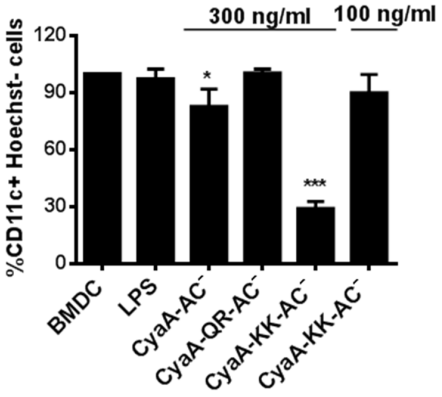
a

CyaA mutants	Ca^{2+} influx	Specific cell-permeabilizing activity (K^+ efflux)
CyaA-AC$^-$	+++	++
CyaA-E570Q-K860R-AC$^-$	+++	-/+
CyaA-E509K-E516K-AC$^-$	-/+	++++









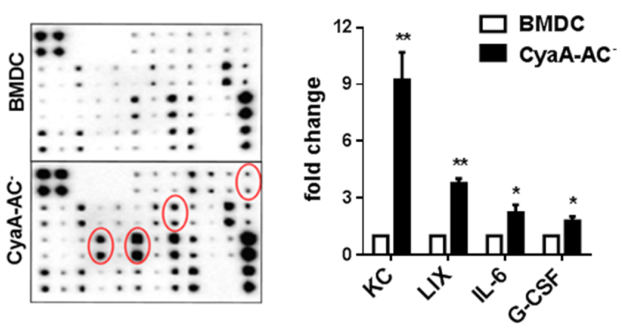


Fig. 3

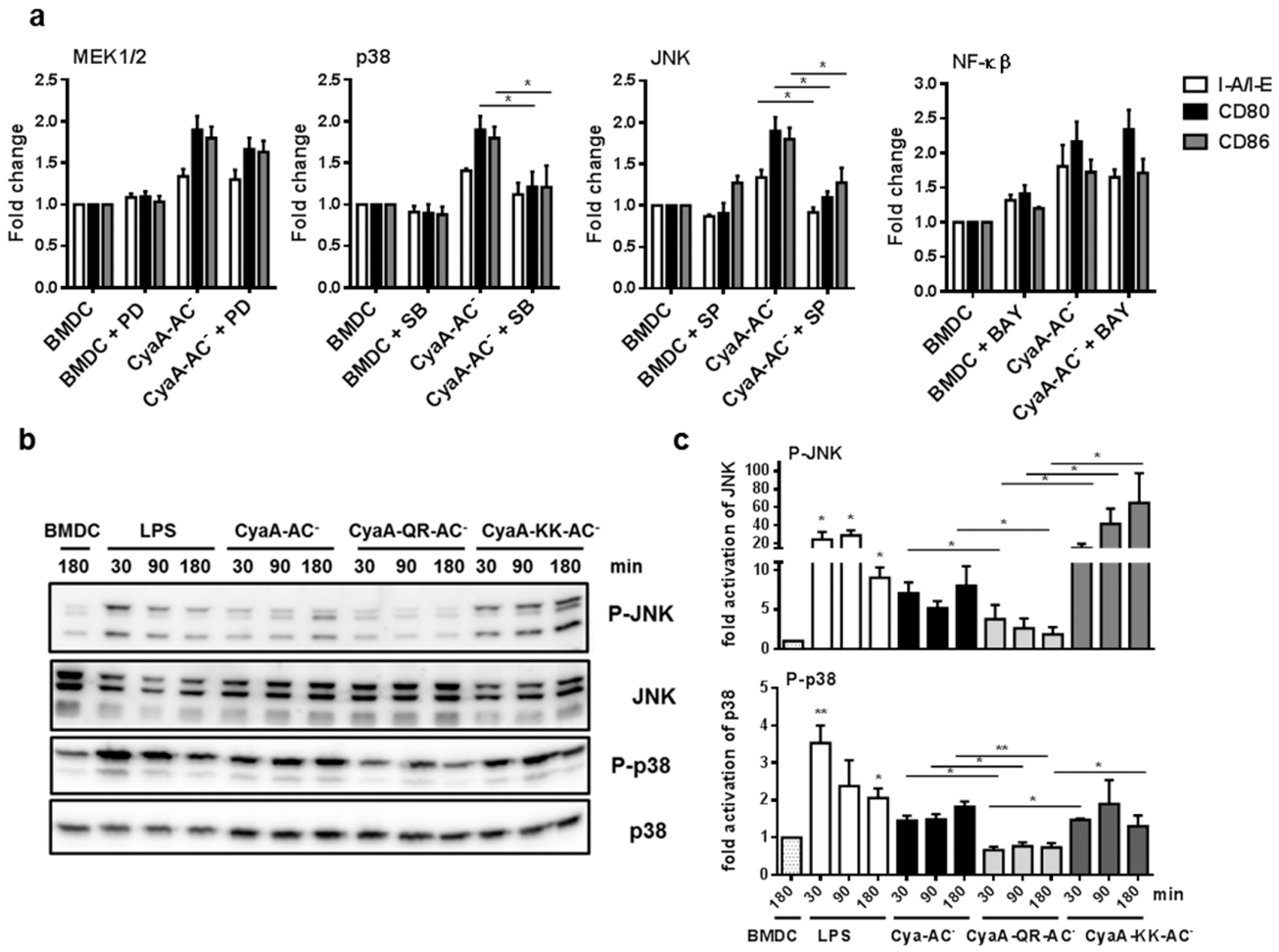

a
MEK1/2
p38
JNK
NF-κβ
I-A/I-E
CD80
CD86
Fold change
BMDC
BMDC + PD
CyaA-AC-
CyaA-AC- + PD
BMDC + SB
CyaA-AC- + SB
BMDC + SP
CyaA-AC- + SP
BMDC + BAY
CyaA-AC- + BAY
b
BMDC
LPS
CyaA-AC-
CyaA-QR-AC-
CyaA-KK-AC-
180 30 90 180 30 90 180 30 90 180 30 90 180 min
P-JNK
JNK
P-p38
p38
c
P-JNK
fold activation of JNK
P-p38
fold activation of p38
180 30 90 180 30 90 180 30 90 180 30 90 180 min
BMDC
LPS
Cya-AC-
CyaA-QR-AC-
CyaA-KK-AC-

Fig. 4

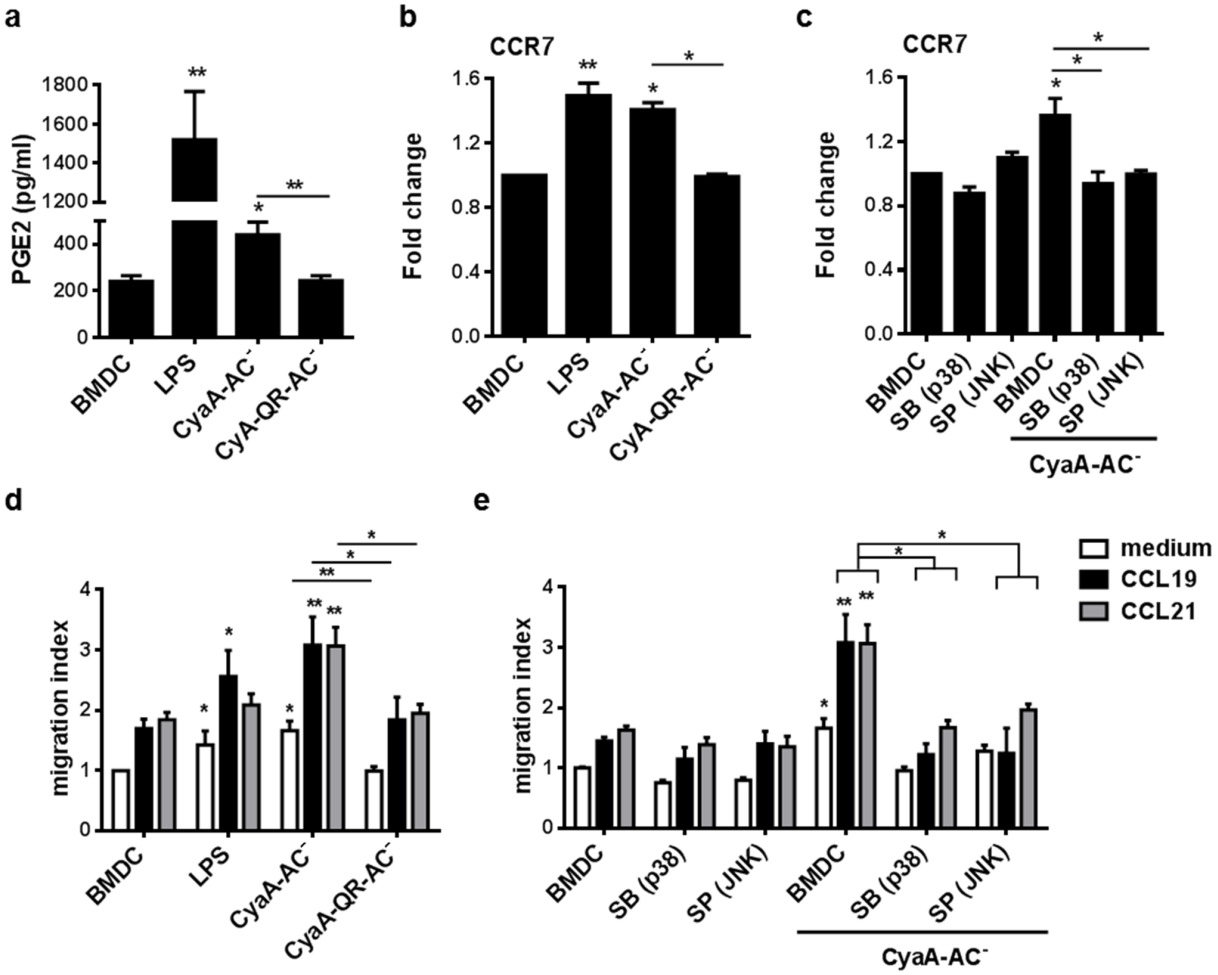

a
PGE2 (pg/ml)
1800
1600
1400
1200
400
200
0
BMDC
LPS
CyaA-AC-
CyA-QR-AC-
b
CCR7
Fold change
1.6
1.2
0.8
0.4
0.0
c
CCR7
Fold change
SB (p38)
SP (JNK)
CyaA-AC-
d
migration index
e
medium
CCL19
CCL21

Fig. 5

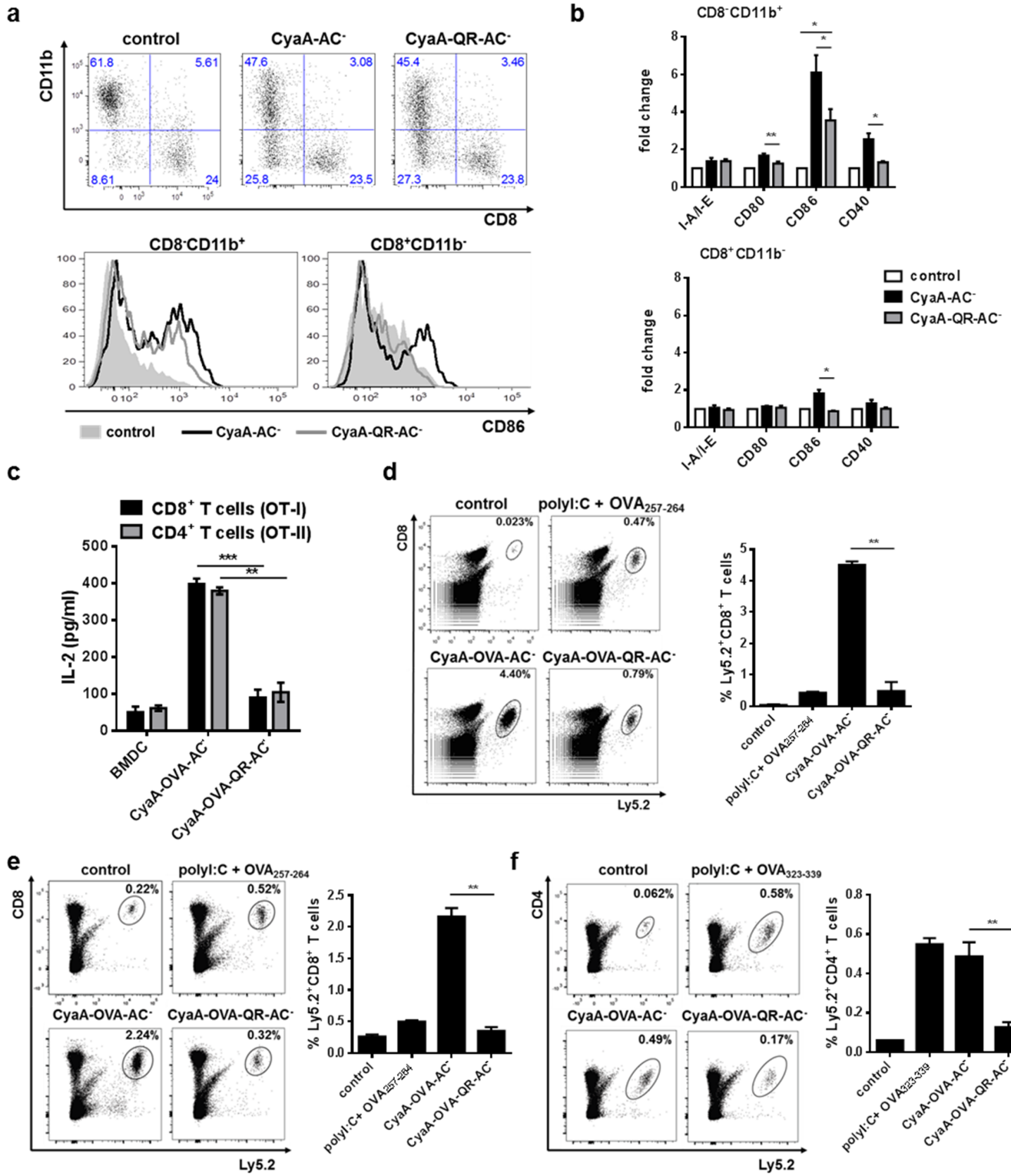
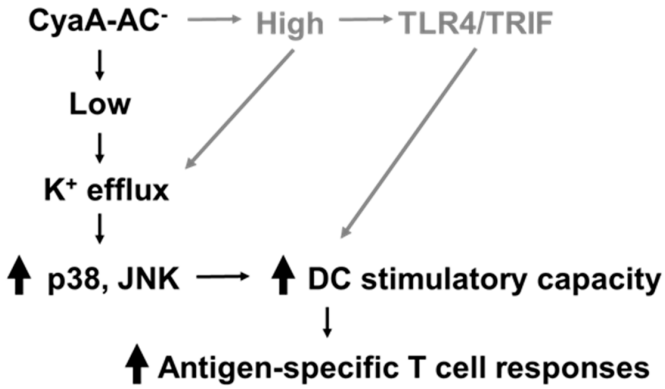

a
control
CyaA-AC-
CyaA-QR-AC-
CD11b
CD8
61.8
5.61
8.61
24
47.6
3.08
25.8
23.5
45.4
3.46
27.3
23.8
CD8-CD11b+
CD8+CD11b-
CD86
control
CyaA-AC-
CyaA-QR-AC-
b
CD8-CD11b+
fold change
I-A/I-E
CD80
CD86
CD40
CD8+CD11b-
control
CyaA-AC-
CyaA-QR-AC-
c
CD8+ T cells (OT-I)
CD4+ T cells (OT-II)
IL-2 (pg/ml)
BMDC
CyaA-OVA-AC-
CyaA-OVA-QR-AC-
d
control
polyI:C + OVA257-264
CD8
0.023%
0.47%
CyaA-OVA-AC-
CyaA-OVA-QR-AC-
4.40%
0.79%
Ly5.2
% Ly5.2+CD8+ T cells
control
polyI:C+ OVA257-264
CyaA-OVA-AC-
CyaA-OVA-QR-AC-
e
control
polyI:C + OVA257-264
CD8
0.22%
0.52%
CyaA-OVA-AC-
CyaA-OVA-QR-AC-
2.24%
0.32%
Ly5.2
% Ly5.2+CD8+ T cells
control
polyI:C+ OVA257-264
CyaA-OVA-AC-
CyaA-OVA-QR-AC-
f
control
polyI:C + OVA323-339
CD4
0.062%
0.58%
CyaA-OVA-AC-
CyaA-OVA-QR-AC-
0.49%
0.17%
Ly5.2
% Ly5.2+CD4+ T cells
control
polyI:C+ OVA323-339
CyaA-OVA-AC-
CyaA-OVA-QR-AC-

Fig. 6


CyaA-AC-
High
TLR4/TRIF
Low
K+ efflux
p38, JNK
DC stimulatory capacity
Antigen-specific T cell responses