**Translating pore forming proteins-induced innate immune responses into memory CD8+ T cells for vaccine development**

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**ABSTRACT**

Induction of CD8+ T cell responses against pathogens and cancers is an important goal of modern immunology. Notably, increased ratios of effector/central memory T cells (Tef)/(Tcm) have been described for highly efficacious vaccines. One current approach of translational interest is the use of liposomes encapsulating pore-forming proteins (PFP), such as Listeriolysin O (LLO), which are very effective at priming strong and sustained CD8+ T cell responses. We have been recently shown that Sticholysin II (StII), a PFP from the sea anemone *Stichodactyla helianthus,* stimulated CD8+ T cells against antigens co-encapsulated into liposomes composed of dipalmitoylphosphatidyl choline and cholesterol (DPPC:Chol). In this work, we demonstrated for the first time that StII shows similar abilities to LLO to induce activation and cross-presentation of OVA in dendritic cells*.* Remarkably, using an *ex vivo* ELISPOT assay to monitor INF-γ producing T CD8+ cells with similar properties to Tef, liposomal formulations containing the PFPs induced a strong response of T CD8+ cells specific for OVA-MHC-I. On the other hand, liposomal formulations with equal molar quantities of both PFPs induced comparable frequencies of Tcm-like proliferating INF-γ producing cells to those observed in mice immunized with PFP-free OVA/liposomes. However, mice immunized with liposomes with a different DPPC:Chol molar ratio and containing a higher amount of StII induced increased levels of Tcm-like responses. These results point to the capacity of StII/liposomes to induce T CD8+ responses with properties of both Tef and Tcm supporting their use for the rational design of T cell vaccines against pathogens and cancers.

**Introduction**

Induction of CD8+ T cell responses against pathogens and cancers is an important goal of modern immunology (1, 2). Particularly, induction of a mixed population of memory T cells including effector memory T cells (Tef) (which circulate the periphery and have a more immediate effector function) and central memory T cells (Tcm) (with the potential to rapidly proliferate and differentiate into effector T cells) has been proposed be the most effective (3). The vaccine platform used to deliver immunogens has a profound influence on the quality of the memory CD8+ T cell responses produced, due to differences in the innate signalling pathways stimulated and the persistence and amount of antigen after vaccination (4). One current approach of translational interest is the use of liposomes, which are very effective at priming strong and sustained CD8+ T cell responses (5). Especially relevant has been the use of pore-forming proteins (PFP) of bacterial origin, such as Listeriolysin O (LLO), to delivery heterologous antigens co-encapsulated in liposomes to the cytosol for processing and presentation in the MHC-I and to induce CD8+ T cell responses (6, 7). We have been recently shown that Sticholysin II (StII), a PFP from the sea anemone Stichodactyla helianthus, is an efficient adjuvant to stimulate cytotoxic CD8+ T lymphocytes (CTLs) responses against antigens co-encapsulated into liposomes (8). In this work, we aimed to compare StII with LLO regarding their capacity to induce activation and cross-presentation of the model antigen OVA by dendritic cells to CD8+ T cells. In addition, we compared their ability to generate CD8+ T cell responses with functions similar to either Tef or Tcm in mice when co-encapsulated with OVA into liposomes comprised of dipalmitoylphosphatidyl choline and cholesterol (DPPC:Chol) at different molar ratios.

**Body of the paper**

***Materials and Methods***

*Bone-marrow-derived dendritic cells (BMDCs) maturation after LLO and StII stimulation:*Immature BMDCs were stimulated with each PFP (1μg/mL) and Polymixin B (pmxB). On the other hand, cells were either treated with LPS (1 μg/mL) (positive control) or LPS+pmxB (negative control), or left untreated. 18 hours later, BMDCs expressing the plasma membrane maturation markers (CD40, CD80, CD86 and MCHII) were detected by flow cytometry.

*OVA cross-presentation in LLO-, StII-stimulated BMDCs:* BMDCs were stimulated with mixed OVA (25 μg/mL) and StII or LLO (0,03-0,5 μg/mL), just OVA 25 μg/mL OVA SIINFEKL peptide (1μm) or no stimulation (C-, negative control). After 3 hours, BMDCs were collected and co-cultured with the CD8+ T cell line, B3Z for 24 hours. B3Z recognize the OVA peptide SIINFEKL presented at the MHC-I and its activation was evaluated by measuring the reporter gen activity of β-Galactosidase (OD at 570), which is expressed under the IL-2 promoter.

*Ex vivo response of IFN-γ producing splenocytes specific for OVA SIINFELK peptide* *(Tef)***.** C57BL/6 mice were immunized s.c. twice at days 0 and 12 with 50 μg of OVA co-encapsulated with 6.25 μg StII into DPPC:Chol liposomes at 1:1 (Lp1:1) or 2:1 (Lp2:1) ratio (4.25 μg of StII or 12.05 ug of LLO to equal molar quantities of both PFPs). In addition, mice were injected with either PBS or Liposomes containing only OVA (Lp2:1/OVA) (formulations Lp1:1/StII/OVA; Lp2:1/StII/OVA; Lp2:1/LLO/OVA; Lp2:1/OVA; PBS). Ten days after the second immunization, mice were sacrificed and splenocytes were isolated and seeded in ELISPOT plates (MSHAS4510) with or without stimulation with the SIINFELK peptide for 20h using the Mouse IFN-g ELISpotBasic kit from Mabtech Ab (Sweden).

*IFN-γ producing splenocytes specific for OVA SIINFELK peptide restimulated in vitro (Tcm)***.** After splenocytes´ isolation as described above, cells were restimulated for 3 days with the SIINFELK peptide. Then, splenocytes were seeded in ELISPOT plates with or without stimulation with the SIINFELK peptide for 20h using the Mouse IFN-g ELISpotBasic kit from Mabtech Ab.

*Statistics:* One way ANOVA followed by the Pairwise Multiple Comparison Procedure using the Holm-Sidak method.

***Results and discussion***

A key feature for priming antigen-specific CD8+ T cells is the activation of dendritic cells increasing the expression of co-stimulatory molecules that are essential for T cell activation. Interestingly, activation assays of BMDCs in vitro, as measured through increased expression of CD40, CD80, CD86 and MCHII, revealed that both StII and LLO effectively induced BMDCs maturation (Figure 1). In addition, StII and LLO stimulated OVA cross-presentation in MHC-I to T cells (Figure 2). These results illustrate the capacity of StII to induce a dendritic cell-mediated innate immune response able to activate specific CD8+ T cells which is similar to that observed for the strong adjuvant LLO.

Notably, similar liposomal formulations carrying OVA with either StII or LLO induced equivalent CTLs responses against OVA in immunized mice. Remarkably, using an ex vivo ELISPOT assay to monitor INF-γ producing T CD8+ cells with similar properties to Tef, liposomal formulations containing the PFPs induced strong frequencies of T CD8+ cells specific for OVA-MHC-I (Figure 3). On the other hand, the use an ELISPOT assay based on in vitro expansion of OVA-specific T CD8+ cells with similar properties to Tcm, revealed that liposomal formulations with equal molar quantities of both PFPs induced comparable frequencies of INF-γ producing cells to those observed in mice immunized with PFP-free OVA/liposomes (Figure 4).

This result is different to that observed for the Tef-like response in figure 3 suggesting that while Lp2:1 liposomes promoted a Tcm-like response against OVA, the presence of PFPs on these formulations potentiated the Tef-like response (Figure 4). However, mice immunized with liposomes with a different DPPC:Chol molar ratio (Lp1:1) and containing a higher amount of StII induced increased levels of both proliferating INF-γ producing T CD8+ cells (Tcm) and Tef-like INF-γ producing T CD8+ cells. Notably, increased ratios of Tef/Tcm that are associated with life-long protection have been previously described to be elicited by potent CD8+ T cell vaccines against hepatitis C virus and simian immunodeficiency virus (9, 10) and the highly efficacious vaccines for yellow fever and smallpox (3). Consequently, results shown in this work support the value of including StII in liposomal compositions to induce strong and functional CD8+ T cell responses against heterologous antigens and thus for T cell vaccines against chronic infection and diseases.

***Conclusions***

Our results demonstrate for the first time that StII, a PFP from an eukaryotic organism, shows similar abilities to LLO to induce activation and cross-presentation of heterologous antigens in APCs in vitro and strong T CD8+ responses in vivo. Particularly relevant is the capacity of StII/liposomes to induce T CD8+ responses with properties of both Tef and Tcm supporting their use for the rational design of T cell vaccines against pathogens and cancers.



**Figure 1: BMDCs maturation after LLO and StII stimulation.** The graph shows the percent of CD11c+ cells expressing each maturation marker (CD40, CD80, CD86 and MCHII) (average ± SD) after stimulation with either StII+pmxB, or LLO+pmxB, or LPS or LPS+pmxB. Letters indicate significant difference among evaluated groups. Average comparison according to simple class ANOVA, p<0,001 followed by Holm-Sidak test. **CD40**: a>b,c, p<0,001; b>c, p<0,05. **CD80**: a>b, p<0,01; a>c, p<0,001; b>c, p<0,001. **CD86**: a>b>c>d, p<0,05; a>c,d, p<0,01. **MHCII (IAb)**: a>b, p<0,05; a>c, p<0,001; b>c, p<0,001.



**Figure 2: OVA cross-presentation in LLO-, StII-stimulated BMDCs.** Results are shown as OD at 570 (average ± SD) after stimulation with either mixed OVA (25 μg/mL) and StII or LLO (0,03-0,5 μg/mL), just OVA 25 μg/mL OVA SIINFEKL peptide (1μm) (positive control) or no stimulation (C-, negative control). Letters indicate significant difference among evaluated groups. Average comparison according to simple class ANOVA, p<0,001 followed by Holm-Sidak test. a>b>c, p<0,001; \*>a, b,c; p<0,001. ´



**Figure 3. Ex vivo response of IFN-γ producing splenocytes.** The graph shows the number of IFN-γ producing splenocytes that are specific for the complex MHC-I/SIINFEKL in each group of mice immunizated with different formulations (Lp1:1/StII/OVA; Lp2:1/StII/OVA; Lp2:1/LLO/OVA; Lp2:1/OVA; PBS). Letters indicate significant difference among evaluated groups. Average comparison according to simple class ANOVA, p<0,001 followed by a>b>c, p<0,001.



**Figure 4. Response of IFN-γ producing splenocytes after in vitro restimulation with the SIINFEKL peptide.** The graph shows the number of in vitro restimulated IFN-γ producing splenocytes that are specific for the complex MHC-I/SIINFEKL in each group of mice immunizated with different formulations (Lp1:1/StII/OVA; Lp2:1/StII/OVA; Lp2:1/LLO/OVA; Lp2:1/OVA; PBS). Letters indicate significant difference among evaluated groups. Average comparison according to simple class ANOVA, p<0,001 followed by a>b>c, p<0,001.

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