Orchestrating the immune system to initiate adaptive anti-tumor immunity

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Chapter 3

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Bacterial inclusion bodies function as vehicles for dendritic cell-mediated T cell responses

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Immunogenic antigens for vaccination are often created through the production of recombinant proteins using *Escherichia coli* (*E.coli*)[1]. As an often-undesired side-product, aggregates called inclusion bodies (IBs) are formed, containing largely misfolded forms of the overexpressed recombinant protein[2]. Since bacterial inclusion bodies are rarely formed in unmodified natural bacterial strains, their effect on the immune system has not been studied. The non-native conformation of proteins upon accumulation in IBs is expected to abrogate the use as vaccines aimed at generating high affinity antibodies[3]. However, they exhibit unique properties, including mechanical and thermal stability, biocompatibility, high antigenic content, low toxicity and relative resistance to proteases. Whether IBs can trigger adaptive cellular responses, initiated via uptake by dendritic cells for presentation to T cells, is unknown.

To efficiently produce inclusion bodies (IB) containing recombinant antigen from the E.coli strain, we generated IBs containing the ovalbumin sequence using the TorA signal sequence (Figure 1A), as previously described[4]. Since dendritic cells are the primary cell type responsible for the activation of T cells, we first analyzed the inherent immunogenic capacity of inclusion bodies directly derived from E.coli by membrane disruption and centrifugation (Crude), as well as, IBs additionally processed by sequential washing (Processed; Figure 1B). Bone marrow-derived dendritic cells (BMDCs) were cultured from bone marrow of C57Bl6 WT mice, as previously described[5]. Immunogenic maturation of BMDCs was measured by the expression of co-stimulatory markers CD70, CD80, CD86, and the MHC Class I and -II complexes using flow cytometry. Both unprocessed (Crude) and processed IBs induced the expression of CD70, CD80, CD86 and MHC class I in a concentration-dependent manner, whereas MHC class II was downregulated by IBs at higher (100nM) concentrations, like LPS (Figure 1C). Additionally, splenic $CD11c^+$ dendritic cells show similar concentration-dependent maturation by IBs as BMDCs. Even though IBs are particulate aggregates that are structurally resistant to detoxification methods like Triton-mediated phase separation (removing free LPS), IBs are rarely endotoxin free[6]. Since endotoxins like LPS are sensed by TLR4 on innate immune cells resulting in MyD88-mediated NFκB activation[7], we tested the effect of IB-induced maturation on MyD88-deficient BMDCs. Interestingly, loss of MyD88 in BMDCs completely abrogated IB-induced maturation (Figure 1D), suggesting intact TLR signaling is required for IBs to induce maturation in DCs. Even though free LPS is effectively absent in processed IBs, the maturation of BMDCs by processed IBs remains MyD88-dependent and is independent of antigen content, since both ovalbumin (Figure 1D; Green and Red) and GFP-containing IBs (Blue; Figure 1B) induce MyD88-dependent DC maturation. In summary, IBs show an inherent capacity to induce DC maturation that is dependent on the presence of MyD88, downstream of TLR signaling.

Dendritic cell maturation is required for the effective priming of naïve $CD8^+$ and $CD4^+$ T cells, however, it is unknown whether dendritic cells can process IB-included antigens for antigen presentation. Therefore, we tested the capacity of IBs to induce antigen presentation by dendritic cells to $CD8^+$ and $CD4^+$ T cells. BMDCs were cultured in the presence of OVA-IBs, engineered to include a

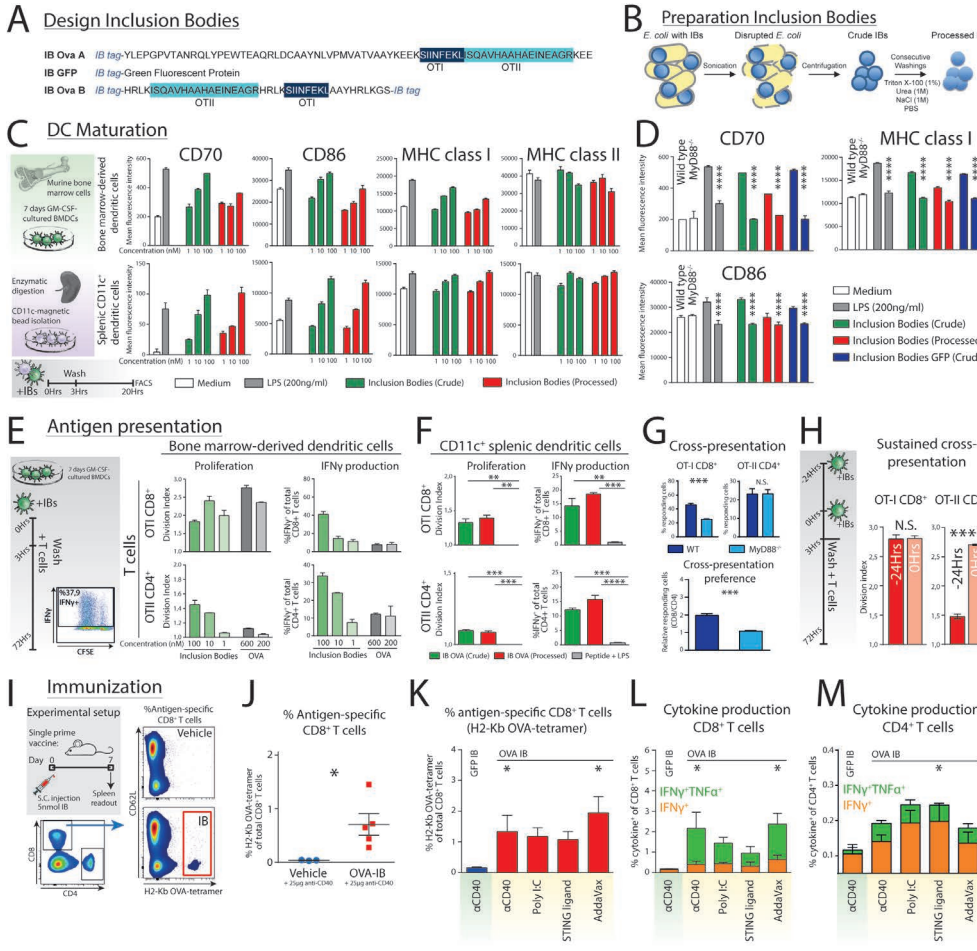

Figure 1 | A. Ova A polypeptide containing indicated OTI-OTII epitopes and GFP were produced in Inclusion bodies (IBs) upon N-terminal fusion to IB tag ssTorA(3X)[4]. Ova B polypeptide was produced in IBs upon fusion of a truncated ssTorA(3X) derivative (Jong, Luirink, *manuscript in preparation*) at both termini. **B.** IB production and processing as described[4]; includes sequential washing with Triton X-100 to remove remove excess membrane material including LPS, urea to break low affinity protein interactions and NaCl and high-salt to break potential electrostatic protein interactions (Processed). **C.** Bone marrow-derived dendritic cells (BMDCs, from C57BL/6 WT mice, 7 days GM-CSF cultured5) and splenic CD11c$^+$ dendritic cells, isolated by magnetic beads (MagniSort™/ThermoFisher; 8802-6861-74), pulsed with IBs (both crude and processed) show concentration-dependent maturation as measured by FACS. **D.** DC maturation induced by IBs in BMDCs from WT or MyD88-deficient mice. **E.** DC-mediated antigen presentation was measured as proliferation (CFSE dilution as described previously10) and IFNγ production (intracellular staining FACS) by antigen-specific CD8$^+$ OTI and CD4$^+$ OTII transgenic T cells after 3 days of co-culture with product-pulsed BMDCs. **F.** CD11c$^+$ splenic dendritic cells purified by magnetic CD11c-bead isolation were tested for antigen presentation to CD8$^+$ and CD4$^+$ T cells. **G.** MyD88-/- BMDCs show significantly decreased levels of CD8$^+$ T cell activation but not CD4$^+$ T cells compared to WT BMDCs. **H.** BMDCs were cultured with product either 24 or 0 hours before co-culture with CD8$^+$ OTI and CD4$^+$ OTII transgenic T cells to test the long-term antigen presentation capacity of IB-pulsed BMDCs. The effect of 24 hours of processing had little effect on cross-presentation of IB-pulsed BMDCs to CD8$^+$ T cells, but highly reduced CD4$^+$ T cell activation. **I.** C57BL/6 WT mice were immunized with 5nmol OVA-IB with 25μg agonistic CD40, subcutaneously. After 7 days, mice were sacrificed and splenocytes were analyzed by flow cytometry to detect OVA antigen-specific CD8$^+$ T cells by H2-Kb OVA-tetramer staining. Representative of experiments with both OVA-A and OVA-B IBs. **J.** OVA-IBs induced *de novo* antigen-specific CD8$^+$ T cell responses *in vivo*. **K.** Mice (N=9 per group) were immunized with 5nmol OVA-B IBs in combination with a

variety of adjuvants and antigen-specific CD8+ T cells in splenocytes were measured byFACS using H2-Kb OVA-tetramer staining. **L.** Splenocytes from immunized mice were re-stimulated for 6 hours with 10ug/ml OVA-derived SIINFEKL short peptide in the presence of brefeldin A and IFNγ/TNFα production by CD8+ T cells was measured by intracellular FACS staining. **M.** Similarly, CD4+ T cells were restimulation with OVA-derived ISQAVHAAHAEINEAGR short peptide for 48 hours and IFNγ was measured by intracellular FACS staining. All data representative of at least 2 individual experiments. *In vitro* experiments performed as biological triplicates and OVA-A IBs were used. *In vivo* experiments were performed using both OVA-A and OVA-B IBs, yielding comparable results. Graphs show mean +-SEM. Statistics applied by Graphpad PRISM 7.0; D/H/J unpaired Fisher t-test, F/G/K/L/M one-way ANOVA with Tukey post-hoc comparison; *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001

sizable ovalbumin fragment, or ovalbumin protein for three hours, extensively washed and co-cultured with antigen-specific CFSE-labeled OTI CD8+ or CD4+ OTII T cells purified from transgenic mice for three days (Figure 1E). We observed concentration-dependent proliferation and production of IFNγ by both antigen-specific OTI CD8+ T cells and OTII CD4+ T cells after co-culture with IB-pulsed BMDCs (Figure 1E). Purified splenic CD11c+ DCs loaded with OVA-IBs were also capable of inducing antigen-specific proliferation and IFNγ production in both CD8+ and CD4+ T cells (Figure 1F). Proliferation and IFNγ production was significantly higher than equal amounts of synthetic long peptide containing OTI and OTII sequences. Hence, both cultured BMDCs and isolated CD11c+ splenic DCs take up, process and present IB-derived antigens to CD8+ and CD4+ T cells.

The uptake, processing and presentation of exogenous antigen on MHC class I to CD8+ T cells is a process called cross-presentation[8]. TLR4 engagement, MyD88 signaling and DC maturation by LPS has previously been shown to enhance cross-presentation[9]. Therefore, we tested the capacity of MyD88-deficient BMDCs to cross-present IB-derived antigen to CD8+ T cells. MyD88-deficient BMDCs showed a reduction in CD8+ T cell activation, while CD4+ T cell activation was unaffected (Figure 1G). Quantification of the preference for cross-presentation (calculated percentage of activated CD8+ T cells divided by CD4+ T cells) showed a significant decrease in cross-presentation when MyD88 signaling was lost. Hence, intact TLR-MyD88 signaling is required for optimal cross-presentation of IB-derived antigen. Lastly, we hypothesized that the increase in antigen presentation of IB-derived antigens was due to depot-formation and slow release of antigen from internalized IBs. To test this hypothesis, we incubated BMDCs with IBs (for 3 hours and extensive post-incubation wash) 24 hours before T cell co-culture or without the 24-hour pre-incubation (Figure 1H). Interestingly, no difference in CD8+ T cell activation induced by IBs was observed when IBs were pre-incubated for 24 hours, suggesting intact and a continued cross-presentation (Figure 1H). In contrast, CD4+ T cell activation was significantly decreased in conditions of pre-incubation for 24 hours, suggesting that MHC class II-mediated antigen presentation to CD4+ T cells occurs mostly within the first 24 hours after IB incubation.

Having verified the capacity of IBs to induce DC-mediated antigen presentation to T cells, we next investigated the use of IBs in generating *de novo* cellular responses *in vivo*. First, we injected OVA-IBs with agonistic CD40 subcutaneously in mice and after 7 days measured the percentage of antigen-specific CD8+ T cells in splenocytes by flow cytometry using an H2-Kb OVA-tetramer (Figure 1I). A significant induction in antigen-specific CD8+ T cells can be found in splenocytes of immunized mice after 7 days (Figure 1J). To test whether the induction of cellular responses could be further optimized, we vaccinated mice with IBs combined with a selection of adjuvants known to boost CD8+ T cell responses, including agonistic CD40, Poly I:C, AddaVax (MF59) and STING ligand (DMXAA). AddaVax and agonistic CD40 induced the highest frequency of CD8+ T cells as measured by tetramer staining (Figure 1K). Functionally, the splenocytes with the highest frequency of CD8+ T cells producing the effector cytokines IFNγ and TNFα after peptide restimulation were found when IBs were combined with agonistic CD40 or AddaVax (Figure 1L). Surprisingly, the frequency of CD4+ T cell producing IFNγ upon peptide restimulation shows in inverse correlation compared to the CD8+ T cell responses (Figure 1M). Hence, the adjuvants may determine the size of the CD8+ or CD4+ T cell response to IBs. In summary, we experimentally show the potency of IBs to be used as a vaccine to induce strong antigen-specific CD8+ T cell responses.

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Author contributions

S.T.T.S., W.S.P.J., J.L., Y.K. conceived the study, S.T.T.S., W.W.J.U., J.M.M.H. designed the experiments, S.T.T.S., L.K., S.E., H.B.B.S., D.H. performed and analyzed experiments, S.T.T.S., W.S.P.J., J.L., Y.K. wrote the manuscript, W.W.J.U., J.M.M.H., J.L., Y.K. supervised the work

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