Protein immunogenicity: impact of aggregate morphology and innate immune response modulating impurities on local immune activation

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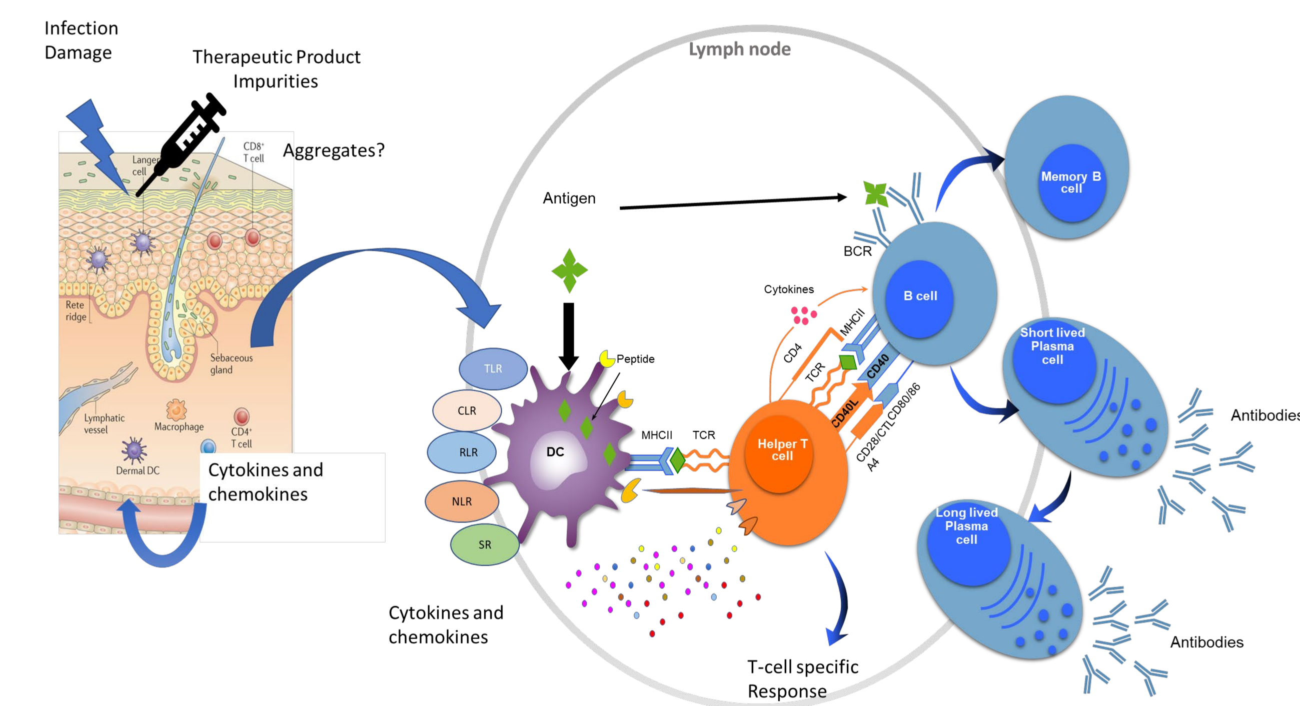
Abstract

Immunogenicity has been shown to negatively impact safety, PK, and efficacy. A critical quality attribute that has been associated with immunogenicity risk is protein aggregation. Multiple factors such as mechanical stress, light, metal ions, silica particles, pH, and liquid-solid interphases can impact on the formation of aggregates and it is generally accepted that the stress impacts on the size, charge, and cohesion of the protein aggregates. Despite advances in aggregate characterization, it is still unclear what are the critical attributes of protein aggregates that impact on their immunogenicity risk. For example, it is not known whether particles in the low nm size are more likely to induce an immune response than those in the um range. Correlating the properties of protein aggregates with their immunogenicity has been difficult because it is hard to isolate specific types and sizes of protein aggregates. To address this, we generated aggregates using different stress conditions (stirring, end over end rotation and heat) and characterized the resulting aggregates for size and shape as well as the innate immune response they elicited using in in vitro cell-based assays and in vivo. We found that stirring and rotational mixing stress yielded distinct aggregates capable of eliciting a defined pattern of innate immune activation in vitro and in vivo. Further, we demonstrate that the response to protein aggregates is magnified by the presence of trace amounts of microbial impurities resulting in increased ADA rates in macaques. This studies provide evidence that both the quantity and quality of protein aggregates should be considered when performing a immunogenicity risk assessment as some types of aggregates are more immunogenic than others.

Background



FDA Mission Relevance Statement

This work demonstrates an effect of protein aggregation on aggregate's ability to induce a local immune response and its impact on immunogenicity.

Materials and Methods

A model monoclonal antibody bevacizumab was used in this study. Bevacizumab was diluted to 1mg/mL and stressed under several defined stress conditions stirring or rotational stress (Endo over end) for 24 hours. Samples were allowed to rest for at least an additional 24 hours to allow reversible aggregates to revert monomeric form. The degree of aggregation was measured using FlowCam™ imaging.

The impact different stresses on the immunogenic potential of Bevacizumab was assessed in primary human PBMC. The expression of inflammatory genes (IL-1β and IL6) was measured after 24 hours. Induction was shown as the fold increase over media along treated cells.

The immune response in vivo was assessed in the skin 6 hours post injection at the site of injection. Potential synergy was assessed by injecting trace levels of a TLR2/6 agonist (FSL-1) or TLR4 (LPS). RNA was extracted from the tissue harvested and induction of proinflammatory genes was assessed. The magnitude of the response in shown as fold change over Saline alone(murine) or baseline(NHP).

The levels of anti-bevacizumab antibodies were assessed in the serum of NHP following three injections of Bevacizumab (+/- aggregation 30 days a part) and 30 days following the last injection. Levels were measured using a bridging Elisa format and levels of bevacizumab were quantified in same samples using HPLC.

Type of Stress and Formulation Determine Aggregate Frequency and Morphology

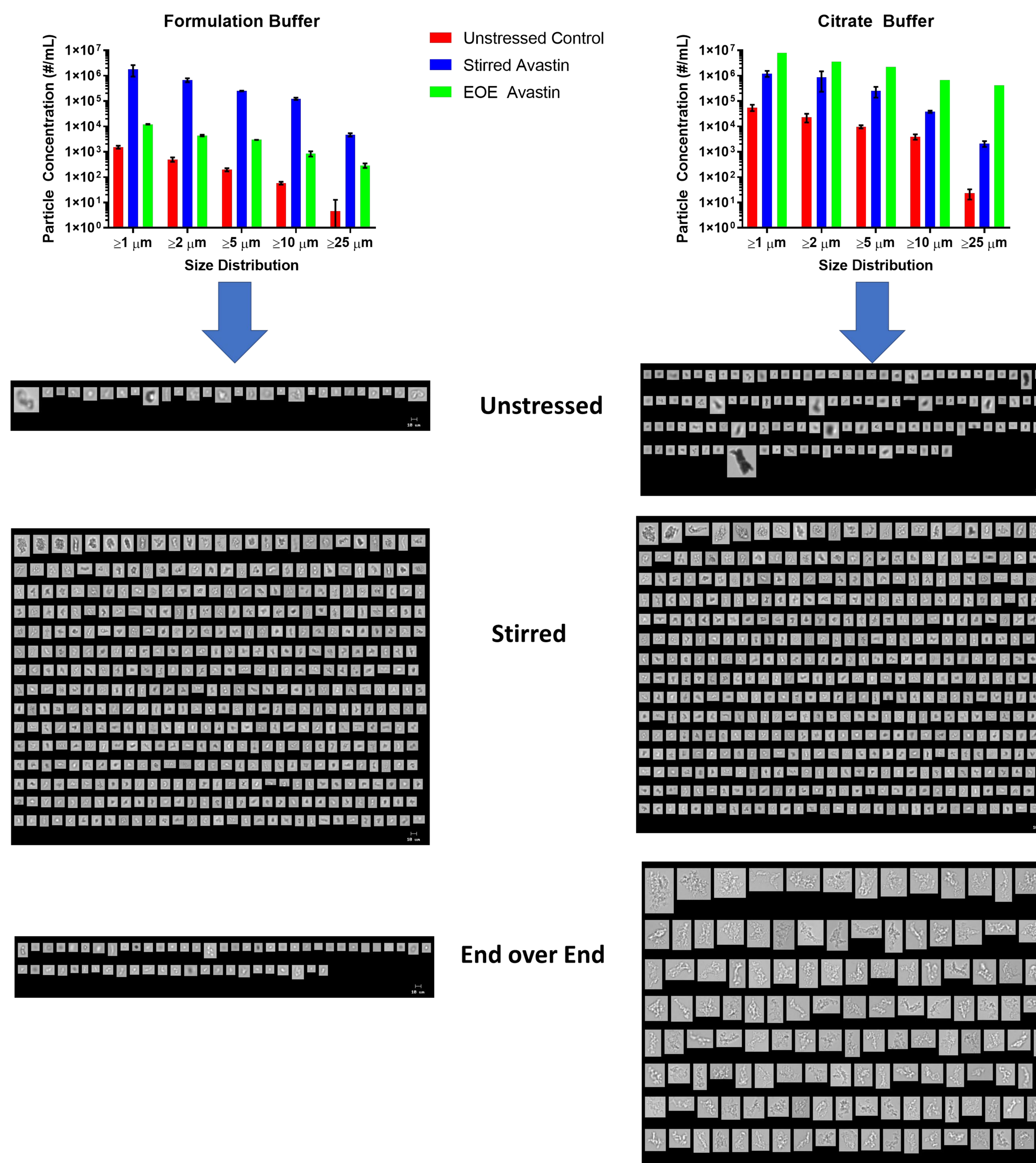


Figure 1. Characterization of Protein aggregates generated by Stirred and End over End Stress. Aggregates in Bevacizumab 1mg/mL were generated by stressing the mAbs in their formulation buffer or in citrate buffer and then quantified by FlowCam ™ Imaging. Representative images obtained by FlowCam of the aggregate size and morphology shown for each condition.

Results and Discussion

Aggregate morphology and numbers impacts the ability to induce an inflammatory response

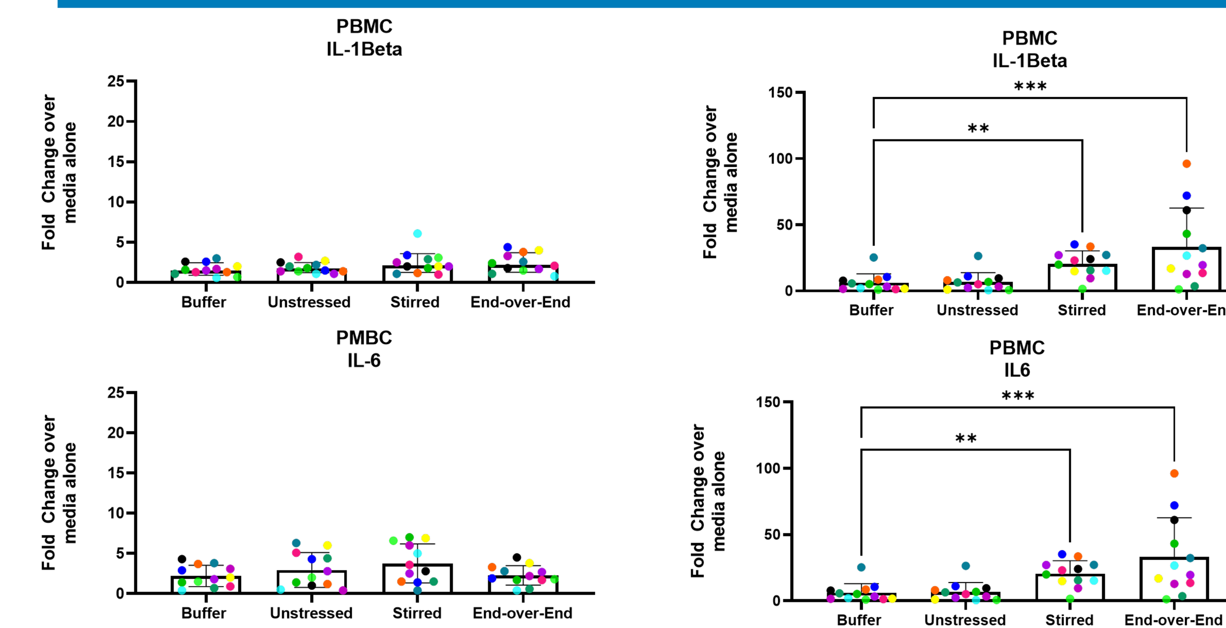


Figure 2. Inflammatory response in human PBMC exposed to aggregates.
Human PBMC were exposed to 100ug/mL of Bevacizumab (+/- aggregation) for 24 hours. Induction of IL-1β and IL-6 compared to media alone was assessed. PBMC exposed to aggregates generated in citrate buffer significantly upregulated inflammatory genes compared to buffer alone. The end over end stress tended to have a higher induction of IL-1β and IL-6. Stat analysis: Non-parametric ANOVA.

In vivo Effects of Protein Aggregates

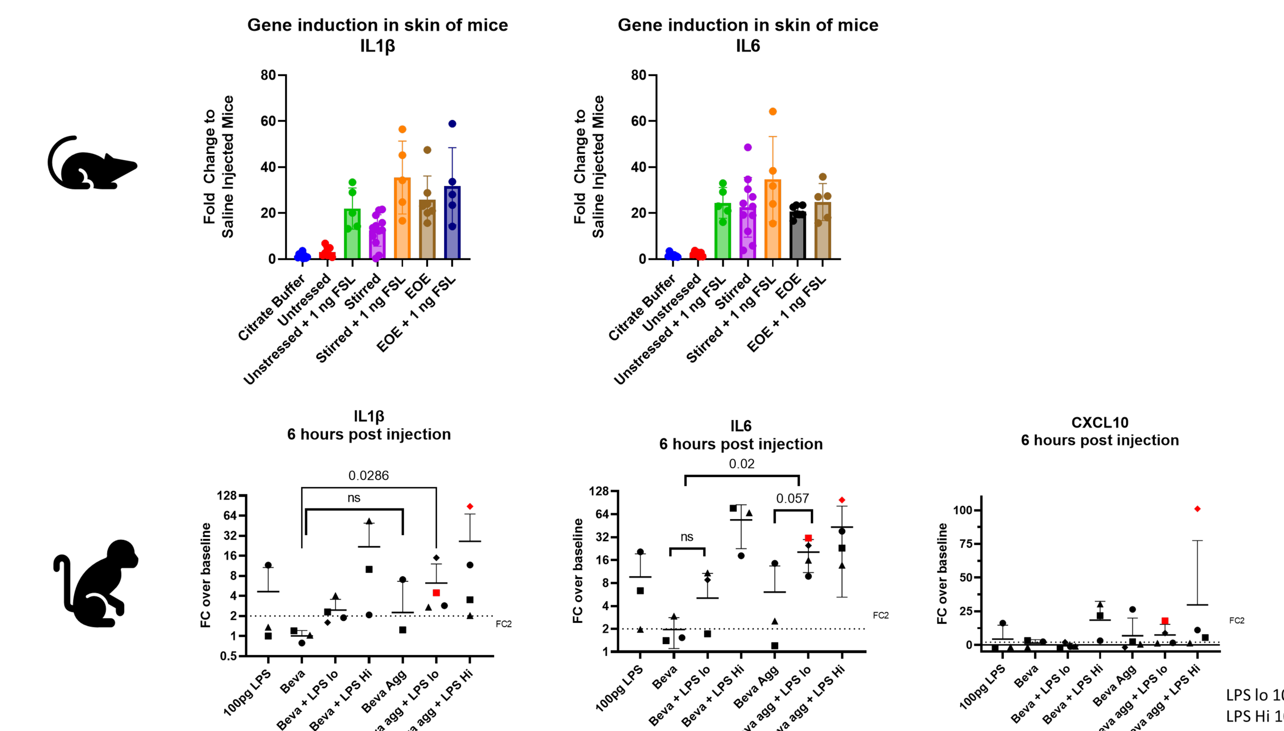


Figure 3. *In vivo* induction of proinflammatory genes by protein aggregates.
The in vivo impact of aggregates was assessed in the skin of C57/6 mice and NHP six hours after injection. Aggregated product induce a robust induction of IL-1β and IL-6. Despite the difference in response observed in vitro for stirred and EOE aggregates, SC administration in mice resulted in modest differences in local IL-1 and Il-6 mRNA expression at 6 h post inoculation. Addition of an TLR 2/6 agonist increased the response of aggregates in murine skin.
In NHP SC administration of stirred aggregated mAbs showed a significantly increased response only when coupled with low levels of LPS suggesting that LPS and aggregates have additive effects on the local response. Animals that developed a positive ADA response are shown in red. Stat analysis: unpaired T test.

In vivo Effects of Protein Aggregates continued

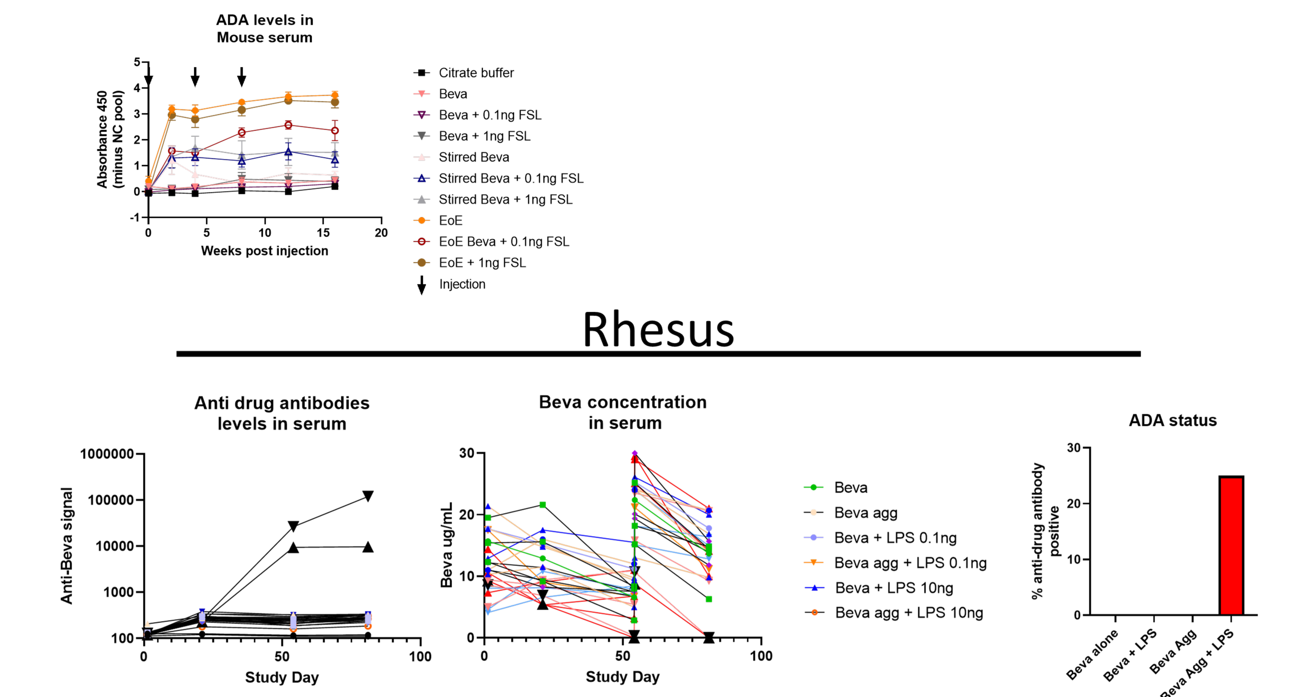


Figure 4. Anti-Bevacizumab and Bevacizumab level in serum of mouse and NHP.
In murine samples serum was collected every four weeks and mice were injected 3X with the indicated aggregate and FSL amount.
ADA status and levels of Bevacizumab were assessed immediate before each treatment and 30 days following the last injection. Two animals (designated with large black symbols) developed a robust and persistent ADA response to Bevacizumab. These animals also showed evidence of accelerated clearance of bevacizumab in circulation. Aggregation of bevacizumab and presence of LPS resulted in a prevalence of ADA of 25% compared to 0% in all other conditions in the study.

Conclusion

- Type of stress impacts on aggregate morphology, which impacts on the inflammatory activity of protein aggregates.
- In primates, spiking a therapeutic with low immunogenicity potential with protein aggregates and trace levels of IIRMI was associated with increased ADA. The ADA elicited macaques had reduced PK.
- These findings link innate immune activation with ADA development.
- Further research is needed to identify which markers of biological activation in vitro are associated with increased immunogenicity in vivo.

Regulatory impact

- Protein aggregates and innate immune response modulating impurities appear to increase the immunogenicity risk. However not all protein aggregates are equal and improved aggregate characterization may enhance immunogenicity risk assessment.
- These studies help establish the importance of controlling IIRMI and aggregation and improve our understanding of the correlation between in vitro assays examining immunogenicity risk and clinical outcomes.