Innate immune activation depends on the characteristics of protein aggregates

Cheng Her, Seth G Thacker, Logan K Baker, and D. Verthelyi

Laboratory of Immunology, OBP, CDER, FDA

Abstract and Background

Biologics, whether recombinant, synthetic or naturally-derived have the potential to induce an immune response in the host that can impact on the safety and efficacy of the product. Predicting with certainty whether a product will be immunogenic and the potential clinical consequences of the immune responses to the product is difficult but can help streamline clinical trials and hasten availability of therapeutic products. Many proteins and peptides form some level of aggregation at release or during storage. Product aggregation is considered a risk factor for product immunogenicity, partly because they can induce an inflammatory response at the site of inoculation that facilitates an immune response. In addition, previous studies have shown that aggregates can synergize with IIRMIs[1]. However the characteristics that make those aggregates immunogenic remain unclear. Using a combination of biophysical analytics and innate immune response assays we explore the response induced by different types of peptides and aggregates in an effort to correlate aggregate type, size, charge and shape with innate immune activation.

Experimental Design

To determine the in vitro effects of aggregated drug product on innate immune activation, we used an aggregated monoclonal antibody; Avastin® (bevacizumab – 25 mg/mL; Genentech, San Francisco, CA), and peripheral blood mononuclear cells (PBMCs, obtained from NIH). Avastin® was aggregated at formulation concentration through stirring (1100 RPM for 4 hours), heating (65ºC for 30 minutes) and end-over-end rotation (40 RPM for 4 hours). Before running immune response assays, the aggregates were characterized using Flow Imaging Microscopy (FlowCam™; Fluid Imaging Technologies, Scarborough, ME) for size and morphology. Avastin was then diluted into PBMCs at 1 mg/mL for 24 hour incubations. Cell viability was assessed for each experiment using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.). Quantitative PCR was done on PBMCs for expression of IL-1b and IL-6 in order to assess cell activation as a function of aggregated Avastin®.

Results – Particulate Formation and Storage Stability

Figure 1 Comparison of IL-1b and IL-6 Expression in PBMCs for 80 µg/mL Avastin under Different Stresses for unstimulated PBMCs in different buffers. Mean of means calculated from N = 2 measurements across 5 donors.

Innate immune activation induced by Avastin aggregates (Fold Change over GAPDH)

Experimental Condition (IL-1B Expression)	Formulation Buffer	Citrate Buffer
Avastin Buffer Control	1.9 ± 0.6	9.0 ± 8.3
Unstressed Avastin Control	2.1 ± 0.5	9.7 ± 8.7
Stirred Avastin	1.8 ± 0.6	**23 ± 7.1**
Heated Avastin	**8.9 ± 8.3**	12 ± 6.1
End-over-End Avastin	2.6 ± 1.2	**47 ± 28**
Experimental Condition (IL-6 Expression)	**Formulation Buffer**	**Citrate Buffer**
Avastin Buffer Control	2.6 ± 1.5	9.0 ± 5.1
Unstressed Avastin Control	3.4 ± 2.4	13 ± 7.2
Stirred Avastin	3.3 ± 3.9	**39 ± 17**
Heated Avastin	**6.3 ± 5.8**	14 ± 6.4
End-over-End Avastin	2.8 ± 1.4	**128 ± 121**

- Heated Avastin lead to a more robust expression of IL-1B and IL-6 in formulation buffer than in citrate buffer
- Stirred and Avastin that underwent end-over-end rotation lead to a more robust expression of IL-1B and IL-6 in citrate buffer than in formulation buffer

Formulation impacts on aggregation

RED is Avastin in Formulation

BLUE is Avastin in Citrate Buffer

Avastin In Formulation | **Avastin In Citrate Buffer**

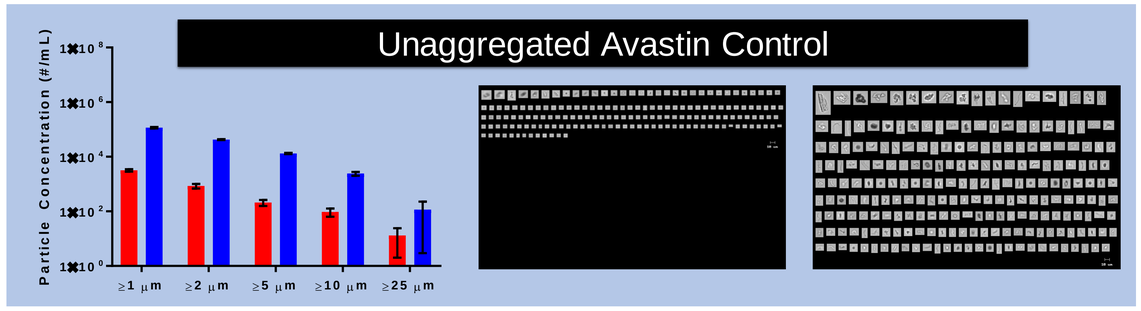


Figure 3 Size Distribution and Morphology for 1 mg/mL Avastin® in Different formulations when Unstressed, Stirred, Heated and End-over-End Rotated Avastin via Flow Imaging Microscopy (FlowCam™).

- Avastin aggregates (stirred and end-over-end) in formulation synergize additively with 100 pg LPS to induce Innate immune activation in citrate buffer, but not formulation buffer for IL-1B and IL-6
- Heated Avastin showed slightly additive synergy in both buffers for IL-1B and IL-6

Figure 2 Comparison of IL-1B and IL-6 Expression in PBMCs for 80 µg/mL Avastin under Different Stresses for PBMCs stimulated with 100 pg of Lipopolysaccharide (LPS) in different buffers. Mean of means calculated from N = 2 measurements across 5 donors.

Experimental Condition (IL-1B Expression)	Formulation Buffer	Citrate Buffer
LPS Control	83 ± 44	83 ± 44
Avastin Buffer Control with LPS	80 ± 43	85 ± 44
Unstressed Avastin Control with LPS	73 ± 35	84 ± 46
Stirred Avastin with LPS	53 ± 50	**127 ± 64**
Heated Avastin with LPS	90 ± 50	91 ± 53
End-over-End Avastin with LPS	64 ± 52	**180 ± 81**

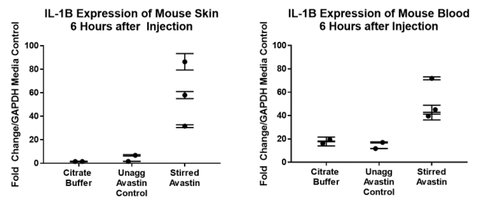
Experimental Condition (IL-6 Expression)	Formulation Buffer	Citrate Buffer
LPS Control	87 ± 37	87 ± 37
Avastin Buffer Control with LPS	79 ± 30	81 ± 27
Unstressed Avastin Control with LPS	75 ± 25	78 ± 31
Stirred Avastin with LPS	35 ± 27	**183 ± 90**
Heated Avastin with LPS	105 ± 39	97 ± 46
End-over-End Avastin with LPS	81 ± 74	**479 ± 233**

Mice Studies

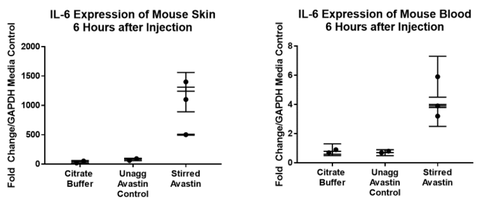
Mice were shaved 24 hours prior to injection. The loading volume was 100 µL per injection. For the data below, mice were injected with placebo (PBS), a sodium citrate buffer control, an unstressed Avastin control in a sodium citrate buffer and Avastin in a sodium citrate buffer that had been stirred for 24 hours at 1100 RPM. 6 Hours after injection, the mice were sacked and skin, blood and spleen were harvested. Skin and blood data shown below.

Figure 4 Comparison of IL-1B and IL-6 Expression in mice for 80 µg/mL Avastin Stirred for 24 hours at 1100 RPM and Formulated in 10 mM sodium citrate and 5% in mice skin and blood.

In vivo Data: Fold Change/GAPDH of Placebo Mice (IL-1β)



In vivo Data: Fold Change/GAPDH of Placebo Mice (IL-6)



- IL-1B Expression for mice injected with stirred Avastin was similarly robust in skin and blood
- IL-6 Expression for mice injected with stirred Avastin was significantly more robust in skin than in blood
- Cytokine analysis of Spleen and lymph nodes is currently being done

Conclusions

1. Different Stresses can result in different immune activation by different aggregates
2. Formulation differences can result in different IIRMI synergies between the same stresses
3. Early mice studies correlate a relationship to PBMC results when using Stirred Avastin and looking at IL-1B and IL-6 Expression
4. Particle concentration, but not necessarily total protein concentration may be important when looking at innate immune activation and IIRMI synergy

Future Work

1. Look at IIRMI synergy in mice studies
2. Look at different stresses on IL-1B and IL-6 expression in mice
3. Look at different properties of aggregates that can potentially give rise to different innate immune responses

References

1. Polumuri, SK, Haile, LA, Ireland, DC and Verthelyi, D. Aggregates of IVIG or Avastin, but not HAS, modify the response to model innate immune response modulating impurities. Sci Rep (2018); 8 11477. PMID: 30065306