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Title: Treatment with anti-cytokine monoclonal antibodies can potentiate the target cytokine rather than neutralize its activity.

Short Title: Antibody complexes can increase cytokine activity.

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Abstract: Type 2 cytokines are critical mediators of allergic airway disease. Therefore, it has been theorized that developing monoclonal antibodies that neutralize these cytokines should ameliorate allergic rhinitis and asthma; however, the majority of these antibodies have demonstrated minimal clinical efficacy. It is well-established in mice that the formation of cytokine/ anti-cytokine complexes can potentiate, rather than neutralize, cytokine activity. Emerging evidence suggests that this

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phenomenon may exist in humans. We highlight this evidence and suggest approaches that circumvent the problem of cytokine/ anti-cytokine complex formation in treated subjects.

Airway diseases such as allergic rhinitis and allergic asthma are due to a Th2 response to innocuous environmental antigens. Animal models have shown that the antibody mediated neutralization of Th2 cytokines can greatly diminish airway inflammation (1-3); these results lead to the development and clinical investigation of humanized anti-Th2 cytokine antibodies for the amelioration of asthma (4-8). Unfortunately, clinical trials of these biologics have not been a resounding success, with inabilities to significantly improve clinical symptoms being common and successful trials requiring strict patient stratification (9). These failures have spawned numerous plausible explanations, such as a requirement to neutralize more than one Th2 cytokine to achieve a disease modifying effect or that antibodies with a greater affinity for the target cytokine may be required. Alternatively, a well-documented phenomenon may be hampering clinical efficacy, namely the potentiation of cytokines through the formation of cytokine/ anti-cytokine immune complexes.

The formation of cytokine/anti-cytokine immune complexes does not guarantee cytokine neutralization, as these complexes can actually increase the potency of a cytokine. Due to their bivalent nature, antibodies form immune complexes when neither the antibody nor the antigen is in excess. Murine studies have shown that cytokine/ anti-cytokine immune complexes can potentiate the activity of numerous cytokines. For instance, IL-4/anti-IL-4 complexes are much more efficient than IL-4 alone at stimulating B cells, increasing their production of IgE and inducing the proliferation of CD8 T-cells (10-12). Similarly, IL-2/anti-IL-2 complexes are much more potent in their ability to expand memory CD8 T cells, NK cells or regulatory T cells (Tregs) than IL-2 alone (12-15). An increase in the potency and bioactivity of IL-3, IL-6 and IL-7, when complexed with their respective antibodies, has also been

reported (11, 16-22). Furthermore, recent studies have demonstrated the ability of IL-2/anti-IL-2 complexes to cause type 2 innate lymphoid cells (ILC2s) to expand, produce IL-5 and induce airway eosinophilia (23, 24). Together these studies establish that the formation of immune complexes can potentiate the effects of the cytokine rather than neutralize its activity.

The ability of immune complexes to increase the potency of the cytokine has also been described in humans. Stein *et al.* reported the presence of IL-5/anti-IL5 complexes in the circulation of subjects treated with mepolizumab and that these immune complexes facilitated increased production of IL-5 by CD4 T cells (25). Indirect evidence that immune complexes can worsen disease comes from a dose finding study using lebrikizumab, an anti-IL-13 antibody, in subjects with moderate to severe asthma in which there was a direct correlation between the exacerbation rate and the dose of lebrikizumab in periostin-high subjects (26). Although the presence of IL-13/lebrikizumab complexes were not assessed, the seemingly paradoxical dose response of lebrikizumab is readily explained by the hypothesis that higher doses of lebrikizumab led to increased complex formation; this is also in line with the observations from the murine studies outlined above. The potentiation of cytokine activity is not restricted to antibodies but also extends to decoy receptors. For instance, when subjects with ankylosing spondylitis were treated with etanercept, which is a dimerized TNF receptor/Fc fusion protein designed to bind to and neutralize soluble TNF- α , a significant increase in the percentage of CD4 and CD8 T cells producing TNF- α and IFN- γ was reported (27). Collectively, these data extend the findings of murine studies to humans and emphasize that attempting to neutralize free cytokine with antibodies or decoy receptors may be an inefficient approach, as complexes can potentiate cytokine activity rather than neutralize it.

Treatment of subjects with neutralizing antibodies can not only increase the activity of the cytokine but may also increase its levels in the circulation. For example, subjects treated with mepolizumab displayed greater levels of IL-5 in the circulation with the majority of it bound in IL-5/anti-IL-5 complexes (17), while treatment of asthmatic subjects with anti-IL-13 antibodies directly increased the levels of IL-13 in the serum (7). Similarly, the treatment of patients with metastatic breast cancer with etanercept increased TNF- α levels in the plasma, demonstrating that this effect is not restricted to Th2 cytokines and diseases (28). Studies in mice using antibodies to IL-2, 4, 6 and 7 have paralleled these clinical findings as greater bioactive levels of the targeted cytokine have been found following antibody treatment (12, 14, 16). Despite these observations, there appears to be little importance placed upon the formation of cytokine/anti-cytokine complexes in subjects treated with monoclonal antibodies as it is largely assumed that cytokines present in immune complexes are neutralized. However, as we have discussed, studies have demonstrated that the neutralization of a cytokine by its binding antibody is not a foregone conclusion and further studies in humans are required.

The formation of immune complexes is prevented when the neutralizing antibody is present in excess. This concept is very clearly exhibited in early studies on the stimulatory potential of cytokine/anti-cytokine complexes where an approximate 2:1 ratio of antibody to antigen is required to form cytokine/anti-cytokine complexes. Typically, the addition of greater amounts of neutralizing antibody prevents the formation of immune complexes. In line with these original observations is the ability of IL-6/anti-IL-6 complexes to stimulate an IL-6 dependent cell line which is then inhibited when additional anti-IL-6 is added to the assay (13, 19). Further complicating matters is evidence demonstrating that for certain cytokines, the addition of more antibody simply induces greater cytokine potentiation. For instance, titrations of an anti-IL-2 antibody (clone S4B6) in the presence of a fixed concentration of IL-2 have demonstrated that increasing levels of S4B6 induce greater CD8 T-cell

stimulation (12). Therefore, efforts to use biologics to target free cytokine might be sabotaged by the development of complexes, again highlighting a possible inefficiency of this approach.

The mechanisms by which anti-cytokine antibodies increase the circulatory levels and potency of the target cytokine are incompletely defined. The most obvious explanation is that cytokine ligation by an anti-cytokine antibody prevents cytokine degradation thereby greatly increasing the half-life of the cytokine from a few hours to a few days (11, 13, 21). Indeed, an increase in the half-life of some cytokines has been shown, in mice, to be dependent upon the neonatal Fc receptor (FcRn), which rescues antibody/antigen complexes from endosomes and recycles them to the cell surface (12, 22). The fusion of targets to Fc domains, an analogue of immune complexes, has been used by the pharmaceutical industry for several years as it utilizes normal physiological mechanisms (namely, the FcRn receptor) to facilitate drug trafficking across membranes and enhances half-life (29). Evidence has begun to accumulate indicating that immune complexes may directly potentiate cytokines independently of FcRn mediated half-life prolongation. In studies investigating the mechanism of cytokine potentiation by FcRn, IL-2-IgG Fc fusion proteins (IL-2-FP) were constructed. Interestingly, at equivalent doses, IL-2-FP was significantly less effective than IL-2/anti-IL-2 complexes in its ability to induce expansion of CD8 T-cells, despite similar *in vivo* life spans (13). The co-administration of an anti-CD25 antibody with the IL-2-FP rescued the ability to robustly stimulate T-cell proliferation. These data indicate that the antibody portion of the cytokine/anti-cytokine complex may enhance proliferation by preventing cytokine binding to scavenger receptors. Importantly, this mechanism of potentiation is not limited to the relatively unique IL-2 receptor as similar findings have been noted for IL-7. Murine studies investigating the use of anti-IL-7 antibody have indicated that the presence of an immune complex may protect the complexed cytokine from receptor-mediated consumption (22). Clearly, cytokine potentiation by anti-cytokine antibodies is a complex process that spans numerous disciplines from

pharmacology to immunology. These mechanisms of potentiation highlight the potential risks of treating subjects with anti-cytokine antibody and may indicate that a modification to the approach is required.

Given the large amount of neutralizing antibody that is required to effectively neutralize a cytokine, we propose that it may be more effective to focus efforts on developing humanized antibodies that target cytokine receptors. In contrast to experimental asthma models in inbred strains of animals, effective dosing of individuals is complicated by the heterogeneity of human asthma. The level of Th2 cytokines produced by an individual is determined by both the severity of their asthma and whether the subject is exposed to the allergen. Thus, a dose of neutralizing anti-cytokine antibody that is high enough to avoid the formation of immune complexes in one individual may form cytokine/anti-cytokine complexes in other individuals. Moreover, in a given patient, the ratio of cytokine to antibody will increase during exacerbations, thereby increasing the risk of immune complex formation.

Targeting the receptor avoids the complication of forming cytokine/anti-cytokine complexes while still preventing the target cytokine from initiating a signal. The efficacy of targeting the cytokine receptor is evident from the impressive results observed in the clinical trials of dupilumab, which targets the alpha subunit of the IL-4 receptor, in subjects with severe asthma (30) and atopic dermatitis (31). Similarly encouraging clinical trial results were obtained with benralizumab, an anti-IL-5 receptor antibody, in subjects with severe asthma (32).

We have outlined a series of observations that may explain why targeting cytokines may be clinically inefficient in some cases and suggest that much larger quantities of anti-cytokine antibodies be used in clinical trials, with this dose being guided by the assessment of immune complexes in the subjects. We also suggest that targeting of cytokine receptors, rather than the cytokine itself, may be a more fruitful avenue of research since it avoids the complication of immune complex formation and may

require lower levels of antibody since a blocked receptor is insensitive to increases in cytokine levels that occur following exposure to allergen.

Statement of Contribution.

The idea for this manuscript was conceived by C.D.R. and developed by C.D.R, M.L and D.M. The manuscript was written by C.D.R, M.L., and D.M.

Statement of Conflict of Interest

C.D.R and D.M. have no conflict of interest to declare. M.L. is a co-founder, stockholder, and consultant of Circassia Ltd. and a founding scientist of Adiga Life Sciences Inc. and has received research support from both of these companies.

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