Title Page

Title:

TLR-3/9 agonists synergize with anti-ErbB2 mAb-Letter

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Charlebois et al recently reported that the anti-tumor activity of anti-ErbB2 mAb is enhanced by local polyI:C and CpG administration in murine breast tumor models (1) and concluded that this activity was dependent on IFNs, $CD8^+$ T and NK cells. The requirement for NK cells was based on *in vivo* depletion using anti-asialo-GM1 (ASGM1) Ab.

ASGM1 is a glycolipid highly expressed on the surface of mouse NK cells, but also expressed on T cells, NKT cells, eosinophils, basophils and macrophages (2). Treatment with anti-ASGM1 Ab reduces NK cell cytolytic ability *in vitro*, and *in vivo*, results in NK depletion by an unexplained mechanism (3). Anti-ASGM1 is available as polyimmunoglobulin (pIg) or IgG preparations. Most published studies, including Charlebois et al's work, use the pIg. Our studies show that anti-ASGM1 pIg binds to macrophages at a higher level than anti-ASGM1 IgG (Fig. 1A and B), and that the IgG preparation is more efficient at depleting NK cells (Fig. 1C and D). When mice were treated with 50, 25 or 10 μl anti-ASGM1 on day 1 (D1), 10 μl of anti-ASGM1 IgG was as efficacious as 50 μl of pIg in depleting peripheral and splenic NK cells. Despite anti-ASGM1 binding to myeloid cells, we did not observe any significant changes in the number of these cells (Fig. 1E). Next, we examined the effects of anti-ASGM1 on macrophage function. Anti-ASGM1 pIG, but not IgG (at 10 μg/ml or a super-saturating level of 100 μg/ml), impaired macrophage-mediated phagocytosis of anti-CD20-opsonized human CD20 transgenic B cells in vitro (Fig. 2A-B). This effect was absent with deglycosylated anti-ASGM1 pIG, indicating that FcγR-dependent effector function was impaired. In vivo, anti-ASGM1 pIg reduced anti-CD20 mediated B-cell depletion to 60% in some mice (Fig. 2C).

In contrast, the IgG preparation induced a markedly less profound effect, especially at 10μl, reducing depletion by only 10% at 48h.

In summary, we demonstrate that the anti-ASGM1 Ab used by Charlebois et al can disrupt macrophage function. Whilst we do not dispute that NK cells might have a role in the described antitumor activity, our data suggests that the authors may have inadvertently excluded the contribution of macrophages, which can be affected by pIg anti-ASGM1 and which are known to be critical mediators of mAb function (4). Therefore, we highlight an important caveat of using the pIg preparation of anti-ASGM1 to deplete NK cells, and recommend the use of a pure IgG preparation.

References

1. Charlebois R, Allard B, Allard D, Buisseret L, Turcotte M, Pommey S, *et al.* PolyI:C and CpG Synergize with Anti-ErbB2 mAb for Treatment of Breast Tumors Resistant to Immune Checkpoint Inhibitors. Cancer Res 2017;77:312-9
2. Nishikado H, Mukai K, Kawano Y, Minegishi Y, Karasuyama H. NK cell-depleting anti-asialo GM1 antibody exhibits a lethal off-target effect on basophils in vivo. J Immunol 2011;186:5766-71
3. Kawase I, Urdal DL, Brooks CG, Henney CS. Selective depletion of NK cell activity in vivo and its effect on the growth of NK-sensitive and NK-resistant tumor cell variants. Int J Cancer 1982;29:567-74
4. Gordan S, Biburger M, Nimmerjahn F. bIgG time for large eaters: monocytes and macrophages as effector and target cells of antibody-mediated immune activation and repression. Immunol Rev 2015;268:52-65

5. Beers SA, Chan CH, James S, French RR, Attfield KE, Brennan CM, *et al.* Type II (tositumomab) anti-CD20 monoclonal antibody out performs type I (rituximab-like) reagents in B-cell depletion regardless of complement activation. Blood 2008;112:4170-7

Figure Legends

Figure 1.

Characterization of anti-ASGM1 pIg and IgG on NK and myeloid cells

(A) BALB/c and C57BL/6J mice splenocytes were FcγR blocked for 10 mins at RT, then untreated or treated with anti-ASGM1 pIg (pIg, 1/80 dilution, Wako Chemicals) or anti-ASGM1 IgG (IgG, 10 µg/ml, BioLegend) for 30 mins, 4ºC and washed twice. Cells were then stained with an Alexa 488-conjugated anti-rabbit IgG for 15 mins, RT, and washed twice before analysis on a flow cytometer. The histograms and MFI values are representative of 6 mice per strain.

(B) The graph shows the cumulative data described in (A). MFI values of individual 'pIg' and 'IgG' samples were divided by the average MFI value of 'secondary only' samples in each experiment. *P* values were calculated using the t test, $n = 6$ per group.

(C) BALB/c mice were treated with rabbit serum (RS), anti-ASGM1 pIg or anti-ASGM1 IgG i.p. using the volumes shown. Tail blood was drawn immediately prior to injection (D1) and from D4-6 to stain for NK cells. $n = 3$, means and S.D. shown, (t test)

(D) The spleens of the mice treated in (B) were harvested and NK cells analyzed by flow cytometry. $n = 3$, means and S.D. shown, (t test)

(E) Cells from (C) were also stained for macrophages, monocytes and neutrophils. $n = 3$, means and S.D. shown, (t test)

Figure 2.

Anti-ASGM1 reduces *in vitro* and *in vivo* macrophage-mediated antibody-dependent cellular phagocytosis

(A) Murine bone marrow-derived macrophages were incubated in the presence of RS, anti-ASGM1 pIg or anti-ASGM1 IgG (native or deglycosylated) overnight and then co-cultured with target human CD20 transgenic purified splenic B-cells. Target cells were CFSE-labeled before opsonization with Ritux-m2a (produced in house as previously described(5)) or an isotype control. Phagocytic cells were identified by double positive $CFSE^{+}F4/80\text{-}APC^{+}$ staining, using flow cytometry. n = 4-13, means and S.D. shown, (t test)

(B) The phagocytosis assay described in (A) was performed. To exclude differences in effect due to disparities in IgG concentrations in pIg and IgG preparations, murine bone marrow-derived macrophages were treated with RS, 10 µg/ml or a supersaturating concentration of 100 µg/ml anti-ASGM1 IgG. n = 4-6, means and S.D. shown

(C) BALB/c mice were treated i.p. with 2 doses of 50 µl RS, 50 µl anti-ASGM1 pIg, 50 µl or at the equally efficacious dose of 10 µl IgG, 4 days apart, to deplete NK cells. 24h after the second dose of anti-ASGM1, 100 µg anti-CD20 mAb was administered i.p. to deplete B cells. Absolute peripheral blood B cells counts were enumerated using a Coulter Counter and by flow cytometry at 24h and 48h post anti-CD20 administration. % B cell depletion was derived by comparing absolute B cell counts at individual conditions and time points over pre-anti-CD20 B-cell counts in respective mice. =9-12, means shown, (t-test)