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# Comment on "KIR2DL4 Does not Mediate NK Cell IFN-γ Responses to Soluble HLA-G Preparations"

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Previous studies on the killer cell Ig-like receptor 2DL4 (KIR2DL4, CD158d) have shown that this unusual receptor is not detectable at the cell surface, and resides in endosomes of resting NK cells (1). Although direct in vitro binding of HLA-G to KIR2DL4 has not been demonstrated, soluble HLA-G accumulates in KIR2DL4-positive endosomes in NK cells and transfected cells (1). Soluble agonists of KIR2DL4 trigger an endosomal signaling pathway involving DNA damage response signaling, p21 expression and NF-κB activation to promote senescence in primary NK cells (2, 3). A physiological consequence of this signaling pathway is a senescence-associated secretory phenotype (SASP), involving proinflammatory and proangiogenic factors (including IFN-γ, IL-1β, IL-6, IL-8), that promotes tissue remodeling and angiogenesis (3). As soluble HLA-G is secreted by fetal trophoblast cells in early pregnancy, activation of NK cells in response to soluble HLA-G may contribute to remodeling of the maternal vasculature.

We read with interest the article by Le Page et al. (4), who did not find evidence of functional interaction between soluble HLA-G and KIR2DL4, and question the validity of earlier reports. The conclusion that their findings "raise serious doubts about many published results suggesting that IFN-γ production by NK cells can be directly stimulated through KIR2DL4 by sHLA-G or soluble Abs" is premature, given that their work differed from other studies in several important ways:

1. The response of NK cells to soluble HLA-G, as measured by IFN-γ, was shown to be dependent on contaminating DCs in NK cells purified using the Rosette Sep technique (Stem Cell Technologies). More highly purified NK cells using the EasySep method (Stem Cell Technologies) did not respond (4). Earlier studies on KIR2DL4 have used NK cells purified with the EasySep method (3) or the similar MACS NK negative isolation kit (Miltenyi Biotech) (1, 2).
2. Contamination by bacterial products in the soluble HLA-G prepared after expression in *E. coli* was proposed as the basis for the stimulation of DC, which in turn activated NK cells to secrete IFN-γ (4). To avoid this problem, previous functional studies of resting NK cells used soluble HLA-G expressed by mammalian cell lines, and affinity purified with mAb W6/32 in order to enrich for properly folded HLA-G (1–3). The presence of multiple forms ($\beta_2$m, free heavy

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chain, monomer, dimer, and high m.w. forms) of HLA-G in the bacterial preparation used by Le Page et al. (4) may have contributed to the failure to detect an NK cell response.

3. NK cells produced IFN-γ in response to “unpurified” anti-KIR2DL4 mAb #33, but not to commercial LEAF (low endotoxin azide free) mAb #33 (BioLegend) (4). In our studies (1–3), we used our own purified preparations of mAb #33. To avoid the harsh acid elution from protein A affinity columns, we purified mAb #33 by ion exchange chromatography and size exclusion (1).

4. HLA-G tetramers bound only to the contaminating DC (presumably through receptors ILT2 and ILT4) and not to resting NK cells (4). However, KIR2DL4 is not readily detectable at the cell surface, even when using Abs. KIR2DL4 is detectable in endosomes of NK cells after incubation with soluble Ab. In addition, colocalization of soluble HLA-G with KIR2DL4 in endosomes of transfected cells is KIR2DL4-dependent (1).

In summary, Le Page et al. have shown that the combination of partially purified NK cells with soluble HLA-G derived from bacteria results in nonspecific and indirect activation of NK cells (4). Their study does not invalidate previous work carried out with purified NK cells and with properly folded, soluble HLA-G isolated from transfected mammalian cells.

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