SHORT COMMUNICATION

In Vitro Evidence Demonstrating the Nondepleting Mechanism of Action of Amlitelimab, an OX40 Ligand Monoclonal Antibody

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The OX40 ligand (OX40L)/OX40 signalling pathway is a critical driver of atopic dermatitis (AD)-associated inflammation. OX40L, expressed on antigen-presenting cells (APCs), binds to OX40 on T cells, inducing T-cell proliferation and survival, while suppressing anti-inflammatory activity of regulatory T cells (Tregs). In AD, OX40L is upregulated on APCs as well as other cells involved in inflammation, potentiating the T-cell response and secretion of pro-inflammatory cytokines. Therefore, OX40L/OX40 interaction is a critical co-stimulatory signal that amplifies and sustains the immune response (1–4). Inhibition of OX40L/OX40 binding may attenuate the immune response and limit chronic inflammation (5).

Several monoclonal antibodies targeting the OX40L/OX40 signalling pathway have completed phase 2 trials in AD (4, 6, 7). Differences in molecular structure and binding among these antibodies may influence their safety and efficacy (4, 6, 7). Amlitelimab is a fully human, anti-OX40L human immunoglobulin G4 (hIgG4) S228P L235E (PE) antibody that binds to OX40L on APCs, preventing interaction with OX40 on activated T cells (3). The IgG4 backbone and the accompanying L235E mutation have been shown to suppress antibody-dependent cellular cytotoxicity (ADCC), a mechanism by which antibodies induce target-cell death through engagement of Fc receptors on effector cells, whereas the S228P mutation increases stability and prevents Fab-arm exchange (8, 9). In contrast, rocatinlimab and telazorlimab target OX40 on T cells and have an immunoglobulin G1 (IgG1) backbone, which has been shown to lead to reductions in T cells via increased ADCC (6, 7, 10). Results from the phase 2b STREAM-AD study demonstrated clinically meaningful improvements and off-therapy maintenance in AD with amlitelimab vs placebo (4). It is currently unknown whether ADCC occurs via blockade of OX40L on APCs with an IgG4 antibody and whether it differentially impacts OX40L-expressing cells, OX40-expressing pro-inflammatory effector T cells, and immune-suppressing OX40-expressing Tregs. The current study aimed to evaluate *in vitro* the potential nondepleting mechanism of action of amlitelimab and other monoclonal antibodies targeting the OX40L/OX40 pathway using 2 approaches: first, by assessing ADCC in cluster of differentiation (CD)4$^+$ T cells with anti-OX40 vs anti-OX40L (amlitelimab) antibodies; and second, by evaluating ADCC activity in hOX40L-expressing cells with anti-OX40L antibodies with different backbones (IgG4 vs IgG1).

MATERIALS AND METHODS

An ADCC assay was established using activated CD4$^+$ T cells as target cells and autologous natural killer (NK)-containing peripheral blood mononuclear cells (PBMCs [Cellero]; see Table SI for list of reagents and vendors) derived from healthy donors as effector cells. Target cells were labelled with CellTrace™ Violet (Thermo Fisher Scientific, Waltham, MA, USA) and co-cultured with effector cells in the presence of the following antibodies: anti-OX40L (amlitelimab), in-house-generated anti-OX40 hIgG1 [anti-OX40 hIgG1 (A)], in-house-generated fucosylated anti-OX40 hIgG1 [anti-OX40 hIgG1 (B)], and isotype control antibodies, hIgG4(PE) and hIgG1. Isotype control antibodies were matched to the antibodies being evaluated. Changes in live cell numbers were analysed using flow cytometry to assess ADCC against activated CD4$^+$ effector T cells (CD4$^+$CD25$^+$Foxp3$^-$) or Foxp3$^+$ Tregs (CD4$^+$CD25$^+$Foxp3$^+$CD127$^-$) utilizing 3 independent donors with 2 replicates for each (Fig. S1).

OX40L was expressed in human embryonic kidney (HEK) cells (HEK hOX40L). Labelled target cells (HEK hOX40L) and effector cells (primary NK cells isolated from PBMCs obtained from leukoreduction chambers) were incubated with the following anti-OX40L antibodies: amlitelimab hIgG4(PE), anti-OX40L hIgG1 (C), anti-OX40L hIgG1 (D), and isotype controls, IgG4(PE) and IgG1. The 2 anti-OX40L hIgG1antibodies had similar binding affinities to Fc gamma receptors (FcγRs), but different antigen binding site sequences compared with one another, with anti-OX40L hIgG1 (D) having a similar sequence to amlitelimab. Antibody titres were matched across the 5 antibodies. OX40L surface expression on HEK cells was confirmed by flow cytometry with fluorescently labelled amlitelimab. HEK hOX40L cell lysis by NK cells was assessed using the DELFIA™ cell cytotoxicity kit (Revivity Life and Analytical Sciences, Llantrisant, Wales), which utilizes fluorescence to evaluate ADCC activity. Three independent donors were used (Fig. S2).

RESULTS

In ADCC co-cultures of activated OX40-expressing CD4$^+$ T cells with NK-cell–containing PBMCs as effector cells, the percentages of OX40-expressing activated CD4$^+$ T cells and Foxp3$^+$ Tregs were decreased in the presence of anti-OX40 IgG1 antibodies (anti-OX40

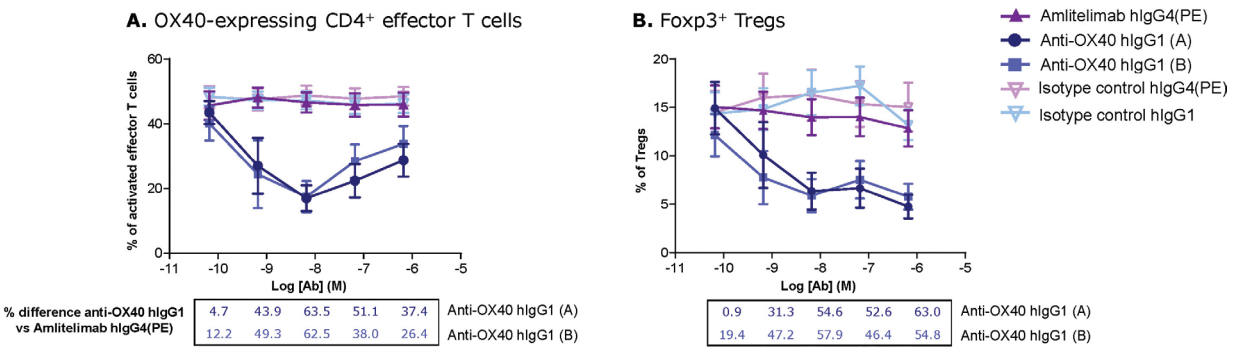

Fig. 1. ADCC activity against activated OX40-expressing CD4⁺ effector T cells and Foxp3⁺ Tregs by amlitelimab and anti-OX40 IgG1 antibodies. Flow cytometry analysis quantifying (A) the percentage of activated CD4⁺ effector T cells and (B) Foxp3⁺ Tregs following incubation with amlitelimab hIgG4(PE), anti-OX40 hIgG1 (A), anti-OX40 hIgG1 (B) and isotype controls at various concentrations, with the percentage difference between CD4⁺ T cells and Tregs with anti-OX40 hIgG1 antibodies compared with amlitelimab hIgG4(PE). Ab: antibody; ADCC: antibody-dependent cellular cytotoxicity; CD: cluster of differentiation; h: human; Ig: immunoglobulin; PE: S228P L235E; Treg: regulatory T cell.

hIgG1 [A] and anti-OX40 hIgG1 [B]; range: effector T cells, 17.0–43.5%; Tregs, 4.8–14.9%). In contrast, the percentages of activated CD4⁺ T cells and Foxp3⁺ Tregs remained unchanged with amlitelimab and isotype controls IgG4(PE) and IgG1 (range: effector T cells, 45.7–48.8%; Tregs, 12.8–17.2%), indicating absence of ADCC activity (**Fig. 1**). The percentage difference in activated CD4⁺ T cells between anti-OX40 IgG1 antibodies and amlitelimab across various concentrations ranges from 4.7% to 63.5%, and in Foxp3⁺ Tregs; the difference ranges from 0.9% to 63.0% (Fig. 1).

Following co-culture of hOX40L-transfected HEK cells with purified NK cells, specific lysis of hOX40L-HEK cells increased significantly in the presence of anti-OX40L IgG1 antibodies (anti-OX40L hIgG1 [C] and anti-OX40L hIgG1 [D], percentage specific lysis [standard error of the mean (±SEM)], 58.4% [±5.5] and 59.3% [±3.0], respectively). Conversely, cell lysis was not significantly increased with amlitelimab (22.7% [±6.5]), with percentages similar to those observed with isotype control hIgG4(PE) (16.7% [±4.1]) and isotype control hIgG1 (17.4% [±3.2]), suggesting amlitelimab did not mediate ADCC against hOX40L-expressing target cells (**Fig. 2**).

DISCUSSION

Results of this *in vitro* study indicate that amlitelimab, an anti-OX40L hIgG4(PE) monoclonal antibody, does not deplete OX40-expressing T cells or OX40L-expressing cells via ADCC. In contrast, anti-OX40 hIgG1 and anti-OX40L hIgG1 antibodies exhibited ADCC activity against CD4⁺ T cells and Tregs, and OX40L-expressing cells, respectively. The differences observed align with prior studies demonstrating that IgG4 antibodies exhibit decreased binding affinity to Fc gamma receptors (FcγRs) on effector cells, reducing their ability to deplete immune cells via ADCC. Furthermore, PE mutations (S228P and L235E) within the IgG4 backbone of amlitelimab reduce Fab-arm exchange, improve stability, and diminish residual FcγR binding, respectively (8, 9, 11).

AD is a chronic inflammatory skin disease characterized by dysregulated immune responses often driven by T-cell activation (2, 12). Current therapeutic approaches target specific cytokines or intracellular signalling pathways downstream of T-cell activation (13). By binding to OX40L on APCs to prevent interaction with OX40 at the level of T-cell co-stimulation, amlitelimab prevents activation and expansion of a broader range of pathogenic T-cell subsets involved in AD to modulate the immune

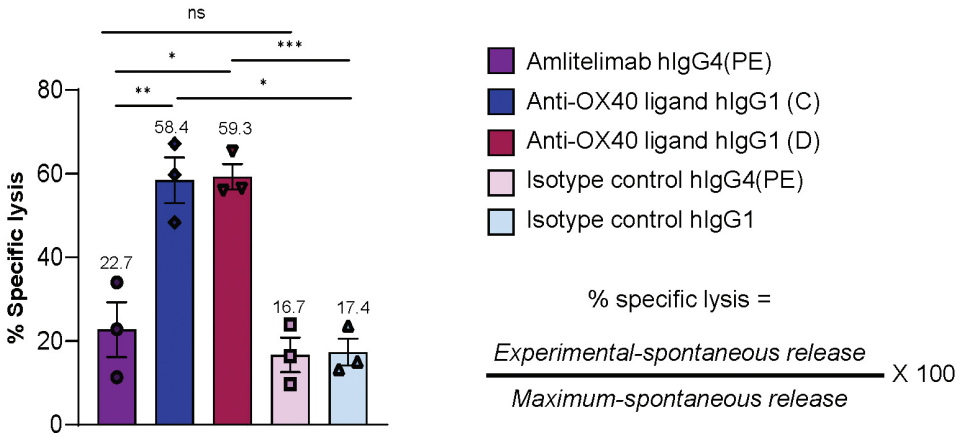

Fig. 2. ADCC activity against hOX40L-expressing HEK cells by anti-OX40L antibodies. Percentage of specific lysis of hOX40L-expressing HEK cells following ADCC assay with anti-OX40L antibodies quantified by detection of fluorescent label. Data plotted are mean ± SEM; $n = 3$ from 3 independent donors; $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.0001$. RM one-way ANOVA with Tukey's post hoc multiple comparison test. ADCC: antibody-dependent cellular cytotoxicity; ANOVA: analysis of variance; h: human; HEK: human embryonic kidney; Ig: immunoglobulin; ns: not significant; PE: S228P L235E; RM: repeated measures; SEM: standard error of the mean.

response without substantial T-cell depletion (3, 4). T cells are essential for normal immune function. Their depletion may affect the ability to respond to infections. (2, 7, 12, 14). Tregs help regulate immune responses and can act to prevent excessive immune system activation. Treg depletion could potentially lead to unregulated T-cell activation and exacerbated inflammation (1, 2, 12, 15). Furthermore, in AD, the OX40L/OX40 pathway potentiates chronic inflammation by suppressing Treg function (2). Targeting OX40L/OX40 signalling while preserving OX40-expressing cells, including Tregs, and OX40L-expressing cells, such as innate lymphoid type 2 cells, potentially allows for restoration of immune homeostasis. In turn, this may attenuate the amplification of the inflammatory process involved in cytokine production and immune dysregulation central to AD chronicity (2, 4, 12).

This *in vitro* study provides insights into the nondepleting mechanism of amlitelimab; however, several limitations should be acknowledged. While HEK cells engineered to express OX40L were used as a proxy for APCs, they likely do not fully mimic APC biology. Alternative cell types may serve as better physiological models. Experiments were conducted with a limited number of donors, which may affect generalizability of the findings. Although the effect of an anti-OX40L IgG4 antibody on T-cell depletion was evaluated, additional studies are needed to better understand the effects of OX40L/OX40 pathway modulation with amlitelimab on cytokine production. This may further elucidate how targeting OX40L and maintaining T-cell populations shapes the immune response in AD.

Notably, multiple trials evaluating anti-OX40L and anti-OX40 antibodies in AD are ongoing and will provide further insight into their efficacy and safety profiles (12). The availability of multiple therapies would enable physicians and patients to select treatments tailored to individual needs.

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