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Design and characterization of Zweimab and Doppelmab, high affinity dual antagonistic anti-TSLP/IL13 bispecific antibodies

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ABSTRACT

Patients with severe Th2 type asthma often have a steroid resistant phenotype and are prone to acute exacerbations. Current novel therapies have only marginal therapeutic effects. One of the hypotheses for lack of major efficacy in most patients is targeting only one redundant pathway leaving others active. Hence, we have designed and developed novel highly potent bispecific anti-TSLP/IL13 antibodies called Zweimabs (monovalent bispecific) and Doppelmabs (bivalent bispecific) that concurrently inhibits the signaling by these two cytokines.

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1. Introduction

Severe asthma is characterized by persistent inflammation of the airways, airway hyperreactivity and pronounced airway remodeling, involving increased fibrosis and smooth muscle thickening, mucus overproduction and sub epithelial thickening. Patients with this asthma phenotype (~30%) often exhibit a steroid resistance and are prone to acute exacerbations, which can result in the death of the patient. Despite a myriad of potential drug targets, only anti-IgE mAb therapy (Omalizumab) and recently an anti-IL5 mAb [1] have made it to the market [2–5]. Unfortunately, many 'proof of concept (POC)' targets derived from preclinical studies in mouse models failed when they were tested in patients including anti-CCR3 antagonists, anti-IL17, anti-IL4, anti-OX40L, anti-TNF α , anti-IL1 β , PDE4 inhibitors, and TLR agonists amongst numerous others [3,6,7]. Roche provided mixed results of two identical phase

III studies (LAVOLTA I-II) of the anti-IL13 antibody lebrikizumab in patients with severe asthma. Surprisingly the initial positive data seen in phase 2 trials could not be confirmed [8–10].

The results with Lebrikizumab are particularly surprising since overexpressing IL13 in the lung of mice alone causes all features of severe asthma including fibrosis and smooth muscle thickening [11]. Furthermore, numerous studies in mice using IL13 knock-out mice or mice treated with anti-IL13 mAb or soluble IL13R-Fc clearly demonstrated that inhibiting IL13 in mouse asthma models strongly reduces the allergic phenotype [12–14]. Dendritic cells (DC) pulsed with allergen secrete IL13, which, in turn induces DC maturation, contribute to Th2 polarization and IL4 secretion by CD4⁺ T cells [15]. A recent report also showed that IL13 induced epithelial barrier dysfunction may contribute to airway inflammation in allergic asthma [16]. A possibility why anti-IL13 as a monotherapy at least partly failed is that inhibiting only one pathway may not be sufficient to achieve the desired efficacy because of the redundancy of the underlying pathology. This may also explain why the efficacy of Dupilumab (inhibiting both IL4 and IL13 signaling) appears to be superior to inhibiting only IL13 (Lebrikizumab) [17]. Nevertheless, taken together anti-IL13 therapy shows a clear signal in humans and we attest the lack of better efficacy to redundant pathways being active.

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We decided to combine an anti-IL13 mAb with an anti-TSLP mAb in one bispecific format. The reason we chose TSLP is that there is a great overlap in the downstream effects induced by both cytokines. Furthermore, it was shown that severe asthma patients co-express IL13 and TSLP protein in the airway epithelium and lamina propria of the lung, suggesting that both cytokines are simultaneously contributing to disease in patients, attesting to redundant pathways [18]. Similar to IL13, TSLP transgenic animals overexpressing TSLP develop AHR and show strong eosinophil influx into the lung and elevated serum IgE [19,20], typical signs of Th2-driven asthma. In addition, strong profibrotic effects are observed. In contrast, TSLP-receptor Knock Out (KO) mice show reduced allergic responses to Ovalbumin [21]. Similar effects were shown when using an anti-TSLP antibody [22]. RSV-induced TSLP expression was found to be critical for the development of immunopathology [23] indicating a strong link to viral induction of Th2-responses. In a recent report on a phase I proof of concept clinical trial of AMG157 [24], suppressive effects of this anti-TSLP antibody on allergen-induced asthmatic responses in patients including FEV1 and the late asthmatic response were demonstrated. Furthermore, in recent phase 2 study severe asthmatics treated with long-acting beta-agonists and medium-to-high dose of inhaled glucocorticoids, had lower rates of clinically significantly reduced asthma exacerbations after treatment with the same antibody (now termed tezepelumab) than those who received placebo, independent of baseline blood eosinophil counts [24]. These are the first clinical trials showing efficacy of TSLP inhibition in asthmatic patients, possibly also in non-atopic asthmatics.

Bispecific antibodies that bind to two different targets are attractive formats to enhance the potential of drug delivery and disease treatment. There are many bispecific antibodies that are in clinical development, advanced stages and some FDA approved for oncology, inflammation and respiratory and cardio metabolic diseases [25]. Most challenging factors in the development of bispecific antibodies are meeting the requirement for upstream and downstream processes. Also, such a bispecific antibody should maintain the function and exhibit excellent biophysical properties such as solubility and stability that will not hinder the manufacturability of these molecules. Attempts have been made before to target two cytokines by bispecific antibody format to show dual targeting has better effect and is a promising approach for asthma and allergy [26,27]. We have designed, developed and characterized a monovalent bispecific antibody format called Zweimab and a bivalent bispecific antibody scaffold termed Doppelmab that are utilized for targeting both TSLP and IL13. Zweimabs are heterodimers with knob-in-hole mutations on the heavy chain. Doppelmab scaffold (was inspired based on [28]) has scFv (single chain fragment variable) moiety that is tethered to the CH₃ domain by a short polypeptide linker. Both binding and functional data are in good correlation and demonstrates that humanized anti-TSLP and anti-IL13 antibodies as Zweimabs and Doppelmabs have stronger affinity to the human targets compared to parental antibody formats.

2. Materials and methods

2.1. Identification of anti-TSLP and anti-IL13 leads

Lead mouse antibodies were either derived from conventional hybridoma or single B-cell cloning methods. The former has been described elsewhere [29]. For the latter, antigen-specific B cells were sorted singularly into 384-well plates and cultured for 7 days in Jurkat-conditioned medium with supplements as described [29]. Supernatants were then screened for antigen reactivity. All antigens used were expressed and purified in-house and are of high

quality. Cells were lysed for RT-PCR to amplify VH and VL genes. The genes were then cloned in frame with human Igγ and κ genes, expressed as recombinant human IgG1/κ and further tested. Mouse leads were converted to a chimeric antibody consisting of the mouse variable domain and a human constant IgG1 Fc with effector function knock-out mutations (L234A, L235A). Humanization was performed following our typical M-13 based fab expression system method [30]. Sequence optimization also involves removing potential liabilities such as *in silico* predicted deamidation sites, aspartate isomerization sites and oxidation residues.

2.2. Bispecific formatting of lead molecules

The variable region sequences from humanized anti-IL13 (clones: 5D5 and 242) and anti-TSLP (clone: I019) antibodies were formatted either as monovalent IgG-like scaffold (Zweimab, ZM) or as IgG-like bivalent scaffold (Doppelmab, DM). Cloning was accomplished by standard molecular biology technologies on to a CMV(cytomegalovirus)-promoter based vector. Both orientations (i.e. Vk-linker-VH and VH-linker-Vk) for the scFv modules in the Doppelmab scaffold to identify scFv's (single chain fragment variable) with optimal molecular attributes. All these clones were formatted as human IgG1 isotypes with effector function deficient mutations (Leu234Ala and Leu235Ala) called as "IgG1KO" that eliminate ADCC (Antibody-Dependent Cell Mediated Cytotoxicity) and CDC (Complement Dependent Cytotoxicity) activity by reducing effector functions such as FcγR and complement binding.

2.3. Molecular affinity to the targets

2.3.1. General biosensor methods

ProteON XPR36 (Bio-Rad) sensor chips (GLM) and amine coupling reagents (EDC, sulfo-NHS and 1 M Ethanolamine-HCl) were purchased from BioRad. Recombinant Protein A/G was purchased from Thermo Fisher Scientific and Human Fab Capture Kit was purchased from Ge Healthcare Life Sciences. All interaction analysis was performed at 25 °C in a running buffer of phosphate buffered saline (PBS), pH 7.4, 0.005% Tween 20, 3 mM EDTA. Kinetic data was double-referenced and fit globally to a simple 1:1 Langmuir model using the ProteON Manager software.

2.3.2. Molecular affinity of chimeric and humanized leads

Recombinant Protein A/G was immobilized to the dextran matrix of a GLM sensor chip along 6 horizontal channels using an amine coupling kit at a surface density of approximately 5000 resonance units (RU). Each antibody was captured on the Protein A/G surface along 6 vertical channels at a surface density of approximately 800 RU for the TSLP antibodies and 1800 RU for the IL13 antibodies. The binding kinetics was determined by the global fitting of human and cyno TSLP injections at 6 dilutions (20, 10, 5.0, 2.5, 1.25 and 0 nM) at a flow rate of 30 μL/min for 300 s with a dissociation time of 3600s. The binding kinetics of human and cyno IL13 for the chimeric and sequence optimized antibodies were determined by the global fitting of human and cyno IL13 injections at 6 dilutions (5.0, 2.5, 1.25, 0.625, 0.3125 and 0 nM) at a flow rate of 25 μL/min for 900s with a dissociation time of 7200 s. Same method was used for measuring the affinity of Zweimab molecules.

2.3.3. Molecular affinity of TSLP-IL13 doppelmabs (DM)

Due to the location of the scFv on the TSLP-IL13 DM constructs, both Protein A/G and human Fab capture-based assay formats were used to ensure that capture via Protein A/G did not impact the binding kinetics of the scFv. In the first method, recombinant Protein A/G was immobilized to the dextran matrix of a GLM sensor chip at a surface density of approximately 5000 RU and each DM

construct was subsequently captured on the Protein A/G surface at a surface density of approximately 1500 RU. In the second method, anti-human Fab antibody was amine-coupled to the dextran matrix of a GLM sensor chip along 6 horizontal channels at a surface density of about 7000 RU and each DM construct was captured on the anti-human Fab surface at a surface density of about 900 RU.

2.4. Isolation of primary human dendritic cells and stimulation by TSLP/IL13

Primary human blood dendritic cells (DCs) were isolated from buffy coats of healthy adult volunteer blood donors with informed consent (German Red Cross blood bank, Ulm, Germany). Briefly, PBMCs were obtained by centrifugation on 50 ml Leukosep tubes (Greiner Bio-one) and untouched myeloid DCs were isolated by MACS using the Myeloid Dendritic Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany). DCs were cultured immediately after isolation in 96 well flat bottom plates at 5×10^4 cells/well in RPMI1640 containing 10% FCS, 1% pyruvate, and 1% HEPES and were stimulated with human or cyno TSLP (0.5 ng/ml), or with human or cyno IL13 (3 ng/ml; Sino Biological), or a combination of TSLP and IL13. For potency determinations of lead molecules, dose-response curves were generated by adding increasing concentrations of antibodies (typically ranging from 0.05 ng/ml to 50 µg/ml) to the DCs in parallel with respective stimuli. After 72 h, concentrations of TARC (CCL17) in culture supernatants were determined by ELISA (R&D Systems).

3. Results and discussion

3.1. Identification of potent anti-TSLP and anti-IL13 lead molecules

TSLP and IL13 are key cytokines that trigger Type 2 immunity and are promising targets to treat airway diseases. AMG157 is so far, the major anti-TSLP player that showed greater efficacy in patients with severe asthma [31]. Unlike TSLP, there are several anti-IL13 neutralizing antibodies that have been tested in clinical trials for patients with severe asthma, but unfortunately exhibited poor efficacy in Phase 3 trials. We have identified potent lead molecules that bind and antagonizes both cytokines effectively. The SPR based affinity data shows that both chimeric and humanized mono anti-TSLP lead molecules have double digit pM binding values to the human and cyno targets (Table 1A). Similarly, anti-IL13 lead molecules exhibited tight binding to the human IL13 target while the K_D values to the cyno-IL13 were within acceptable range for monkey PK/PD dosing studies (Table 1B). In the in-vitro functional assays, molecules are highly potent with EC50 values in picomolar in inhibiting the production of TARC, a chemoattractant of Th2 cells and Eotaxin, chemoattractant for eosinophils that plays a key role in airway inflammation (Table 1C).

Humanized anti-TSLP (BI763871) has 1 potential liability which is isoaspartic isomerization site (DG) in the heavy chain CDR3 (Complementarity Determining Region 3). Anti-IL13 BI763870 (5D5) and BI763869 (242) have 3 deamidation (NY) hot spots in both heavy and light chain CDR1. There are several literature references that have shown that deamidation or isomerization in the CDRs have resulted in the loss of function of monoclonal antibodies [32,33] but only at high levels. Stressed molecules showed deamidation and isomerization below 5% in all CDR hot spots as analyzed by Mass spectrometry for the humanized antibodies (Table 3). Such a low level of modification may not have significant impact on in-vivo biological function. It is important to note that both Lebrikizumab and AMG157 have similar sequence liabilities in CDRs.

Table 1

(A) Molecular affinity of anti-TSLP (I019, chimeric) and (BI763871, humanized) lead molecules to human and cyno TSLP targets; AMG157 (Anti-TSLP) competitor antibody is included. (B) *Mouse/human chimeric; Molecular affinity of anti-IL13 (5D5B9 and 242H3H1, chimeric) and (humanized) lead molecules to human and cyno IL13 targets; Lebrikizumab (Anti-IL13) competitor antibody is included. (C) Potency values measured for both chimeric and humanized lead molecules against the targets by Dendritic cell assay (TSLP) and Eotaxin release assay (IL13).

A		
Molecule	K_D (pM) to HuTSLP	K_D (pM) to CyTSLP
I019	≤ 15.0	≤ 15.0
BI763871	23.4	24.0
AMG157	≤ 15.0	27.6
B		
Molecule	K_D (pM) to HuIL13	K_D (pM) to CyIL13
BI763870	40.7	613.4
BI763869	20.1	215.2
*242H3H1	45.0	362.2
*5D5B9	< 15.0	24.2
Lebrikizumab	155.0	21.8
C		
Molecule	IC ₅₀ (pM) to HuTSLP	IC ₅₀ (pM) to HuIL13
I019	71	N/A
BI763871	135	N/A
5D5B9	N/A	36
BI763870	N/A	110
242H3H1	N/A	232
BI763869	N/A	50

3.2. Epitope mapping of lead molecules

Epitope mapping was performed on the lead molecules by hydrogen-deuterium exchange mass spectrometry as explained elsewhere [34] and shown in the crystal structures of both targets (Fig. 2A and B). Recent binding studies have shown that the binary complex formation between TSLP and TSLPR is a mechanistic prerequisite for recruiting IL7R α and hence enabling TSLP signaling [35]. AMG157 is shown to bind to an epitope on TSLP that effectively blocks the binding to TSLPR but remains clear of the IL7R α binding site on TSLP. Our lead molecule is shown to bind in an analogous way but to a distinct epitope on TSLP that antagonizes the activity of TSLP (Fig. 2A). Likewise, it is well known that IL13 signals through heterodimeric complex that is formed by IL13R α -1 and IL4R α . Another signaling mechanism of IL13 is through direct interaction with IL13R α 2 which is also termed as decoy receptor. Clone 5D5B9 binds to the region of IL13 in such a way that there is a steric hindrance caused to diminish the binding of IL4R α to IL13 (like Lebrikizumab) while clone 242H3H1 has a distinct epitope that blocks the direct interaction of IL13R α 2 to the target (like Tralokinumab) (Fig. 2B). One of the recent studies identified similar anti-IL13 mAbs with distinct epitopes exhibiting different mechanism of actions for inhibiting IL13 signaling [36]. Their first-in-human single dose pharmacokinetic study showed that both mAbs had similar PK profile however very different total IL13 profiles. While there is no clear data exists so far regarding the preferred mechanism of action for anti-IL13 molecules, our current data shows that both mAbs exhibit similar binding and functional profiles.

3.3. Zweimabs and Doppel-mabs

Zweimabs and Doppel-mabs (Fig. 1A and B) were constructed, expressed and purified to high quality. For details, please refer to "Multi-specific binding proteins" patent published recently by Rajkumar Ganesan et al. (WO/2017/004149). Briefly, Zweimabs

Table 2
(A) Molecular affinity of human and cyno TSLP and IL13 soluble proteins for Zweimab molecules are shown in this table as measured by SPR. (B) Molecular affinity of human and cyno TSLP and IL13 for Doppelmab constructs are shown above. Affinity values determined by both *Protein A/G capture format* and *Human FAB capture format* are shown for all molecules. BI733871/BI763870: anti-TSLP as HulgG1KO/anti-IL13 as scFv; BI763869/BI763871: anti-IL13 as HulgG1KO/anti-TSLP as scFv. (C) IC₅₀ values measured from in-vitro functional assay for ZM and DM constructs.

A				
Molecule	K _D (pM) to HuTSLP	K _D (pM) to CyTSLP	K _D (pM) to HuIL13	K _D (pM) to CyIL13
BI763871/BI763870	23.5	25.7	17.2	388.0
BI763871/BI763869	≤15.0	31.0	≤15.0	121.0
B				
Molecule (Fab/scFv)	K _D (pM) to HuTSLP	K _D (pM) to CyTSLP	K _D (pM) to HuIL13	K _D (pM) to CyIL13
BI763870/BI763871	23.5	25.7	17.2	388
BI763869/BI763871	≤15.0	31.0	≤15.0	121
BI763871/BI763869	47.4	43.9	≤15.0	440
BI733871/BI763870	40.5	45.4	≤15.0	134
BI763870/BI763871	85.7	111	53.3	306
BI763869/BI763871	72.3	88.9	≤15.0	88.5
BI763871/BI763869	37.6	41.2	≤15.0	72.8
BI733871/BI763870	36.1	39.7	38.3	242
C				
Molecule (Fab/scFv)	IC ₅₀ (pM) to HuTSLP	IC ₅₀ (pM) to CyTSLP	IC ₅₀ (pM) to HuIL13	IC ₅₀ (pM) to CyIL13
BI763871/BI763870	124.5	171.7	109.0	930.0
BI763871/BI763869	96.9	107.0	93.0	980.0
BI763871/BI763869	152.0	159.0	47.0	1327.0
BI763871/BI763870	247.0	132.0	112.0	1834.0
BI763869/BI763871	630.0	479.0	66.0	1647.0
BI733870/BI763871	2847.0	583.0	135.0	1109.0

Table 3
(A) Sequence liabilities in anti-TSLP lead molecule CDRs are shown here. Also, % deamidation and isomerization determined from Mass spectrometry for both unstressed and day 7.0 stressed samples are provided. (B) Deamidation liabilities in anti-IL13 lead 242H3H1 in CDRs are highlighted along with % deamidation from Mass spectrometry analysis. (C) Deamidation liabilities in anti-IL13 lead 5D5B9 in CDRs are highlighted along with % deamidation from Mass spectrometry analysis.

A			
Liability	Site (s)	Unstressed (%)	Day 7 (%)
Deamidation	HC-CDR2 (NY, NQ)	1.1	2.2
Deamidation	LC-CDR1(NI)	0.5	0.8
Iso-Asp	HC-CDR2 (DP, DT, DT)	ND	ND
Iso-Asp	HC-CDR3 (D101, D104)	0.6	4.0
B			
Liability	Site (s)	Unstressed (%)	Day 7 (%)
Deamidation	LC-CDR1 (NY)	ND	<5%
Deamidation	HC-CDR2 (NP)	ND	ND
C			
Liability	Site (s)	Unstressed (%)	Day 7 (%)
Deamidation	LC-CDR1 (NY, NW)	4.0	4.5
Deamidation	HC-CDR1(NY)	ND	<5%
Deamidation	HC-CDR3 (NF)	ND	ND

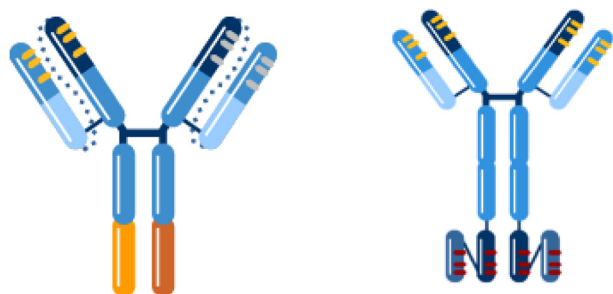


Fig. 1. (A) both light and heavy chains are maintained by a polypeptide linker. (B) The scFv moiety is tethered to CH₃ domain by a short polypeptide linker in DM.

(Zwei means two in German representing two target binding) are asymmetric IgG-like bispecifics that are obtained via the

heterodimerization of the Fc domain. The binding units are monovalent for each antigen. These bispecifics have Fc-based scaffold that enables traditional Protein-A purification and 2-chain for expression that ensures ease of manufacturability and minimizes heterogeneity such as light-chain homo-dimers. On the other hand, Doppelmabs (*Doppel* means *double* in German representing bivalent binding) are symmetric IgG-like bispecifics that retain the canonical Fc domain like the traditional monoclonal antibodies. Often, the binding units are bi-valent for each antigen. There is a scFv fusion to the light chain of Fab and to the Fc domain. Doppelmabs are purified by standard Protein-A purification and bivalency would aid in improved “avidity driven” binding affinity. Both Zweimabs and Doppelmabs were expressed in CHO-E (Chinese Hamster Ovarian-E) cells with an average titer of 40 mg/L. Final purified antibodies were >99% monomer and were stored in 60 mM Sodium Acetate, 150 mM NaCl, pH 5.0 at 4 °C.

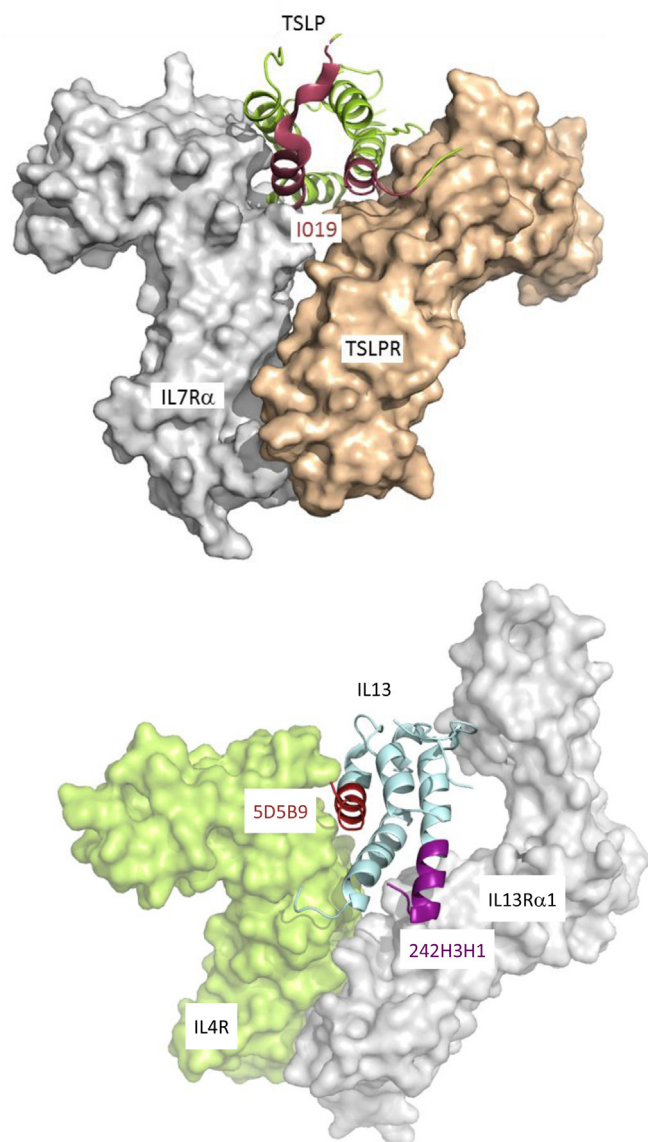


Fig. 2. (A) Epitope mapping information for clone IO19 (anti-TSLP) is highlighted in the crystal structure of TSLP/TSLPR/IL7Rα complex (PDB Code: 5J11). TSLP (Green), TSLPR (Gold) and IL7Rα (Grey) are shown here. Clone IO19 (MAROON) binds directly to TSLP neutralizing the activity of the target. (B) Epitope mapping information for clones 5D5B9 and 242H3H1 highlighted in the crystal structure of IL13-IL4R-IL13Rα ternary complex (PDB: 3BPO) [37]. IL13 is shown in cyan; IL13Rα1 is shown in grey while IL4R is shown in green. The mechanism of action for clone 5D5B9 (RED) is through steric hindrance towards the binding of IL4R to IL13 while the mechanism of action for clone 242H3H1 (PURPLE) is the blockade of IL13Rα1 binding to IL13.

Molecular affinity and potency of all bispecific antibodies is provided in Table 2. For Doppelmabs, irrespective of the capture format, strong affinity is shown by the antibodies to the targets. It is well known that initial cytokine-receptor binding followed by the recruitment of the second receptor ends up forming a tight ternary complex on the cell surface to trigger signaling in both TSLP and IL13 pathways [37,38]. Bispecific molecules have shown strong affinity and inhibition of target mediated cytokine and chemokine release in the dendritic cell assays meeting the desired set criteria for further development. However, loss of potency was observed for cyno IL13 (both Zweimab and Doppelmab). Species cross-reactivity is important to select the pharmacologically relevant species for toxicology studies since drug is expected to trigger similar

responses in the species that is tested as in humans. However, a drug with relatively lower potency for cyno targets may still be used if the antibody can provide complete inhibition of the target at each dosing interval in the monkey pharmacokinetics studies.

In summary, both TSLP and IL13 have emerged as important players in cellular signaling in asthma that is characterized by Th2 inflammation. Due to great overlap of downstream signaling effects of both cytokines in severe asthma we decided to make bispecific antibody against these two attractive targets. Our lead molecules have shown tight binding and function to the targets when formatted as both ZM and DM. Interestingly, when anti-TSLP was formatted as scFv in Doppelmab, we observed a four-fold reduction in the binding and function to the human target. Inherently scFvs are known to have an impact on binding to the target due to structural flexibility as compared to Fab. At this point, we did not choose to further investigate the reason behind this since we have successfully shown that dual targeting is highly possible without compromising tight engagement of the targets even as monovalent bispecific molecules.

Author contributions

K.J.E, B.W. are the project leaders. S.L is the co-project leader. K.J.E wrote the manuscript. P.G was responsible for TSLP and R.G was responsible for IL13 antibody engineering. R.G did the modeling for Fig. 2. D.D was responsible for antibody generation. K.J, M.Z.M and R.K.B designed, developed and ran all SPR experiments. L.F provided Mass Spectrometry experimental support. All authors provided contributions by commenting on the manuscript at all stages. R.G and S.S conceptualized and designed the ZM and DM constructs.

Disclosure statement

At the time when this research work was conducted, all authors were employees of Boehringer Ingelheim Pharmaceuticals Inc.

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Transparency document

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