Introduction

The value of combining treatments to impact multiple targets in a disease pathway has been the focus of much recent interest. Bispecific antibodies (bsAbs) are dual-targeting antibodies that bind two distinct proteins and can minimize the regulatory and commercial issues that stem from administration of multiple therapeutic molecules (Hoggenboom, 1997; Fischer and Léger, 2007; Lum and Al-Kadhimi, 2008; Chames and Baty, 2009; Dimassi et al., 2009; Parren and Burton, 2009). bsAbs are commonly based on the structure of immunoglobulins (Ig). Incorporation of an Fc domain from IgG improves the half-life in serum and can provide antibody-like effector function, if desired. An extensive array of formats have been developed and tailored for affinity, valency, expression, stability and pharmacokinetics (Plückthun and Pack, 1997; Todorovska et al., 2003a). In recent reports, these bispecific molecules have been widely anticipated, attractive building blocks for the construction of bsAbs. The primary advantage of scFvs is the production of a single polypeptide chain, which obviates the difficulties that arise in pairing appropriately the separate chains of a heteromultimeric protein (Coloma and Morrison, 1997). Although engineered bsAbs have been reported for over two decades, difficulties with manufacturing related primarily to stability remain a significant obstacle to their development as therapeutics (Kontermann, 2005; Fischer and Léger, 2007; Demarest and Glaser, 2008). Typically, the construction of bsAbs has been approached through reconfiguration of monoclonal antibodies (mAbs) and antibody fragments, generating molecules which may not possess the same physical properties as the parental antibodies. This reformatting frequently creates issues related to affinity, stability and production (Holliger et al., 1993; Coloma and Morrison, 1997; Lu et al., 2003b) that may be resolved by additional engineering of the antibody moieties (Wörn and Plückthun, 2001; Demarest et al., 2006), a process that can prove labor-intensive and costly. Thus, it has been proposed that the construction of bsAbs requires the use of stable monomeric units as building blocks (Holliger and Winter, 1993; Demarest and Glaser, 2008; Saerens et al., 2008).

Because of their modular properties, antibody fragments based on variable light and heavy chains tethered by an amino acid linker [single-chain variable fragments (scFvs)] are attractive building blocks for the construction of bsAbs. The primary advantage of scFvs is the production of a single polypeptide chain, which obviates the difficulties that arise in pairing appropriately the separate chains of a heteromultimeric protein (Coloma and Morrison, 1997). Although therapeutic applications of scFvs have been widely anticipated, their development has been severely limited due to issues associated primarily with aggregation and serum stability (Jung et al., 1999; Lu et al., 2003a). In recent reports, these problems have been addressed by applying techniques designed to enhance stability through additional engineering steps that have successfully yielded clones with optimized
physical properties (Jung et al., 1999). However, attempts to identify stable, fully human scFvs in the absence of additional grafting or maturation techniques have resulted in few therapeutic candidates. A method for the early selection of stable, potent scFvs from large human libraries may serve as an alternative to rigorous engineering of the therapeutic candidate.

Phage display is the most widely used display platform for engineering therapeutic antibodies. Libraries have been constructed in several formats using variable regions isolated from a human repertoire (Marks et al., 1991) and/or synthetic complementary determining regions (CDRs) grafted onto human framework regions (Söderlund et al., 1995; Kobayashi et al., 1997; Knappik et al., 2000; Sidhu et al., 2004; Völkel et al., 2004; Hoet et al., 2005). These phage libraries facilitate the isolation of human antibodies, reducing the risk of immunogenicity associated with chimeric mAbs and avoiding the rigors of humanizing murine antibodies. Advances in library construction have resulted in theoretical diversities surpassing $1 \times 10^{10}$ unique sequences (Hoet et al., 2005; Rothe et al., 2007). Even so, the use of display technologies frequently fails to produce antibody fragments with the desired affinity, potency and/or stability. As a result, selection from phage libraries is often complemented with mutagenesis, shuffling, additional display technologies and other combinatorial procedures between iterative rounds of selection to improve affinity (Kang et al., 1991b; Jirholt et al., 1998; Maynard et al., 2002; Harvey et al., 2004; Groves and Osbourn, 2005). These techniques for affinity maturation typically focus on optimization of a single antibody fragment through the generation of new libraries for additional selections against target molecules. Although this process frequently yields binding entities with improved properties, the process is lengthy and labor-intensive. Selection of the desired properties in batch mode may expedite the engineering of bsAbs, bypassing affinity maturation of single clones. The early identification of stable scFvs that bind with high affinity to the target would result in more efficient generation of stable bsAbs for development of human therapeutics.

Stable bispecific molecules targeting two soluble cytokines offer a novel approach to enhance the therapeutic efficacy for inflammatory diseases. TH17 cells are a recently identified subset of T helper cells associated with several inflammatory diseases including multiple sclerosis, psoriasis, rheumatoid arthritis (RA) and inflammatory bowel disease (Dong, 2008; McGeachy and Cua, 2008). These cells produce a number of inflammatory mediators, including two homologous cytokines, IL-17A and IL-17F. Both molecules are found as homodimers (IL-17A/A and IL-17F/F) and as a heterodimer (IL-17A/F), all of which appear to mediate inflammatory responses associated with disease pathology (Fossiez et al., 1998; Wright et al., 2007). The cytokine IL-23 plays an important role in the differentiation and regulation of TH17 cells, resulting in production of a number of cytokines including IL-17A/A and IL-17A/F (McGeachy and Cua, 2007). IL-23 is a heterodimer consisting of a unique p19 subunit and a p40 subunit which is shared with IL-12. Inhibition of the activity of IL-23 on the TH17 pathway, while avoiding interruption of pathways mediated by IL-12, requires a reagent that interacts specifically with the p19 subunit (also referred to as IL-23A). A therapeutic molecule targeting both an expansion and a survival factor for TH17 cells, such as IL-23 and TH17 effector cytokines (IL-17A/A and IL-17A/F), could be more efficacious in treating a number of inflammatory diseases than molecules that inhibit activity mediated by a single cytokine.

In the construction of molecules targeting human IL-17A/A and the p19 subunit of IL-23, we addressed the challenges of bsAb generation by focusing on the identification of stable scFvs and improving the affinity of antibody fragments in batch mode. The resulting bispecific molecules exhibit the ability to bind and neutralize both cytokines, possess stability typical of mAbs and display serum half-lives similar to human IgGs. Assembly of bsAbs with stable antibody fragments as building blocks proved to be an effective method for the development of stable, dual-targeting therapeutic molecules directed against these two attractive targets.

**Materials and methods**

**Protein reagents**

IL-17A assay reagents, anti-IL-17A/A mAb (#MAB317) and IL-17A/A pAb (#AF-317 NA), were purchased from R&D Systems (Minneapolis, MN, USA). IL-23 assay reagents, IL-23 (#1290-IL/CF), IL-12 (#219-IL), IL-23R-Fc (#1400-IR-050), IL-23p19 mAb (#MAB1290), IL-23p19 pAb (#AF1716) and anti-IL-23p40 mAb (#MAB1510), were also purchased from R&D Systems. The IL-23p19 specific antibody was purchased from eBiosciences (#34-823895, San Diego, CA, USA). Recombinant human (h) proteins IL-17RA-Fc, IL-17A/A, IL-17A/F, IL-17F/F, IL-23R-Fc, IL-12 and IL-23 were produced at ZymoGenetics Inc. (Seattle, WA, USA).

**Phage panning and conversion of Fabs to scFvs**

General methods of panning against target antigen using the Dyax Fab libraries have been described previously (de Haard et al., 1999). Human IL-23 and IL-17A/A cytokines were used as single targets for screening the Dyax 310 human Fab library (Hoet et al., 2005). For immunotube panning, human IL-17A/A or IL-23 at a concentration between 10 and 100 µg/ml in PBS was coated in a polystyrene immunotube (#444474, Nalge Nunc International, Rochester, NY, USA). After rotating overnight at 4°C, immunotubes were blocked with the addition of 2 ml of blocking buffer (2% milk, PBS, 0.1% Tween 20) for 1 h at room temperature (RT) with rotation. Uncoated immunotubes were also blocked for depletion of phage binding non-specifically. For the first round of selections, aliquots of the Dyax library were added to blocking buffer to a volume of 1 ml and incubated at RT for 1 h with rotation. Phage were then added to one uncoated immunotube and incubated for 1 h at RT with rotation. Phage were transferred to the immunotube coated with target and incubated for 10 min to 1 h before washing. Phage were eluted from the immunotubes by the addition of 100 mM Triethylammonium, followed by neutralization with 1 M Tris–HCl, pH 7.4. Iterative rounds of panning (2–4 rounds) were performed for enrichment against each target with an increase in the number of wash steps (between 8 and 10 washes with PBS, 0.1% Tween 20) and variations in incubation times (5 min–1 h).

Phage output from each round was used to infect TG1 cells (TG1) (#200123, Stratagene, La Jolla, CA, USA) in...
a 4 ml culture containing 2 × YT supplemented with ampicillin (100 μg/ml). Cultures were shaken at 37°C to a final OD600nm of 0.5. For phage production, 0.3 ml of helper phage (#18 311-019, Invitrogen, Carlsbad, CA, USA) was added and the mixture was incubated at 30°C for 30 min without shaking. The mixture was then added to 44 ml of culture media (2 × YT supplemented with ampicillin at 100 μg/ml) and incubated at 37°C for 6 h with shaking. The temperature was adjusted to 30°C and the cultures were grown overnight with shaking. The following day, a 40 ml aliquot of the culture was removed and precipitated with PEG for phage purification. Phage pellets were resuspended in 1 ml of PBS, quantified and used as an input for iterative panning rounds or frozen away in 15% glycerol for later use.

For scFv panning, the selections were performed as described above with the integration of thermal treatment. Prior to each selection, the phage input (in PBS) was subjected to 1 h incubation at 60°C. Phage samples were then spun down at 16K rpm for 10 min and supernatants transferred to fresh 1.5 ml tubes. Phage were then blocked as described above prior to each panning round. One to two additional rounds of panning were performed after batch conversion of Fab clones (see Fab to scFv conversion).

**Preparation of bacterial supernatants for screening**

For soluble expression of Fabs in scFvs, DNA representing the phage output against each target was digested to remove the gIII fusion partner and expressed as a soluble Fab protein in the pMID21 vector (GenBank AY75424). Re-ligated vectors were transformed into TG1 cells (TG1) (#200123) and plated at several dilutions. Single colonies were picked (QP Display, Genetix, Boston, MA, USA) into a 96-well plate (Corning, #3359) containing 100 μl of media [2 × YT media, 2% glucose, ampicillin (100 μg/ml)]. Plates were grown overnight at 30°C with shaking. The next day, each plate was subcultured into a new plate (Corning, #3956) containing 800 μl of fresh media [2 × YT media, 0.1% Triton X 100, ampicillin (100 μg/ml)] per well and grown for 2.5 h at 30°C with shaking. Cultures were induced with 1 mM IPTG for expression and cultured for 15 h at 30°C with shaking. Plates were then centrifuged at 6000 rpm for 10 min, and supernatants containing secreted scFv or Fab were transferred to new 96-well plates for analysis.

**ELISAs for primary screening of bacterial supernatants**

For binding ELISAs, Costar (#9018) 96-well plates were coated with 50 μl of anti-human IgG Fcγ-specific antibody (#109-005-098, Jackson Immunology) at 1 μg/ml in 0.1 M NaHCO3, pH 9.6 overnight at 4°C. The next day, plates were washed three times with 0.1% PBST, and the volume of the supernatant/target complex transferred from the pre-incubation plate to the assay plate, followed by incubation at RT for 1 h. Plates were then washed three times with PBST, and the volume of the supernatant/target complex incubated at 37°C for 30 min. For blocking ELISAs, Costar (#9018) 96-well plates were coated with 50 μl of anti-human IgG Fcγ-specific antibody (#A7058, Pierce) diluted 1:4000 in 2% milk/PBST, and the volume of the supernatant/target complex transferred from the pre-incubation plate to the assay plate, followed by incubation at RT for 1 h. Plates were then washed three times with PBST. Fifty microliters of TMB (TMBW-1000-01, BioFX Laboratories) were added to each well and the reaction was allowed to develop for 20–30 min, followed by the addition of 50 μl of stop buffer (STPR-1000-01, BioFX Laboratories) to quench the reaction. Plates were read at 450 nm on a plate reader.

For blocking ELISAs, Costar (#9018) 96-well plates were washed three times with PBST. Fifty microliters of TMB (TMBW-1000-01, BioFX Laboratories) were added to each well and the reaction was allowed to develop for 20–30 min, followed by the addition of 50 μl of stop buffer (STPR-1000-01, BioFX Laboratories) to quench the reaction. Plates were read at 450 nm on a plate reader.

**Fab to scFv batch conversion**

Once specific binding was verified for each round, Fab cDNA served as a template for batch amplification of the light and heavy chain variable regions. Lambda, kappa and heavy chain variable regions were amplified from pooled DNA taken from rounds 2 and 3 of phage panning. The DNA served as a template in a three-step process using primers directed against framework sequences for each subtype. The primers were designed to complement the terminal sequences of all identified human framework regions 1 and 4 (www.kabatdatabase.com). The first PCR step amplified each of the variable frame work regions and added appropriate overhangs to facilitate round 2 PCR reactions. The second PCR step added appropriate gly/ser linker sequences to the ends of the appropriate products from the first and step 3 PCR reactions overlapped the variable light chain lambda, variable light chain kappa and variable heavy chain products to create scFv products in both LH and HL orientations with a 25 mer G4S linker between the V regions. Assembled scFvs were cloned into a phagemid vector as a gIIp fusion. DNA ligations were transformed into TG1 cells (TG1) (#200123) and added to a 200 ml culture of 2 × YT supplemented with ampicillin (100 μg/ml) to a final OD600nm of 0.2. For phage production, 2 ml (>2 × 1011 pfu) of helper phage (#18311-019, Invitrogen) was added and the culture incubated at 37°C for 6 h with shaking. The temperature was then adjusted to 30°C and the cultures were grown overnight with shaking. The next day, 2 × 50 ml
 aliquots of culture were taken from phage culture and precipitated with PEG for phage purification. Phage pellets were resuspended in 1 ml of PBS, quantified and used as phage input for iterative panning rounds or frozen away in 15% glycerol for later use.

**Protein expression and purification**

Selected Fab or scFv clones were expressed from a proprietary vector utilizing the phoA promoter in either BL21 (#69449-4, Novagen, Madison, WI, USA) or TG1 cells (#200123). After transformation, colonies were selected for inoculation into 2 ml Luria–Bertani medium supplemented with antifoam at 100 µg/l (#A8311, Sigma-Aldrich, St Louis, MO, USA) and kanamycin (#60615, Sigma-Aldrich) at 25 µg/ml. Cultures were grown overnight at 37°C, shaking at 250 rpm. The overnight cultures were diluted to 0.2% for inoculation into 0.5 l of phosphate-limiting media (Simmons et al., 2002) supplemented with antifoam and antibiotic as above. Cultures were grown in 2 l side-baffled flasks for 24 h at 30°C. Soluble scFv and Fab samples were recovered from wet cell pellets by treatment with periplasmic buffer containing Ready-Lyse lysozyme (#R1802, Epicentre Biotechnologies, Madison, WI, USA) as per manufacturer’s instructions in the ‘Protocol for the Preparation of Periplasmic and Spheroplastic Proteins from >1 ml Bacterial Culture’ (Epicentre Biotechnologies).

For the immobilized metal affinity chromatography enrichment of scFvs, the periplasmic fraction was passed through a 0.22 µm filter and purified by affinity capture with a HiTrap HP column (GE Healthcare, Piscataway, NJ, USA) on a liquid chromatography instrument (Akta Explorer System, GE Healthcare). The bound protein was eluted using 0.4 M NaCl in 50 mM NaPO4 and 500 mM NaCl at pH 7.4. Protein content was by absorbance at 280 nm and quality was assayed by analytical size exclusion chromatography (SEC) and SDS–PAGE. For Fab enrichment, affinity purification was performed using Protein A (#17-1279-04, GE Healthcare) in batch mode. The Protein A resin was added to the periplasmic extract for overnight incubation at 4°C with mixing. The following day, the protein was recovered using a low pH step elution with 50 mM NaH2PO4 pH 2.5, 150 mM NaCl, immediately neutralized with 1 M HEPES pH 7.2, and evaluated as described above.

The three forms of bsAbs were assembled by a combination of PCR and homologous recombination in yeast using a proprietary mammalian expression vector with DHFR as the selectable marker. The expression constructs were electroporated into CHO DXB11 cells, adapted to grow in suspension in a protein-free medium, and subjected to nutrient selection in HT medium followed by selection in methotrexate. Pools of selected cells were scaled up in spinner flask culture for purification and analysis of bsAbs. Conditioned media were harvested, passed through a 0.2 µm filter and adjusted to pH 7.4. The protein was purified from the filtered media using a combination of POROS® A50 Protein A Affinity Chromatography (Applied Biosciences, Foster City, CA, USA) and Superdex 200 SEC (GE Healthcare). Column fractions were analyzed by SDS–PAGE to determine the appropriate pools. Enriched protein was quantified by UV at A280 nm and an analytical SEC method was used to characterize the purified protein.

**Kinetic analysis**

The binding affinities of Fab and scFvs to IL-17A/A, IL-17/F/F, IL-23 and IL-12 were evaluated using surface plasmon resonance (SPR) on a Biacore T-100 instrument (GE Healthcare). For affinity analysis, recombinant human target proteins were diluted to 10 µg/ml in 10 mM acetate (pH 4.0 for IL-17A/A and IL-17/F/F, pH 4.5 for IL23 and IL12) and then immobilized onto CM5 chip (GE Healthcare/ Biacore#BR-1006-68) using amine coupling. Briefly, the carboxymethyl dextran surface was activated with a 7 min injection of a 1:1 ratio of 0.4 M EDC [N-ethyl-N'- (3-diethylamino-propyl) carbodiimide] and 0.1 M NHS (N-hydroxysuccinimide). The final immobilization levels were between 300 and 480 resonance units (RUs). After the immobilization procedure, active sites on the flow cell were blocked with 1 M ethanolamine. The reference cell was activated and then blocked with ethanolamine prior to sample analysis. For measuring association and dissociation kinetics, serial dilutions of the anti-IL-17A/A or IL-23 antibodies were prepared in 1× HBS-EP+ buffer (GE Healthcare/ Biacore, #BR-1006-69). Duplicate injections of each concentration were performed at 30 µl/min with an association time of 300 s and a dissociation time of 600 s. Between experiments, the immobilized IL-17A/A surface was regenerated with a 60 s injection of 10 mM H3PO4 followed by a 60 s injection of buffer at 50 µl/min. The optimal regeneration condition for IL-23 analysis was a 60 s injection of 2 M MgCl2 followed by 60 s of buffer injection at a flow rate of 50 µl/min.

For analysis of crude supernatants, the chip surfaces were prepared as described above. Unless otherwise indicated, all binding experiments were performed at 25°C in 1× HBS-EP+ buffer supplemented with, 0.1% BSA. IL-23 or IL-17A/A supernatants were diluted 1:3, and 100 nM of IL-23R and IL-17RA was prepared. Analyte was injected at 30 µl/min with an association time of 300 s and a dissociation time of 300 s. Regeneration conditions were adapted from previous kinetic analysis of IL-23 and IL-17A/A as described above. Crude samples were ranked first according to their off-rates (slowest to fastest) and then according to their binding levels (highest to lowest binding RUs) during the association phase. These values were compared with positive controls using IL-23R or IL-17RA.

Analysis of tetravalent bsAbs was performed using an Fc capture to obtain binding affinities for the ligands. Goat anti-human IgG Fc-gamma (Jackson ImmunoResearch, West Grove, PA, USA) was covalently immobilized on a CM4 chip (GE Healthcare/Biacore, #BR-1005-30) to a density of ~4000 RU using amine coupling as described above. Each IL-17/IL-23p19 antagonist was captured onto a separate flow cell of the CM4 chip at an approximate density of 80–100 RU. Capture of the antagonist to the immobilized surface was performed at a flow rate of 10 µl/min. For binding studies with IL-17A/A, serial 1:3 dilutions of the IL-17A antigen from 20 to 0.03 nM were injected over the surface. For binding studies with IL-23, serial 1:3 dilutions of the IL-23 antigen from 50 to 0.07 nM were injected over the surface. Duplicate injections of each antigen concentration were performed with an association time of 7 min and a dissociation time of either 10 or 60 min. Kinetic binding studies were performed with a flow rate of 30 µl/min. The
flow cell was washed between cycles with 20 mM hydrochloric acid to regenerate the surface.

For co-binding analysis of bispecific constructs, recombinant human IL-17A/A was immobilized onto a CM5 sensor chip using amine coupling as described above. Each IL-17/IL-23p19 antagonist was diluted to 100 nM and injected over the immobilized IL-17A/A surface at a flow rate of 10 µl/min for 5 min. The capture level of the IL-17A/IL-23 antagonists ranged from 330 to 910 RU. A saturating concentration of 500 nM IL-23 was then flowed over the surface. Binding of IL-23 was performed for 10 min with a flow rate of 10 µl/min. Regeneration conditions were adapted from previous kinetic analysis of IL-17A/A as explained above.

All data were evaluated using Biacore Evaluation software to define either the affinity or the off-rate of the interactions. Baseline stability was assessed to ensure that the regeneration step provided a consistent binding surface throughout the sequence of injections. Binding curves were normalized by double-referencing and the resulting binding curves were globally fit using the Biacore Evaluation Software v1.1.1.

Cell-based assay data analysis

All cell-based assay data were analyzed using GraphPad Prism version 4 software (San Diego, CA, USA) with a sigmoidal (four-parameter logistics) non-linear regression curve fit, and the amount of antagonist needed to neutralize 50% of the activity of each ligand was calculated (IC50).

IL-17A cell-based assays

For short-term analysis of the antibody fragments, NIH 3T3 mouse fibroblast cells stably expressing an NFκB Luciferase reporter were used. Cells were seeded at 10 000 cells/well in 96-well tissue culture plates, in DMEM, 3% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM l-glutamine. After overnight incubation at 37 °C, 5% CO2, plating medium was removed and replaced with DMEM, 0.1% BSA, 1 mM sodium pyruvate, 2 mM l-glutamine, 25 mM HEPES buffer. In a separate plate, hIL-17A/A at a concentration of 1.5 nM was pre-incubated for 30 min at 37 °C with 3-fold serial dilutions of antagonists. The IL-17A/A ligand (0.75 nM final assay concentration) plus antagonists were then added to the cells and incubated at 37 °C, 5% CO2 for 4 h. After incubation, cells were lysed in 25 µl/well of Flash Luciferase lysis buffer (E-1501, Promega Corp, Madison, WI, USA) and read on a Berthold Centro XS3 luminometer (Berthold Technologies, Wildbad, Germany) with addition of 40 µL/well Flash Luciferase substrate (E-1501) and 5 s integration of signal.

For long-term analysis in a primary cell assay, human small airway epithelial cells (SAEC) [cells, SAEC Basal Medium, and SAEC Singlekit kits (Lonza, Walkersville, MD, USA)] were plated at 8000 cells/well in 96-well flat bottom tissue culture plates and incubated overnight at 37 °C, 5% CO2. The following day, cells were treated with a dose range of the antagonist in combination with 0.25 mM hIL-17A/A. The dose ranges of the antagonists were 0.001–300 nM for Fabs, 0.002–200 nM for scFvs (except for AP1733 scFv which was tested at 0.004–60 nM) and 0.0018–30 nM for the bispecific molecules. Ligand and antagonist were incubated together for 30 min at 37 °C before adding to the cells. Triplicate wells were set up for each dose. After 24 h, supernatants were collected, and stored at −80°C, if not used immediately. Owing to the sensitivity of primary SAEC to growth conditions and manipulation, prior to collection of supernatants, the health of the SAEC monolayer was assessed by visual evaluation on an inverted microscope. Any wells exhibiting significant cell death were excluded from analysis. Supernatants were assayed using a Bio-Plex singleplex bead-based assay for cytokine huG-CSF (Bio-Rad Laboratories), and read on a Bio-Plex array system utilizing Bio-Plex Manager 3.0 (Bio-Rad Laboratories).

IL-23 cell-based assays

For short-term analysis of the antibody fragments, BaF3 mouse B-cells stably expressing the human IL-23RA and IL-12RB1 receptors were seeded at 50 000 cells/well in 96-well tissue culture plates in RPMI 1640, 10% FBS, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 µM BME. In a separate plate, hIL-23 at a concentration of 0.1 nM was pre-incubated for 30 min at 37 °C with 3-fold serial dilutions of antagonists. The IL-23 ligand (0.05 nM final assay concentration) plus antagonists were then added to the cells and incubated at 37 °C, 5% CO2 for 15 min. After incubation, the reaction was stopped and cell lysates were created using a Bio-Plex Cell Lysis Kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. Cell lysates were analyzed for the presence of phosphorylated STAT3 on a Bio-Plex Array Reader (Bio-Rad Laboratories) according to the manufacturer’s instructions for the Bio-Plex Phospho-STAT3 Assay.

For long-term analysis in a primary cell assay, a single cell suspension of murine splenocytes was prepared from whole spleens harvested from C57BL/6 or BALB/c mice. After red blood cell lysis with ACK buffer (0.010 M KHCO3, 0.0001 M EDTA, 0.150 M NH4Cl, pH 7.2), splenocytes were washed and resuspended in RPMI buffer (containing 10% FBS, 1 mM sodium pyruvate, 2 mM l-glutamine, 0.1 mM non-essential amino acids, 2.5 mM HEPES, 0.00035% 2-mercaptoethanol Pen-Strep and 50 ng/ml human IL-2 (R&D Systems). Cells were seeded at 500 000 cells per well in a 96-well round-bottom plate. In a separate plate, hIL-23 at a concentration of 10 pM was pre-incubated for 30 min at 37 °C with 3-fold serial dilutions of antagonists at concentrations ranging from 0 to 343 nM. The IL-23 ligand plus antagonists were then added to the splenocytes and incubated at 37 °C, 5% CO2 for 24–72 h. The supernatants were collected and frozen at −80°C until ready to process. The levels of murine IL-17A/A, IL-17F/F, IL17A/F and GM-CSF protein in the supernatants were measured using bead-based sandwich ELISAs. A commercial kit (Beadlyte Cytokine Detection System, Millipore Scientific, Bellerica, MA, USA) was used to measure concentrations of IL-17A/A and GM-CSF produced. A bead-based ELISA using an antibody to mouse IL-17F/F (R&D Systems) conjugated to a bead was used to measure murine IL-17F/F. A combination of the commercial beads used to measure IL-17A/A with a secondary antibody to IL-17F/F (R&D Systems) was used to measure murine IL-17A/F heterodimer production.

Protein characterization

SEC multi-angle light scattering (SEC-MALS) analysis was incorporated into the characterization of protein to provide an absolute measurement of molecular weight from static light scattering in-line with SEC. Samples were analyzed
with the three detectors connected in series with UV first (Agilent 1100 HPLC with diode array detector), followed by the LS (Wyatt Technology DAWN EOS Multi-angle laser light scattering detector) and RI (Wyatt Technology Optilab REX Differential Refractometer) detectors. For sample analysis, 150 µg of each sample was injected to the SEC-MALS system (Amersham Pharmacia Superdex-200 SEC column, 10 × 300 mm) in a mobile phase consisting of 25 mM histidine, 150 mM NaCl at pH 6.8 at a flow rate of 0.5 ml/min run at ambient temperature. Data were analyzed with Wyatt Technology Astra software, version 5.3, according to the Three-detector Method (Wen et al., 1996).

Differential scanning calorimetry (DSC) measurements were performed using a single-cell VP-DSC instrument (MicroCal, Northampton, MA, USA) at a heating rate of 1°C/min for bispecific molecules and 1.5°C/min for single chain Fv. Working concentrations of these molecules were 0.35 mg/ml. Samples were buffered exchanged over a Superdex 200 column and a single peak of the target protein was collected, concentrated and filtered. The diluted was collected prior to injection of a bispecific molecule, and was also used as a reference blank for DSC analysis. All samples were stored frozen at −80°C until analyzed. Samples were thawed at RT, diluted to 0.35 mg/ml according to calculated extinction coefficient, and degassed prior to analysis.

The propensity of the antibody fragments and bsAbs to aggregate was evaluated using dynamic light scattering (DLS), a technique that measures the size of molecules in solution as a function of diffusion. Owing to the extreme sensitivity to very large species, DLS can be used to reliably quantify large aggregate down to at least 0.01% by weight in a sample containing a range of masses. Each of the bsAbs was analyzed in a generic buffer solution (25 mM histidine, 125 mM NaCl, pH 6.8). No attempt was made to improve stability through formulation. Samples were stored at −80°C prior to thawing and underwent one freeze–thaw cycle. Each sample was thawed at RT and scanned after two different incubation conditions in consecutive order: overnight incubation at 5°C and incubation for 1 h at 37°C. Sample concentration was normalized to 1.3–1.7 mg/ml for low concentration analysis by dilution in the standard buffer. All samples were centrifuged at 14K rpm prior to DLS analysis (DynaPro Plus Platereader, Wyatt Technology).

**Pharmacokinetics**

Unanaesthetized female CD-1 mice were administered 100 µg of bsAb via intravenous tail injection. Whole blood was collected via cardiac puncture under isoflurane anesthesia at pre-designated time points out to 504 h (3 weeks) post-dose (n = 3 mice/time point). Serum was generated from each sample and stored at −80°C until analyzed. The serum concentration versus time profiles as determined by binding ELISAs (mentioned above) were subjected to non-compartmental pharmacokinetic analysis using WinNonlin 5.0.1 (Pharsight, Inc., Mountain View, CA, USA). The terminal half-life (t1/2,∞) was calculated from the slope of the regression line that best fit the terminal portion of the log-linear concentration versus time curve.

**Results**

**Characterization of antibody fragments**

Identification and kinetic screening Dyax libraries (Hoet et al., 2005) were employed for isolation of Fabs that antagonize the binding of human IL-17A and human IL-23p19 to their respective receptors. Supernatants from bacterial clones expressing soluble Fab antibody fragments enriched through the panning process were screened by ELISA for competition with the binding of IL-23p19 or IL-17A/A to the appropriate receptor. DNA sequence analysis of antibody fragments that blocked the binding of IL-17A/A to the soluble receptor IL-17A/Fc and did not cross-react with human IL-17F/F revealed 104 unique Fabs out of 631 clones examined. A similar analysis of antibody fragments that inhibited the binding of IL-23 (p40p19) to the soluble receptor IL-23RA-Fc without cross-reactivity to human IL-12(p40p35) yielded 144 unique Fabs from 876 clones. Antibody fragments were considered unique based on differences in protein sequence of at least one amino acid in either a framework region or a CDR. Variability in protein sequence of the antibody fragments identified as inhibitors ranged from single amino acid differences to extreme variability within CDRs and/or framework regions. In addition, both light chain isotypes (κ and λ) were represented within the identified set of antibody variable regions. The combined output of panning rounds 2 and 3 for each target represented ~10^5 phage molecules as determined by phage titer. DNA sequence analysis of Fab antagonists demonstrated favorable diversity and validated use of the Fab output as template for the conversion process.

Fabs were converted to scFvs, as described in Materials and methods, to provide the preferred modular units for assembly of bsAbs. scFvs were expressed in bacteria, and supernatants were screened for antagonistic activity. DNA sequence analysis of 355 colonies expressing anti-IL-17A scFvs identified 129 unique antibody fragments, and sequencing of 431 colonies expressing anti-IL-23 scFvs identified 97 unique antibody fragments.

Clonal supernatants containing scFvs or Fabs with unique amino acid sequences were transferred to 96-well plates for evaluation by SPR. The unique fragments were ranked by analysis of off-rates (Supplementary Fig. 1 available at PEDS online). Antibody fragments with the slowest off-rates were selected for expression and purification for analysis of biological activity in cell-based assays.

**Assessment of biological activity**

Four Fabs and four scFvs, targeting IL-17 A, were expressed in Escherichia coli and purified for analysis of biological activity in vitro. Antibody fragments were selected for evaluation in cell-based assays based on neutralization of binding activity in plate assays, relative off-rate determined by SPR and expression level in E.coli. The purified scFv fragments targeting IL-17A were subjected to analysis using primary human SAEC to measure neutralization of G-CSF production stimulated by human IL-17A/A (Fig. 1). Of the purified scFvs, AP1713 and AP1733 displayed the lowest IC50 values, 0.45 and 0.33 nM, respectively, while AP1761 and AP1712 were much less potent. Both AP1713 and AP1733 exhibited neutralization similar to the dimeric, bivalent soluble receptor for IL-17A/A, IL-17RA-Fc (0.21 nM, Table 1). However, none of the
purified Fabs displayed even moderate neutralization of G-CSF production in the assay within the concentration range tested (0.1–100 nM) (Supplementary Fig. 2A available at PEDS online). Neutralization of the activity of the human IL-17A/F heterodimer was also measured in this assay. The IC50 values obtained for AP1733 were similar to those obtained for neutralization of the activity of human IL-17A/F. However, AP1713 was much less potent against the human IL-17A/F heterodimer, as evidenced by IC50 values 10- to 20-fold higher than those observed with AP1733 (Supplementary Fig. 3 available at PEDS online).

Four scFvs and four Fabs, targeting the p19 subunit of IL-23 were expressed from E.coli and purified for analysis of biological activity in vitro. The purified proteins were evaluated in an assay measuring neutralization of the activity of human IL-23 on murine splenocytes. A human version of the soluble receptor for IL-23 (IL-23RA-Fc) inhibited production of murine IL-17A/A with an IC50 of 7–12 nM (Fig. 2). Of the scFvs (Fig. 2, Table I), inhibition by either AP2350 (2.1 nM) or AP2327 (0.3 nM) was more potent than that observed with AP2392 and AP2379 or the soluble receptor. None of the anti-IL-23 Fabs exhibited significant neutralization in the concentration range examined (Supplementary Fig. 2B available at PEDS online).

Table 1. Inhibition of biological activity by anti-IL17A and anti-IL23p19 scFvs

<table>
<thead>
<tr>
<th>Target</th>
<th>Protein</th>
<th>Format</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAEC</td>
<td>IL-17A</td>
<td>Fe-fusion</td>
<td>0.21</td>
</tr>
<tr>
<td>IL-17A</td>
<td>AP1712</td>
<td>scFv</td>
<td>37</td>
</tr>
<tr>
<td>IL-17A</td>
<td>AP1761</td>
<td>scFv</td>
<td>36</td>
</tr>
<tr>
<td>IL-17A</td>
<td>AP1713</td>
<td>scFv</td>
<td>0.45</td>
</tr>
<tr>
<td>IL-17A</td>
<td>AP1733</td>
<td>scFv</td>
<td>0.33</td>
</tr>
<tr>
<td>Murine splenocyte</td>
<td>IL-23R</td>
<td>Fe-fusion</td>
<td>7.0–12.0</td>
</tr>
<tr>
<td>IL-23p19</td>
<td>AP2350</td>
<td>scFv</td>
<td>2.1</td>
</tr>
<tr>
<td>IL-23p19</td>
<td>AP2327</td>
<td>scFv</td>
<td>0.3</td>
</tr>
<tr>
<td>IL-23p19</td>
<td>AP2392</td>
<td>scFv</td>
<td>6.3</td>
</tr>
<tr>
<td>IL-23p19</td>
<td>AP2379</td>
<td>scFv</td>
<td>NA</td>
</tr>
</tbody>
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Four scFvs and four Fabs, targeting the p19 subunit of IL-23 were expressed from E.coli and purified for analysis of biological activity in vitro. The purified proteins were evaluated in an assay measuring neutralization of the activity of human IL-23 on murine splenocytes. A human version of the soluble receptor for IL-23 (IL-23RA-Fc) inhibited production of murine IL-17A/A in response to human IL-23 with an IC50 of 7–12 nM (Fig. 2). Of the scFvs (Fig. 2, Table I), inhibition by either AP2350 (2.1 nM) or AP2327 (0.3 nM) was more potent than that observed with AP2392 and AP2379 or the soluble receptor. None of the anti-IL-23 Fabs exhibited significant neutralization in the concentration range examined (Supplementary Fig. 2B available at PEDS online).

Engineering of stable bsAbs targeting IL-17A and IL-23

Table 1. Inhibition of biological activity by anti-IL17A and anti-IL23p19 scFvs

<table>
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<td>7.0–12.0</td>
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<td>IL-23p19</td>
<td>AP2350</td>
<td>scFv</td>
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<td>IL-23p19</td>
<td>AP2327</td>
<td>scFv</td>
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<td>IL-23p19</td>
<td>AP2392</td>
<td>scFv</td>
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<tr>
<td>IL-23p19</td>
<td>AP2379</td>
<td>scFv</td>
<td>NA</td>
</tr>
</tbody>
</table>

Fig. 1. Neutralization of biological activity stimulated by IL-17A/A (0.25 nM). Four anti-IL-17A scFvs were tested for neutralization of the activity of human IL-17A/A. The assay measures production of G-CSF by human SAEC in response to IL-17A/A. The activity of the antibody fragments is compared with that of the dimeric, bivalent soluble receptor IL-17RA-Fc.

Fig. 2. Inhibition of biological activity stimulated by human IL-23 (10 pM). Murine splenocytes were used to assess inhibition of murine IL-17A/A production stimulated by human IL-23. Murine IL-23R expressed on the splenocyte cells cross-reacts with human IL-23. The activity of the scFvs is compared with that of the dimeric, bivalent soluble receptor IL-23RA-Fc.

Fig. 3. SEC-MALS plot of anti-IL-17A scFv AP1733 (A) and anti-IL23p19 scFv AP2350 (B). SEC-MALS analysis was performed on the selected antagonistic antibody fragments. The absorbance at 208 nm is reported as the black line, and molecular weight is reported in red plotted versus elution time in minutes.

Characterization of antibody fragments produced from E.coli The stabilities of four antibody fragments against each target, selected on the basis of biological activity, were evaluated using SEC-MALS and DSC. SEC-MALS was used to measure the molecular weight and determine the multimerization state of the selected scFvs. Variable regions in this format have been reported to be unstable and prone to aggregation and multimer formation. The absorbance at 208 nm is reported as the black line, and molecular weight is reported in red plotted versus elution time in minutes.
In selecting formats for incorporation of the two binding entities, a variant of the human Fc domain was chosen as the backbone of the molecules. Fusion to Fc is known to improve expression in mammalian systems, enhance the stability of fusion partners (Demarest and Glaser, 2008) and increase retention in vivo by interaction with the neonatal Fc receptor, FcRn (Morrison, 1992). The selected Fc variant contains five mutations that remove effector function by eliminating binding to Fc gamma receptors and C1q, thus preventing recruitment of effector cells and activation of complement (Gross et al., 2001). Effector function was eliminated because the targets were soluble cytokines and cell killing was not desired. Although tetravalency (two binding sites for each cytokine) was not a requirement based on biological considerations, the dimerization of the Fc domain made this format easier to manufacture. If the Fc fusion molecules are not identical, a mixture of products is formed, greatly complicating production and purification.

Bispecific molecules were assembled in three formats: a tandem scFv fusion to the hinge region of Fc [tascFv-Fc (Connelly et al., 1998)], fusion of scFvs to the hinge and C-terminal regions of Fc [scFv-Fc-scFv (Jendreyko et al., 2003)] and fusion of an scFv to the C-terminus of an IgG molecule containing one set of V regions in the IgG format [IgG-scFv (Coloma and Morrison, 1997)] (Fig. 5). The two scFvs, AP2350 and AP1733, were expressed as either N- or C-terminal fusions in each of the three formats resulting in a total of six constructs. However, variation in expression levels resulted in adequate production of only one construct per format. Table II lists the constructs and positions of AP2350 and AP1733 as either N- or C-terminal fusions.

Protein production and characterization The bsAbs constructed with the anti-IL-17A scFv AP1733 and the anti-IL-23p19 scFv AP2350 were expressed from pools of stably transfected CHO cells and harvested for purification. The tascFv-Fc, scFv-Fc-scFv and IgG-scFv exhibited expression levels of 13, 142 and 83 mg/l, respectively. The proteins were purified, as described in Materials and methods, and the stability of the bispecific molecules was assessed.

The propensity of bispecific molecules to aggregate was evaluated using DLS, a technique that measures the size of molecules in solution as a function of diffusion. No large disordered aggregate was detected in any of the three samples incubated at the two temperatures analyzed. All three constructs displayed an increase in polydispersity and average hydrodynamic radius at 5°C, suggesting multimerization which was the most pronounced in the scFv-Fc-scFv and the IgG-scFv (Supplementary Fig. 5 available at PEDS online). The tascFv-Fc displayed a minor increase in
polydispersity at 5°C, accompanied by a small increase in the average hydrodynamic radius. However, for all three constructs, the multimerization observed at 5°C was reversible by brief incubation at 37°C.

The thermal stability of each bsAb was determined using DSC and compared with the stability of the component scFvs (Fig. 6). Thermograms generated for the tascFv-Fc and the scFv-Fc-scFv revealed primary melting temperatures (57°C and 56°C) very similar to the Tm values derived for the AP1733 scFv (54°C). The observed Tm for the IgG-scFv (69°C) was much higher than that of the AP1733 scFv and is closer to the Tm observed for the AP2350 scFv (73°C). Interestingly, the IgG-scFv construct contains the variable regions of AP1733 in an Fab format rather than an scFv, which may have contributed to the overall stability of the molecule.

**Kinetic analysis**

Binding of the bsAbs to human IL-17A/A and IL-23 was compared with that of the individual scFvs using SPR. Table II displays the affinities of the molecules for the corresponding ligands. The anti-IL-17A scFv AP1733 binds with higher affinity (0.33 nM) to the human IL-17A homodimer than the bivalent soluble receptor IL-17RA-Fc (5 nM). The KD for binding of the anti-IL-23p19 scFv AP2350 (0.7 nM) was similar to that of the human-soluble receptor IL-23RA (0.8 nM). There was no direct correlation between the biological activity of the scFvs in vitro and the affinity for the target ligand. The scFv AP1733 and the soluble receptor showed similar biological activity, yet they displayed a difference in affinity for IL-17A/A of more than 10-fold. Conversely, AP2350 is more potent in the cell-based assay than IL-23RA-Fc, despite having roughly the same KD.

The bsAbs display affinities for IL-17A/A (0.09–0.3 nM) that are very similar to the monomeric AP1733 scFv (Table II, Supplementary Fig. 6A–C available at PEDS online). These binding affinities cannot be accepted with absolute certainty, due to the limitations of instrument detection. However, the binding curves suggest a slight increase in affinity to IL-17A/A for the bsAbs, yet binding affinity does not correlate with the neutralizing activity in a cell-based assay, comparable to observations made for the scFvs. The bsAbs were up to 5-fold more potent inhibiting the activity of IL17A/A in the cell-based assay than the scFv AP1733, despite having a similar affinity for the ligand. A significant decrease in affinity for IL-23 was observed for the bsAbs (≈6- to 8-fold) compared with the monomeric scFv. In this case as well, binding affinity does not correlate with the activity observed in the cell-based assay. The bsAbs were up to 10-fold more potent than AP2350 (Table II), yet bound with lower affinity to IL-23 (Supplementary Fig. 6D–F available at PEDS online).

The ability of the bsAbs to bind both ligands simultaneously was tested using SPR as described in Materials and methods. Figure 7 presents examples of co-binding experiments for the three different bispecific molecules. Binding of the bsAb to ligand captured on the SPR chip is indicated by the first curve. The second curve represents

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**Table II. Properties of bispecific molecules**

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Format</th>
<th>SAEC IC50 (nM)</th>
<th>Murine, splenocyte IC50 (nM)</th>
<th>KD IL-17A (nM)</th>
<th>KD IL-23 (nM)</th>
<th>Half-life (t1/2)</th>
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</thead>
<tbody>
<tr>
<td>IL-17A/A</td>
<td>IL-17RA Fc-fusion</td>
<td>0.21</td>
<td>—</td>
<td>5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IL-17A/A</td>
<td>AP1733 scFv</td>
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<td>—</td>
<td>0.3</td>
<td>—</td>
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<tr>
<td>IL-23/A</td>
<td>IL-23R Fc-fusion</td>
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<td>—</td>
<td>0.8</td>
<td>—</td>
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<td>IL-23</td>
<td>AP2350 scFv</td>
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<td>IL-23, IL-17A/A</td>
<td>AP2350/AP1733 TascFv-Fc</td>
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<td>0.69</td>
<td>0.09</td>
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<tr>
<td>IL-23, IL-17A/A</td>
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<td>0.19</td>
<td>0.3</td>
<td>6.4</td>
<td>83.5 h</td>
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<tr>
<td>IL-17A/A, IL-23</td>
<td>AP1733/AP2350 IgG-scFv</td>
<td>0.12</td>
<td>0.70</td>
<td>0.3</td>
<td>6.5</td>
<td>147 h</td>
</tr>
</tbody>
</table>
binding of the second ligand to the bsAb. Off-rates for each binding event were calculated from the co-binding analysis and were identical to the off-rates reported against each target individually (Supplementary Fig. 5 available at PEDS online). These data suggest that the bsAbs are capable of binding both targets simultaneously and that the assembly of two binding entities derived independently into a bivalent molecule had no effect on the binding and neutralization properties of the individual components.

Assessment of biological activity
The purified bsAbs were evaluated for biological activity in cell-based assays. All three molecules neutralized the activity of IL-17A/A in the SAEC assay (Fig. 8A, Table II). IC₅₀s of 0.15, 0.07 and 0.12 nM were measured for the tascFv-Fc, the scFv-Fc-scFv and the IgG-scFv, respectively. These values represent a 2- to 4-fold increase in activity over the AP1733 monomer (0.33 nM). A gain in avidity was anticipated for the bsAbs targeting the homodimeric ligand due to the increase in valency. The bsAb has two sites for IL-17A and can bind to both subunits of the homodimer. The abilities of the bispecific molecules to inhibit the activity of human IL-23 were analyzed in a murine splenocyte assay (Fig. 8B, Table II). All three molecules exhibited neutralization activity superior to the soluble IL-23R receptor (7–12 nM), with the tascFv-Fc, scFv-Fc-scFv and IgG-scFv molecules inhibiting with IC₅₀s of 0.69, 0.19 and 0.70 nM, respectively. At this time, we cannot explain the increase in activity (up to 10-fold) with binding to a monovalent target.

Pharmacokinetic analysis
The retention and stability of the bsAb molecules in vivo were tested in female CD-1 mice. Purified protein (100 μg) was administered intravenously and terminal blood samples were collected at pre-designated time points over a 3-week period.
In vivo, the bsAbs remained intact and active for at least 1 week. Serum half-life values for each bsAb are reported in Table II. Pharmacokinetic profiles are shown in Fig. 9 and terminal serum concentrations were determined by ELISA as described in Materials and methods. The amount of bsAb present in the serum was determined using a non-compartmental pharmacokinetic analysis using WinNonlin 5.0.1. Serum concentration data shown are mean (± SD).

**Discussion**

In selecting a format for dual-targeting molecules, we considered that antibodies have proven to be excellent therapeutic molecules. Construction of an IgG-like molecule that binds two targets generally requires significant engineering and risks decreased activity relative to the parent antibodies, loss of stability and difficulty with production at large scale. In this study, the fusion of scFvs to Fc retained many of the favorable features possessed by IgGs and eliminated the issue of proper pairing of light and heavy chain variable regions by combining them on the same polypeptide. Despite the advantage of retaining highly specific binding to two antigens, molecules with multiple scFv modules could be difficult to produce on a large scale. However, the stable bsAbs created in this study demonstrate expression levels comparable to those of mAbs. The attachment of scFvs to the Fc domain of IgG also has the potential to impart binding to the neonatal Fc receptor, FcRn. The long serum half-life demonstrated by mAbs in vivo is highly dependent on this interaction. The pharmacokinetic properties of the bsAbs determined in vivo imply that the interaction between the Fc and FcRn is maintained and indicate that the terminal half-lives of the bsAbs are comparable to those of mAbs (Datta-Mannan et al., 2007). The relatively large size of the bsAbs (close to that of an IgG) and tissue penetration was not of concern in this study because the target molecules in this case were both secreted, circulating cytokines.

A significant problem with the scFv format has been a lack of stability. Thus, the identification of stable scFvs was central to the construction of therapeutically relevant bispecific molecules. The Fab library utilized for selections was constructed with the heavy variable region that has been reported to be the most stable (Hoet et al., 2005; Honnegger, 2008). The VH3_3-23 was selected as the heavy chain framework region that has been reported to contribute to stability (Ewert et al., 2003). Additional stability was sought by means of conversion from Fabs isolated from a phage display library in batch mode. The conversion process assembled variable light chain domains with variable heavy chain domains in both orientations at random, permitting the association of previously unpaired variable regions and likely increasing the diversity of the library. Although the true impact of the process is difficult to access, this type of chain shuffling has been shown to permit the isolation of antibodies with high affinity (Barbas et al., 1991; Kang et al., 1991a; Schier et al., 1996; Lu et al., 2003b; Blaise et al., 2004; Damschroder et al., 2007). Following the conversion process, the amplified phage were subjected to selective pressure via heat treatment to enrich for stable scFv clones. This technique has also been shown to yield antibody fragments of higher stability (Jung et al., 1999; Jespers et al., 2004). The candidates chosen for construction of the bispecific molecule were stable and demonstrated little propensity to aggregate. In fact, the AP2350 scFv had the highest $T_m$, as determined by DSC, which has been reported for an scFv. When combined in a bispecific framework, the resulting molecule is very stable, a property that should facilitate therapeutic application.

The bispecific molecules presented in this study display a number of other characteristics that support their application.
as therapeutic agents. Analysis of the final candidates by DLS and DSC indicate that the variable region pairs identified by the process associate tightly, reducing the tendency of scFv molecules to aggregate, which has been a concern for the scFv format (Wörn and Plückthun, 2001). The selected scFvs maintained stability when fused to the Fc domain of IgG to create bsAbs. Studies using DLS revealed that the bsAbs also have little tendency to aggregate. Analysis of thermal stability by DSC indicated that the bsAbs possess stability profiles very similar to those of the monomeric units from which they were constructed. This observation substantiates previous reports that suggest simply fusing an antibody fragment to Fc may not be sufficient to overcome the instability in certain scFvs (Demarest and Glaser, 2008). The results in this study indicate that the use of stable antibody fragments as modular building blocks may significantly improve the assembly of bispecific molecules for application as therapeutic entities.

In addition to being stable, the scFvs derived from the conversion process bound their targets with high affinity. Following variable region shuffling, additional panning was performed on the scFv pool, and stable scFvs with high affinity for the target ligands were identified after just one round. The success of the process is indicated by the fact that no affinity maturation was required. The absence of neutralization demonstrated by the Fabs targeting both cytokines may be a result of the higher off-rates observed by SPR analysis of crude supernatants. Faster off-rates have been reported to influence antagonistic activity of antibodies against other soluble targets (Maynard et al., 2002). However, the purified Fabs did exhibit potent neutralization of IL-23-mediated STAT3 phosphorylation in a short-term assay (15 min) performed in human IL-23R-transfected cells (Supplementary Fig. 7 available at PEDS online). Although the data are suggestive, it is not possible to conclude from this study that conversion of Fabs to scFvs with variable region shuffling will necessarily result in antibody fragments with higher affinity. Additional shuffling and panning steps might have produced more potent Fabs from the original library.

The conversion of Fab to scFv yielded molecules in both VHVL and VLVH configurations. Recent reports have revealed the importance of variable region orientation in the identification of antibody fragments with high activity (Lu et al., 2004; Kim et al., 2008). Incorporation of both orientations followed by additional rounds of panning and screening allowed selection of scFvs with optimal properties. ScFvs with antagonistic activity were found in either orientation, but only a select few V region combinations were found in both orientations. The combinations observed revealed a bias for the VHVL domain order in neutralizing scFvs directed against both targets (Supplementary Fig. 8 available at PEDS online). For both IL-17A/A and IL-23p19, there were few VHVL pairs recovered both as scFvs and as Fabs against their respective targets. It is believed that the number of direct Fab to scFv conversions was altered during the conversion process by non-complementary primer sequences that may have induced changes in terminal framework regions and, thus, created unique sequences. While an overall bias for the VHVL orientation was observed, the two top candidates targeting IL-17A and IL-23p19 represented both orientations (AP1733 VLVH, AP2350 VHVL), validating the utility of the shuffling process.

The conversion from variable regions of Fabs to the single-chain format used a 25-amino acid gly-ser linker to join the light and heavy chain variable domains. The linker was designed to limit the number of primers needed in the PCR steps. Although previous reports have demonstrated that different linker lengths can provide increased activity and/or stability to certain variable region pairs (Arndt et al., 1998), the additional stringent panning step selects for candidate scFvs that perform well with this linker length and eliminates any non-functional pairs among the newly paired scFvs by competitive selection against the target protein. Three to four rounds of phage panning were performed to isolate scFvs that met pre-selected target criteria.

The application of bsAbs as therapeutic molecules provides the opportunity to increase the efficacy of mAbs by combining two therapeutic activities in a single entity, either through interaction with two targets in the same pathway or through interaction with targets in different pathways. This application of bsAbs targets the neutralization of both IL-23 and IL-17A in combination to provide a more effective blockade of the pro-inflammatory activity of TH17 cells. Coordinated neutralization of IL-23, the growth and survival factor that maintains TH17 cells, and IL-17A, a key pro-inflammatory cytokine that they produce, interrupts the inflammatory pathway at two points and should offer an advantage over targeting either cytokine alone. The highly stable bsAbs assembled in this study exhibit the ability to bind both targets simultaneously. The potential for tetravalent binding, while not a requirement for biological activity, provides the opportunity to increase activity through enhanced avidity. In fact, the bsAbs exhibit activity more than 2-fold greater than that of the monovalent scFv counterparts. The increase in activity due to tetravalency may result in greater efficacy and potentially lower doses in therapeutic settings.

The addition of dual-targeting biologics to the arsenal of protein therapeutics presents significant engineering challenges. Maintaining affinity, potency, stability and productivity in the construction of bsAbs is a difficult task. However, the tremendous potential of this class of molecules to advance the efficacy of mAb therapy justifies the effort spent on development. The approach described offers an effective means to develop stable, potent bsAbs that have potential as therapeutic molecules.

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We thank Frank Grant, Monica Tackett, Benjamin Pickett, and Shellie Matthews for voluminous DNA sequence analysis and Kristine Swiderek and Jeff Ellsworth for critical reading of the manuscript.

Supplementary data
Supplementary data are available at PEDS online.

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References


