Chemokines are important mediators of the immune response that are responsible for the trafficking of immune cells between lymphoid organs and migration towards sites of inflammation. Using phage display selection and a functional screening approach, we have isolated a panel of single-chain fragment variable (scFv) capable of neutralizing the activity of the human chemokine CXCL10 (hCXCL10). One of the isolated scFv was weakly cross-reactive against another human chemokine CXCL9, but was unable to block its biological activity. We diversified the complementarity determining region 3 (CDR3) of the light chain variable domain (VL) of this scFv and combined phage display with high throughput antibody array screening to identify variants capable of neutralizing both chemokines. Using this approach it is therefore possible to engineer pan-specific antibodies that could prove very useful to antagonize redundant signaling pathways such as the chemokine signaling network.

Introduction

Chemokines constitute a family of small proteins (8-10 kDa) that are produced by immune and non-immune cells in response to inflammation and under homeostatic conditions. Chemokines and their receptors act as key mediators of the immune system by regulating leukocyte trafficking. The human chemokine system is comprised of 50 chemokines and 20 chemokine receptors.1 They are divided into four subclasses (CXC, CC, C and CX3C) according to their conserved N terminal cysteine pattern. Many chemokines are able to bind several receptors and conversely chemokine receptors often bind more than to one chemokine, which accounts for an apparently redundant signaling system.1 Because chemokines and their receptors are involved in the recruitment of cells to inflammatory sites, they have become an attractive class of therapeutic targets.3 Interferon-inducible protein-10 (IP-10, CXCL10) signals through the receptor CXCR3 and selectively attracts T helper 1 (Th1) cells and monocytes. CXCL10 has been reported to be expressed in many inflammatory diseases, such as psoriasis, atherosclerosis, multiple sclerosis and rheumatoid arthritis. In these diseases, CXCL10 levels correlate with the tissue infiltration of T cells, suggesting that CXCL10 signaling plays an important role in the recruitment of these cells to sites of inflammation and autoimmune pathology.4,5

Monoclonal antibodies (mAbs) exhibit specificity and high affinity to their target antigen and are therefore well suited for the neutralization of a specific molecule involved in a biological process. In recent years mAbs have emerged as a quickly expanding class of therapeutic molecules with several targeting chemokines or their receptors.6 However, given the redundancy of the chemokine signaling system, it is conceivable that blockade of multiple chemokines or complimentary receptors might be required to efficiently interfere with this component of the immune response. Here, we have used phage display libraries to select scFv capable of neutralizing human CXCL10. During the initial screening involving an assay to select candidates capable of binding CXCL10, we identified a clone that was also weakly cross-reactive against hMIG/CXCL9, another ligand capable of eliciting migration via CXCR3. Variants of this clone, i.e. E7, were generated and screened in order to identify second generation candidates capable of neutralizing both chemokines. Our results demonstrate that it is possible to broaden the binding and neutralizing capacities of mAbs, and potentially increase their therapeutic range in particular when targeting redundant signaling systems.

Results

Phage display selection of scFv binding to hCXCL10. A phagemid library representing a total diversity of >10^10 human
selected rounds having a hit rate greater than 80% and maintaining high sequence diversity. For those rounds, several hundreds of colonies were picked and stored for primary screening.

Functional screening of scFv to determine neutralizing capacity for hCXCL10 activity. hCXCR3 engagement by hCXCL10 starts a signaling cascade resulting in a transient increase of intracellular calcium, and eventually to rearrangement of the cytoskeleton that leads to cellular chemotaxis. In order to identify scFv capable of neutralizing such activity, primary screening was performed using a high throughput calcium flux assay. Periplasmic extracts from bacteria expressing a single scFv were incubated with hCXCL10, and then added to cells expressing hCXCR3 that were previously loaded with the calcium sensitive fluorescent dye Fura-2. Positive clones expressing scFv capable of blocking the calcium flux induced by hCXCL10 were sequenced, expressed and purified. Their dose-dependent activity was then established in two independent functional assays: calcium flux as well as in vitro chemotaxis (Table 1). From this analysis 12 clones were identified; these had IC50 values ranging from 14 nM to 250 nM and from 10 nM to 144 nM in calcium flux and chemotaxis assays, respectively. The candidates were encoded by different IGHV and IGLV germline genes and contained different CDR3 sequences demonstrating that the selection procedure allowed a variety of scFv that neutralized hCXCL10 to be identified (Table 2).

Binding specificity of selected scFv. We further tested the specificity of the selected scFv in ELISA against a panel of human chemokines belonging to the CC and CXC families. Amongst the hCXCL10 neutralizing scFv candidates, E7 was the only one to show a detectable signal in an ELISA involving hCXCL9 (Table 1 and Figure 1). The neutralizing activity of E7 for hCXCL10 and hCXCL9 bioactivity was determined using the in vitro chemotaxis assay. E7 was unable to inhibit the migration of hCXCR3 expressing cells in response to hCXCL9 (Table 3). In contrast, E7 blocked cell chemotaxis induced by hCXCL10 with an IC50 of 144 nM. These results suggested that either E7 bound a non-neutralizing epitope on hCXCL9, or its affinity for hCXCL9 was too low to compete with hCXCR3 binding. In order to test the latter hypothesis, we aimed at generating variants of E7 with improved neutralizing potential against hCXCL9 while maintaining their activity against hCXCL10.

Library construction and selection of improved variants. A classical approach to improve the affinity and potency of antibodies is to introduce mutations into CDR.10 In particular, targeting the CDR3 of the heavy and light chains has proven to be very successful.11 The CDR3 of the VH of an antibody is located at the center of the antigen combining site and the analysis of antibody-antigen structures indicate that CDR3 often provides most of the interaction surface with the antigen.12 In an effort to maintain this potentially important interaction, we generated

scFv derived from naïve human donors was used for phage display selections against hCXCL10.7,8 Three or four rounds of selection against biotinylated hCXCL10 were performed increasing the stringency of selection in later rounds (see Materials and Methods for details). Successful enrichment for phage binding to hCXCL10 was assessed by picking random colonies after each round, growing them individually in microtiter plates and performing soluble scFv ELISA against hCXCL10 (data not shown). A gradual enrichment in scFv capable of binding specifically to hCXCL10 was observed after each round of selection and reached approximately 80% at round 3. For each round, 24 clones capable of binding to hCXCL10 were sequenced to determine their diversity. We first

Table 1 Functional activity and cross-reactivity of scFv selected against hCXCL10

| scFv | Calcium Fluxb IC50 (nM) | Chemotaxisb IC50 (nM) | Cross-reactivity |
|------|-------------------------|------------------------|-----------------|
| F1   | 36                      | 18                     | -               |
| E5   | 39                      | 36                     | -               |
| H6   | 71                      | 36                     | -               |
| C3   | 14                      | 14                     | -               |
| F4   | 107                     | 36                     | -               |
| E7   | 250                     | 144                    | hCXCL9          |
| A2   | 36                      | 30                     | -               |
| G7   | 46                      | 10                     | -               |
| B9   | 107                     | 50                     | -               |
| D3   | 179                     | 72                     | -               |
| C1a  | 36                      | 108                    | -               |

bThe IC50 value of each scFv represents the concentration of the scFv that is required for 50% inhibition of hCXCL10 mediated calcium flux or chemotaxis. The ability to bind to hCCL5, hCCL11, hCCL3, hCCL4, hCXCL9, hCCL2, hCCL8 and NusA control was assessed in an ELISA. (-) no detectable signal. Results are representative of three independent experiments.
libraries targeting the CDR3 of the E7 VL. The original VH CDR3 of E7 was, therefore, unaltered in order to increase the likelihood of generating variants that maintained the property of hCXCL10 neutralization. The sequence of E7 was diversified using overlapping PCR and degenerate oligonucleotides introducing NNK codons at four positions within the VL CDR3. Four small size phagemid libraries (10^8 to 10^7 independent transformants), designated L3.1, L3.2, L3.3 and L3.4, were generated in which the diversified regions overlapped by one or two amino acids in order to obtain a good mutagenesis coverage of this CDR (Figure 2). The assembly process and successful diversification of the targeted region was controlled by sequencing 80 randomly picked colonies (20 for each library).

Instead of performing phage selection against hCXCL9 that might retrieve scFv having partially lost their neutralization capacity towards hCXCL10, we performed selections against hCXCL10 to maintain or improve the original characteristic of E7, coupled to high throughput screening for binding to hCXCL9. The four phagemid libraries, that is L3.1, L3.2, L3.3 and L3.4, were subjected to three rounds of selection while increasing the stringency of selection at each round by a 10-fold reduction of hCXCL10 concentration.

Screening of cross-reactive candidates by high density scFv arrays. After the third round of selection, scFv capable of binding to hCXCL9 were identified by high throughput screening using high density arrays of bacterial colonies expressing scFv. In total, 12,224 clones were robotically picked and gridded, in duplicate, onto two nitrocellulose filters for parallel array screening against: (1) NusA-hCXCL9 fusion protein, and (2) NusA alone (the control for non-specific binding). As the original selection was for hCXCL10, it was anticipated that the vast majority of the scFv would bind to hCXCL10, and, therefore, results of an array involving a hCXCL10 containing filter would be difficult to interpret. Thus, binding to hCXCL10 was not included in the array screening strategy. A total of 227 clones produced spots of significantly higher intensity when compared to the parental E7 clone (Figure 3A). No signal was observed on the control filter coated with NusA indicating that the spots were the result of a specific interaction between scFv and hCXCL9. The hits originated from libraries L3.2, L3.3 and L3.4 (18, 38 and 44%, respectively). No relevant clone was identified in library L3.1, suggesting that the N-terminal region of VL CDR3 of clone E7 cannot be altered without loss of binding to hCXCL9 (Figure 2). Next, 88 positive scFv were sequenced (five from L3.2 library, 63 from L3.3 and 20 from L3.4) and found to encode different VL CDR3 sequences, indicating that our selection and screening strategy maintained a good diversity and did not introduce major biases. We further confirmed the results obtained in the scFv array format by soluble scFv ELISA directed against hCXCL9 and hCXCL10.

### Table 2

| scFv | VH (%/gene allele) | VL (%/gene allele) | CDR length | CDR sequences |
|------|-------------------|--------------------|------------|---------------|
| E7   | 90.28 / IGHV3-30*30 | 95.19 /IGLV6-57*01 | [8.81]     | ARDGSE ............... | QSYSDS ............... |
|     |                   |                    | [8.31]     | ARAPDG ............... | QSYVS ............... |
|     |                   |                    | [8.81]     | AKDAG ............... | QYWDSS ......... |
|     |                   |                    | [8.91]     | ARLASSG ............... | ANWDS ......... |
|     |                   |                    | [8.81]     | AXDAG ............... | QYWDSSD .. DREPYY |
|     |                   |                    | [8.81]     | AKSLQDLL ............... | ANWDSD ...... |
|     |                   |                    | [8.81]     | ARLQEVYSTGG ............... | ANWDS .......... |
|     |                   |                    | [8.81]     | ARQDEF ............... | QYWDTS .......

### Table 3

| scFv | VL CDR3 | Chemotaxis | IC50 (M) |
|------|---------|------------|----------|
| E7   | L31NGN  | - | 1.44 x 10^-7 |
| J9   | L31SEP  | 1.88 x 10^-6 | 3.79 x 10^-8 |
| P8   | L31QDR  | 7.13 x 10^-7 | 3.98 x 10^-7 |
| C1   | L31ERY  | 1.42 x 10^-6 | 1.65 x 10^-7 |
| F13  | L31IGNY | 1.02 x 10^-6 | 2.33 x 10^-7 |
| J5   | L31ESPA | 3.74 x 10^-6 | 1.59 x 10^-7 |

The residues of amino acid sequence of the mutated VL from scFv were numbered according to reference. The IC50 value of each scFv represents the concentration of the scFv that is required for 50% inhibition mediated by 1 nM of hCXCL10 or 10 nM of hCXCL9. [ ] no detectable signal.
Engineering antibody cross-reactivity and hCXCL9 respectively, indicating also that the high density array screening strategy generated few false positive signals. A panel of scFv candidates was then expressed at larger scale, purified and quantified as described above, and dose-response ELISA were performed for hCXCL9 and hCXCL10 (Figure 4 and supplementary Figure 1). The results demonstrated that the newly selected scFv had higher binding capacity towards hCXCL9 as compared to E7 while maintaining the binding properties towards hCXCL10.

In order to determine if the increased binding obtained for hCXCL9 was selective and not due to an unspecific binding property of the newly derived candidates, the scFv were tested in an ELISA with a selected panel of human chemokines (Figure 5). The results demonstrated that the binding was specific for hCXCL9 and hCXCL10, and a lack of binding to the third ligand for CXCR3, i.e. CXCL11.

Functional characterization of hCXCL9 and hCXCL10 cross-reactive scFv E7 variants. The neutralizing capability of the selected scFv was tested in a chemotaxis assay using cells expressing hCXCR3. While parental E7 neutralized cell migration induced by 1nM of hCXCL10 with an IC50 value of 144 nM, it was unable to neutralize hCXCL9 used at 10 nM (Figure 6A, 6B Table 3). In contrast, the selected candidates were able to block the biological activity of hCXCL9 with IC50 values ranging from 713 nM to 3.7 μM. Interestingly, only scFv derived from the L3.3 library showed a significantly improved neutralization potential against hCXCL9. This
In addition, various studies have shown that inhibiting the CXCR3/CXCL10 interaction is protective in several animal models of human autoimmune disease.\textsuperscript{15-18} Taken together, these observations support the idea that molecules inhibiting CXCL10 signaling via CXCR3 could be used therapeutically to treat autoimmune conditions.

In this study, we have used phage display to identify human antibody fragments directed against human CXCL10. Using direct primary functional screening, we isolated a panel of scFv capable of inhibiting CXCL10 biological activity in calcium flux and cell chemotaxis assays in the low nM range. These scFv represent potential candidates for drug development. However, the therapeutic effect of neutralizing hCXCL10 might in some situations be limited by the presence and activity of the two other CXCR3 ligands Mig/CXCL9 and I-TAC/CXCL11. Antibodies directed

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Specificity of improved scFv variants. Binding of the selected scFv candidates was tested in an ELISA against a panel of human chemokines. NusA fusion chemokine was coated at 20 μg/ml and 5 μg/ml variant scFv in 1% milk-PBS buffer was incubated for 1 hour at room temperature. Coating was controlled using specific mAb for each chemokine and NusA protein was also added to the assay as negative control (data not shown). Results are expressed as mean ± S.D. of duplicates of two representative experiments.}
\end{figure}

\section*{Discussion}

Elevated CXCL10 levels are found in many tissues affected by autoimmune responses such as in the synovial fluid of arthritic patients, the epidermis of patients suffering from psoriasis, in the lamina propria of ulcerative colitis patients and in multiple sclerosis lesions. In addition, various studies have shown that inhibiting the CXCR3/CXCL10 interaction is protective in several animal models of human autoimmune disease.\textsuperscript{15-18} Taken together, these observations support the idea that molecules inhibiting CXCL10 signaling via CXCR3 could be used therapeutically to treat autoimmune conditions.

In this study, we have used phage display to identify human antibody fragments directed against human CXCL10. Using direct primary functional screening, we isolated a panel of scFv capable of inhibiting CXCL10 biological activity in calcium flux and cell chemotaxis assays in the low nM range. These scFv represent potential candidates for drug development. However, the therapeutic effect of neutralizing hCXCL10 might in some situations be limited by the presence and activity of the two other CXCR3 ligands Mig/CXCL9 and I-TAC/CXCL11. Antibodies directed
Engineering antibody cross-reactivity

unwanted. In most cases, cross-reactivity is observed for a closely related protein having a high percentage of sequence identity, e.g., an isoform or a homologue found in another species. This latter feature can be highly desirable as it facilitates toxicology studies for therapeutic antibodies and can be included as criteria for the selection of a therapeutic candidate. The engineering of antibody specificity has already been described in the literature. Recently, a neutralizing antibody directed against botulinum neurotoxin subtype A1 was engineered so that it could also bind and neutralize neurotoxin subtype A2, which shares 90% sequence identity with subtype A1. This modification greatly improved the application potential of this antibody as efficient intervention against botulinum requires the neutralization of both subtypes.

In another example, a mAb was obtained via immunization of mice using human MCP-1 which bound to human MCP-1, human MCP-2, mouse MCP-1 and mouse MCP-5. Interestingly, analysis of the crystal structure of the Fab fragment complexed with hMCP-1 identified key interacting residues, allowing for clear improvement of the neutralization potential of E7 against hMCP10 from no effect to inhibition in the nM range. For two of the scFv, the strategy also led to a 3- to 4-fold improvement in neutralizing activity against hCXCL10. A different strategy could have included alternate phage selections against hCXCL10 and hCXCL9. We deliberately avoided this approach in order to maintain the high neutralization potential on hCXCL10 of the parental clone, which was successfully achieved. The selected candidates were also specific as they did not react to hCXCL11 or to five other human chemokines, thus indicating that these scFv were selectively cross-reactive. This improvement was obtained by a single round of mutagenesis, selection and screening. It is thus anticipated that the generation of further improved therapeutic candidates can be achieved by introducing diversity into other CDR of the light and/or heavy chain and applying the same strategy.

Although a hallmark of antibodies is their exquisite specificity, cross-reactive antibodies are not uncommon, but are generally unwanted. In most cases, cross-reactivity is observed for a closely related protein having a high percentage of sequence identity, e.g., an isoform or a homologue found in another species. This latter feature can be highly desirable as it facilitates toxicology studies for therapeutic antibodies and can be included as criteria for the selection of a therapeutic candidate. The engineering of antibody specificity has already been described in the literature. Recently, a neutralizing antibody directed against botulinum neurotoxin subtype A1 was engineered so that it could also bind and neutralize neurotoxin subtype A2, which shares 90% sequence identity with subtype A1. This modification greatly improved the application potential of this antibody as efficient intervention against botulinum requires the neutralization of both subtypes.

Figure 6. Dose dependant chemotaxis inhibition of improved scFv variants. (A) The assay involves L1.2 cells expressing hCXCR3 which migrate in response to an established chemokine gradient which was visualized and quantified using FMAT. (B and C) Dose response data for the chemokines, CXCL9 (B) or CXCL10 (C) for the scFv E7, P8 and C1. Different concentrations of purified scFv were incubated with either 1 nM of hCXCL10 or 10 nM of hCXCL9. Results are expressed as mean ± S.D. of duplicates of three representative experiments.
Engineering antibody cross-reactivity

this study are able to bind to hCXCL10 and hCXCL9 is not fully elucidated. Given the structural similarity between chemokines, one hypothesis is that these scFv recognize similar epitopes on the two targets. As binding of hCXCL9 and hCXCL10 to hCXCR3 is mutually exclusive, it is likely that these two chemokines share some structural elements that allow them to engage their receptor on the same, or at least overlapping, site. However, it is relevant to underscore the fact that the scFv described here were not able to bind to hCXCL11, the third ligand of hCXCR3. This suggests that this epitope, while common to hCXCL9 and hCXCL10, is not found on hCXCL11, which is consistent with the proposal that hCXCL11 binds to hCXCR3 on a different site.21,22 Another possibility would be that flexibility of the antigen contacting loops allows for the scFv to present two different paratopes as already described for some antibodies.23

Monoclonal antibodies are a successful class of therapeutic molecules used in many areas of human disease.24 Nevertheless, it becomes increasingly clear that, in certain indications, targeting a single protein might not be sufficient to achieve therapeutic efficacy. As combination therapy is a possible but difficult endeavor, especially for investigative drugs, several strategies to circumvent this limitation have been developed. These include the development of several bi-specific antibody formats, recombinant polyclonal antibodies, as well as cross-reactive antibodies such as those described in this study.25-27 Although these innovative strategies represent novel challenges for drug development, they have the potential of dramatically improving the therapeutic efficacy of antibodies.

Materials and Methods

Reagents and antibodies. Biotinylated NusA fusion chemokine proteins were produced as previously described.28 Recombinant hCXCL10 and hCXCL9 used in chemotaxis assays were purchased from Peprotech. Anti-cmyc antibody conjugated or not with horse radish peroxidase (HRP) was purchased from Roche and from Peprotech. Anti-cmyc antibody conjugated or not with

Phage rescue. 50 μl of cell suspension obtained from previous selection rounds were added to 20 ml of 2xTYAG and grown at 37°C (240 rpm) until the OD_{600} reached 0.3 to 0.5. The culture was then super-infected with 3.3 x 10^{10} MK13K07 helper phage and incubated for one hour at 37°C (100 rpm). Culture medium was then changed for 2xTYAK (100 μg/ml ampicillin; 50 μg/ml kanamyacin) and cells were grown overnight at 30°C (240 rpm).

Affinity maturation library construction. Stretches of 4 residues in the CDR3 of the VL of scFv E7 were randomized by overlapping PCR assembly using degenerated oligonucleotide primers. The assembled E7 diversified scFv genes were digested with SfiI and NotI and ligated into the pCANTAB6 phagemid vector (Medimmune). The ligation products were then transformed into supercompetent TG1 by electroporation using a Gene pulser X cell electroporator (Biorad). Library size was estimated from serial dilutions of transformed cells.

scFv sequencing. Clones were individually grown in 2xTYAG overnight at 37°C. Five microliters of culture was diluted in 45 μl H2O and frozen at -80°C. PCR reaction was then performed with 5 μl of thawed cell suspension and PCR products were purified on PCKp96 plate (Millipore). Sequencing reactions were outsourced (Fasters, Geneva, Switzerland) and the sequences analysed using Sequencer 4.8 software (Genes Code). For germline identification and CDR analysis, standardized IMGT unique numbering was used.14

scFv arrays screening. The protocol was adapted from de Wildt et al.13 Picking. Cells from selected selection rounds were plated onto 2xTYAG Bioassay plate and grown overnight at 30°C. Colonies were picked (QPDisplay, Genetix) into 384-well plates containing 2xTYG supplemented with 8% glycerol and grown at 37°C overnight. These were then replicated into working 384-well plates grown at 37°C overnight and the master plates were stored at -80°C. Gridding. Replica plates were gridded (QPDisplay, Genetix) onto a nitrocellulose membrane (Protran BA 85 Schleicher & Schuell, 22x22 cm, 0.45 μm, BioScience) previously blocked in 3% milk for 1 hour at room temperature, briefly washed in PBS and soaked in 2xTY. Each clone was gridded twice in a 4 x 4 pattern. The gridded membranes were transferred onto 2xTYAG Bioassay plate and grown at 37°C overnight. Immunoblotting. The day before the immunoblotting, nitrocellulose membranes were coated with antigen at 2 μg/mL in 100 mL of PBS and incubated at 4°C overnight. Membranes were then washed three times in PBS, blocked in 3% milk-PBS (w/v) for 1 h at room temperature and washed again three times in PBS. These coated membranes were transferred onto Bioassay plates containing 2xTYAI (IPTG at 1 mM) and gridded membranes were placed on top making sure no air was trapped between the two filters. Plates were incubated for 3 h at 30°C to induce scFv expression. After incubation, the coated membranes were washed three times in PBS-Tween 0.05%. Anti-cmyc HRP was added at 1 μg/mL in 3% milk-PBS (w/v) in order to detect the scFv cmyc tag. After incubation and washing, the signals were revealed with ECL chemiluminescence reagents.
(ECLTM Western blotting Detection, Amersham Biosciences) and exposed to photographic film (BioMax Light Film, Kodak). Positive clones identification. Specific binders characterized by high intensity spots on the NusA-hCXCL9 filter and absence of signal on the control NusA filter, were identified by the specific orientation of the duplicated spots.

**scFv periplasmic extracts for functional screening.** Individual clones were grown in 96 deep-well plates in 2xTYAG medium at 37°C for 6 h (250 rpm). scFv expression was induced by IPTG addition (0.02 mM, final concentration) overnight at 30°C (250 rpm). Cells were centrifuged and the pellet was re-suspended in 150 μl TES buffer (50 mM Tris/HCl, pH 8; 1 mM EDTA, pH 8; 20% sucrose, complemented with Complete protease inhibitor, Roche). A hypotonic shock was produced by adding 150 μl of diluted TES buffer (1/5 TES in water) followed by incubation on ice for 30 min. Plates were then centrifuged (4,000 rpm, 10 min) and supernatants were kept on ice for use in calcium flux assays.

**Soluble scFv expression and purification.** A single colony was used to inoculate 400 ml of 2xTYAG culture and grown overnight at 30°C (300 rpm). Next day scFv expression was induced by adding 400 μl of 1M IPTG and incubated for 3 hours. The cells were collected by centrifugation (4,000 rpm, 10 minutes) at 4°C and resuspended in 10 ml of ice-cold TES buffer complemented with Complete protease inhibitors (Roche). Osmotic shock was achieved by adding 15 ml of 1/5 diluted TES buffer followed by incubation for 30 minutes on ice. Cells were centrifuged (10,000 rpm, 20 minutes, 4°C) to pellet cell debris and the supernatant was transferred to a fresh tube containing imidazole (10 mM, final concentration). One milliliter of Ni-NTA resin slurry (Qiagen), washed in PBS was added to each tube and incubated at 4ºC under agitation (20 rpm) for 1 hour. The tubes were centrifuged (2,000 rpm, 5 minutes), the supernatant was removed and the pelleted resin was resuspended in 10 ml cold (4°C) Wash buffer 1 (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). The suspension was added to a polyprop column (Biorad). Eight milliliters of cold Wash Buffer 2 (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) were used to wash the resin by gravity flow. The scFv were eluted from the column with 2 ml of Elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). Protein fractions were analyzed by absorption at 280 nm and pooled after buffer exchange on a PD-10 desalting column (Amersham). The purity of scFv was assessed by SDS PAGE and protein fractions were stored at −20°C.

**In vitro calcium flux assay.** Murine pre B lymphoma L1.2 cells expressing hCXCR3 cells were cultured in RPMI medium supplemented with 10% FCS. Periplasmic extracts containing the scFv were incubated with 2-10 nM of hIL-10 for 30 minutes at room temperature. Cells were washed in PBS and loaded with 2 μM Fura 2/AM (Invitrogen). One-hundred microliters of loaded cells were added to each well of a 96-well black, transparent flat-bottom plate and calcium flux kinetics were recorded by measuring the fluorescence at 514 nm upon excitation at 340 or 380 nm on a Flexstation II instrument (Molecular Devices). The inhibitory activity of each scFv extract was assessed by comparison to an extract containing an irrelevant scFv. The positive scFv candidates were expressed in larger scale as described in soluble scFv expression and confirmed in dose-response experiments in calcium flux assays.

**In vitro chemotaxis assay.** The day before the experiment L1.2 cells expressing hCXCR3 cells were incubated with 0.6 mg/ml of butyric acid (Sigma). Different concentrations of purified scFv were incubated with 1 nM of hCXCL10 or 10 nM of hCXCL9 and placed in the bottom chamber of chemotaxis 96-well plate (Neuroprobe). The filter plate was placed on top of the chemotaxis plate and each well was overlaid with 20 μl of a 10⁶ cells/ml suspension. The plate was incubated for 3 hours at 37°C, 5% CO₂. The bottom wells containing the migrated cells were transferred by centrifugation using a funnel plate to a black 96-well plate with a transparent glass bottom (Costar). Cells that migrated through the filter were stained with 10 μM DRAQ-5 (Alexis Corporation) and counted on an FMI 8200 reader (Applied Biosystems). The IC₅₀ (where 50% of the hCXCL10 induced cell migration is inhibited, i.e., 50% inhibitory concentration), for each candidate antibody was determined. The chemotactic index was calculated by dividing the number of migrated cells by the number of spontaneously migrating cells in the absence of chemokine.

**Note**

Supplementary materials can be found at: www.landesbioscience.com/supplement/ FageteMABS1-3-Sup.pdf

**References**

1. Allen SJ, Crown SE, Handel TM. Chemokine: receptor structure, interactions, and antagonism. Annu Rev Immunol. 2007; 25:787-820.

2. Fernandez EJ, Lolis E. Structure, function, and inhibition of chemokines. Annu Rev Pharmacol Toxicol 2002; 42:469-499.

3. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. Engl J Med 2006; 354:610-621.

4. Mach F, Sauty A, Iarossi AS, Sukhova GK, Neote K, Libby P; et al. Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. J Clin Invest 1999; 104:1041-1050.

5. Singh UP, Singh S, Iqbal N, Weaver CT, McGhee JR, Lillard JW, Jr. IFN-gamma-inducible chemokines enhance adaptive immunity and colitis. J Interferon Cytokine Res 2003; 23:591-600.

6. Johnson Z, Power CA, Weiss C, Rintelen F, Ji H, Ruckle T; et al. Chemokine inhibition—why, when, where, which and how? Biochem Soc Trans 2004; 32:366-371.

7. Vaughan TJ, Williams AJ, Pritchard K, Osbourn JR, Pope AR, Earnshaw JC, et al. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nat Biotechnol 1996; 14:309-314.

8. Lloyd C, Lowe D, Edwards B, Welsh F, Dilks T, Hardman C, et al. Modelling the human immune response: performance of a 1011 human antibody repertoire against a broad panel of therapeutically relevant antigens. Protein Eng Des Sel 2002; 15:199-209.

9. Lefrance M-P, Lefrance G, ed. The Immunoglobulin FactsBook. London, UK: Academic Press, 2001:1-458.

10. Barbas CF, III, Hu D, Dunlop N, Sawyer L, Cababa D, Hendry RM, et al. In vitro evolution of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross-reactivity. Proc Natl Acad Sci USA 1994; 91:3809-3813.

11. Schier R, McCall A, Adams GP, Marshall KW, Merritt H, Yim M; et al. Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. J Mol Biol 1996; 263:551-567.

12. Wilson IA, Stanfield RL. Antibody-antigen interactions. Curr Opin Struct Biol 1993; 3:113-118.

13. de Wildt RM, Mundy CR, Gorick BD, Tomlinson IM. Antibody arrays for high-throughput screening of antibody-antigen interactions. Nat Biotechnol 2000; 18:989-994.

14. Lefrance MP, Pommie C, Ruiz M, Giudicelli V, Poulquier E, Truong L; et al. IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains. Dev Comp Immunol 2003; 27:55-77.
15. Xie JH, Nomura N, Lu M, Chen SL, Koch GE, Weng Y, et al. Antibody-mediated blockade of the CXCR3 chemokine receptor results in diminished recruitment of T helper 1 cells into sites of inflammation. J Leukoc Biol. 2003; 73:771-780.

16. Singh UP, Singh S, Singh R, Cong Y, Taub DD, Lillard JW, Jr. CXCL10-producing mucosal CD4+ T cells, NK cells, and NKT cells are associated with chronic colitis in IL-10(-/-) mice, which can be abrogated by anti-CXCL10 antibody inhibition. J Interferon Cytokine Res 2008; 28:31-43.

17. Mohan K, Isekiyu TB. Blockade of chemokine receptor CXCR3 inhibits T cell recruitment to inflamed joints and decreases the severity of adjuvant arthritis. J Immunol 2007; 179:8463-8469.

18. Sakhivel SK, Singh UP, Singh S, Taub DD, Novakovic KR, Lillard JW, Jr. CXCL10 blockade protects mice from cyclophosphamide-induced cystitis. J Immune Based Ther Vaccines. 2008; 6:6.

19. Garcia-Rodriguez C, Levy R, Arndt JW, Forsyth CM, Razai A, Lou J, et al. Molecular evolution of antibody cross-reactivity for two subtypes of type A botulinum neurotoxin. Nat Biotechnol 2007; 25:107-116.

20. Reid C, Rushe M, Jarpe M, van VH, Dolinski B, Qian F, et al. Structure activity relationships of monocyt chemoattractant proteins in complex with a blocking antibody. Protein Eng Des Sel 2006; 19:317-324.

21. Cox MA, Jerth CH, Goniorek W, Fine J, Narula SK, Zavodny PJ, et al. Human interferon-inducible 10-kDa protein and human interferon-inducible T cell alpha chemoattractant are allotopic ligands for human CXCR3: differential binding to receptor states. Mol Pharmacol 2001; 59:707-715.

22. Clark-Lewis I, Martusoli I, Gong JH, Laetscher P. Structure-function relationship between the human chemokine receptor CXCR3 and its ligands. J Biol Chem 2003; 278:289-295.

23. James LC, Revers P, Tawfik DS. Antibody multispecificity mediated by conformational diversity. Science 2003; 299:1362-1367.

24. Presta L. Antibody engineering for therapeutics. Curr Opin Struct Biol 2003; 13:519-525.

25. de KJ, Bakker AB, Marissen WE, Kramer RA, Thoosy M, Rupprecht CE, et al. A human monoclonal antibody cocktail as a novel component of rabies postexposure prophylaxis. Annu Rev Med 2007; 58:359-368.

26. Fischer N, Leger O. Bispecific antibodies: molecules that enable novel therapeutic strategies. Pathobiology 2007; 74:3-14.

27. Rasmussen SK, Rasmussen LK, Weilguny D, Tolstrup AB. Manufacture of recombinant polyclonal antibodies. Biotechnol Lett 2007; 29:845-852.

28. Magistrelli G, Gueneau F, Mudman M, Ravu U, Kosco-Vilbois M, Fischer N. Chemokines derived from soluble fusion proteins expressed in Escherichia coli are biologically active. Biochem Biophys Res Commun 2005; 334:370-375.