

Report

Specificity tuning of antibody fragments to neutralize two human chemokines with a single agent

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Abbreviations: scFv, single-chain fragment variable; CDR3, complementarity determining region 3; VH, variable domain of heavy chain; VL, variable domain of light chain; Th1, T helper 1; mAb, monoclonal antibody

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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. Those belonging to the same sub-family share an amino acid sequence identity of about 40%, and have a highly conserved three-dimensional structural fold.² 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 complementary 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

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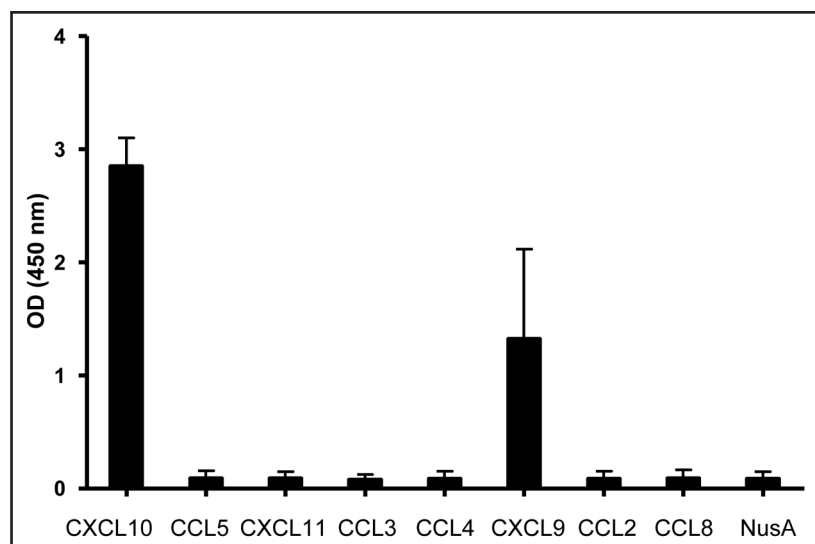


Figure 1. The scFv, E7, binds to hCXCL9 and hCXCL10. Capacity of scFv E7 to bind to a panel of human chemokines was tested in ELISA. NusA fusion chemokine was coated at 20 µg/ml and 5 µg/ml E7 scFv in 1% milk-PBS buffer was incubated for 1 hour at room temperature. Coating was controlled using specific 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.

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

scFv	Calcium Flux ^a	Chemotaxis ^a	Cross-reactivity ^b
	IC ₅₀ (nM)	IC ₅₀ (nM)	
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	-

^aThe IC₅₀ value of each scFv represents the concentration of the scFv that is required for 50% inhibition of hCXCL10 mediated calcium flux or chemotaxis. ^bThe ability to bind to hCCL5, hCXCL11, hCCL3, hCCL4, hCXCL9, hCCL2, hCCL8 and NusA control was assessed in an ELISA. (-) no detectable signal. Results are representative of three independent experiments.

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

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).⁹

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

Table 2 CDR sequences of scFv selected against hCXCL10

scFv	Nucleic acid Identity ^{a,b}		CDR length ^c		CDR sequences	
	VH (% / gene*allele)	VL (% / gene*allele)	VH	VL	VH CDR3	VL CDR3
F1	90.28 / IGHV3-30*03	95.19 / IGLV6-57*01	[8.8.12]	[8.3.10]	ARDGSE.....SEYLDY	QSYDS.....INLWV
E5	98.96 / IGHV3-30*03	95.88 / IGLV6-57*01	[8.8.11]	[8.3.9]	ARAPDG.....HQLDY	QSYVS.....SKWV
H6	93.40 / IGHV3-30*03	94.98 / IGLV3-21*02	[8.8.10]	[6.3.12]	AKDAG.....GPLDY	QVWDSS...IDHSWV
C3	94.50 / IGHV2-70*01	93.33 / IGLV1-44*01	[10.7.14]	[8.3.11]	ARLRAGSG.....PYVFD	ASWDDS...LNGRV
F4	94.44 / IGHV3-30*03	96.06 / IGLV3-21*02	[8.8.10]	[6.3.13]	AKDAG.....GPLDY	QVWDSSS..DHPEVV
E7	95.14 / IGHV3-30*03	99.30 / IGLV1-44*01	[8.8.13]	[8.3.12]	AKDLGDL.....PPGLDY	AAWDDS...LNGNVV
A2	92.98 / IGHV3-66*01	97.19 / IGLV1-44*01	[8.7.22]	[8.3.11]	ARDKEYVTSTGG...AYYFYMDV	AAWDDS...LNGLV
G7	92.39 / IGHV1-69*01	93.55 / IGLV1-44*01	[8.8.27]	[8.3.11]	ARDGRFDVSDLLTDKPKVTINYGMDV	ASWDDS...LNGRV
B9	99.31 / IGHV3-30*03	95.70 / IGLV3-19*01	[8.8.13]	[6.3.11]	AKDGGWY.....DWYFDL	GSRDSS...GYQVV
D3	94.79 / IGHV3-30*03	99.64 / IGLV3-19*01	[8.8.13]	[6.3.11]	TRAGYST.....DWHPDY	NSRDSS...GNHVV
C1a	98.96 / IGHV3-30*03	92.11 / IGLV3-21*01	[8.8.10]	[6.3.11]	AKDEF.....DAFDI	QVWDTS...SGHYV
C1	95.49 / IGHV3-30*03	98.21 / IGLV3-21*01	[8.8.14]	[6.3.11]	AKDWGFSG.....SLTFDY	QVWDSS...SDHVV

^aPercentage of identity to the closest V-gene and allele at the nucleotide level using IMGT/V-QUEST. ^bThe human germline immunoglobulin genes were determined using the nomenclature of reference.⁹ ^cCDR length according to IMGT.

Table 3 VL CDR3 sequence and potency of scFv E7 and improved variants

scFv	VL CDR3 ^a	Chemotaxis IC ₅₀ (M) ^b	
		hCXCL9	hCXCL10
E7	¹¹² LNGN ¹¹⁵	-	1.44×10^{-7}
J9	¹¹² SEPR ¹¹⁵	1.88×10^{-6}	3.79×10^{-8}
P8	¹¹² QDPR ¹¹⁵	7.13×10^{-7}	3.98×10^{-8}
C1	¹¹² YERY ¹¹⁵	1.42×10^{-6}	1.65×10^{-7}
F13	¹¹² IGNY ¹¹⁵	1.02×10^{-6}	2.33×10^{-7}
J5	¹¹² ESPA ¹¹⁵	3.74×10^{-6}	1.59×10^{-7}

^aThe residues of amino acid sequence of the mutated VL from scFv were numbered according to reference.¹⁴ ^bThe 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.

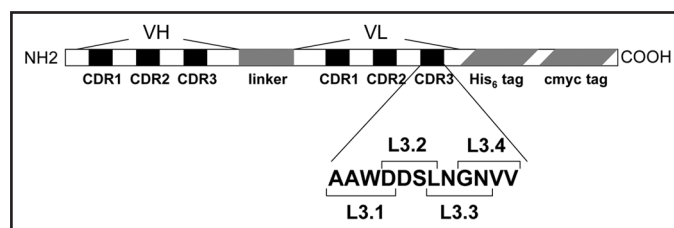


Figure 2. Strategy for library construction and VL CDR3 randomization of the scFv E7. Schematic representation of scFv E7 with complementarity determining regions (CDR) shown in black. The VL CDR3 amino sequence is magnified in order to show the overlapping stretches of four amino acids targeted by the libraries L3.1 to L3.4. The glycine-rich linker between the heavy and light chains as well as the c-terminal hexa-histidine and c-myc tags are indicated.

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 (10^6 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.¹³ 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. As expected, 100 and 93% of candidates were able to bind hCXCL10

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 1 nM of hCXCL10 with an IC_{50} 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 IC_{50} values ranging from 713 nM to 3.7 μ M. Interestingly, only scFv derived from the L3.3 library showed a significantly improved neutralization potential against hCXCL9. This

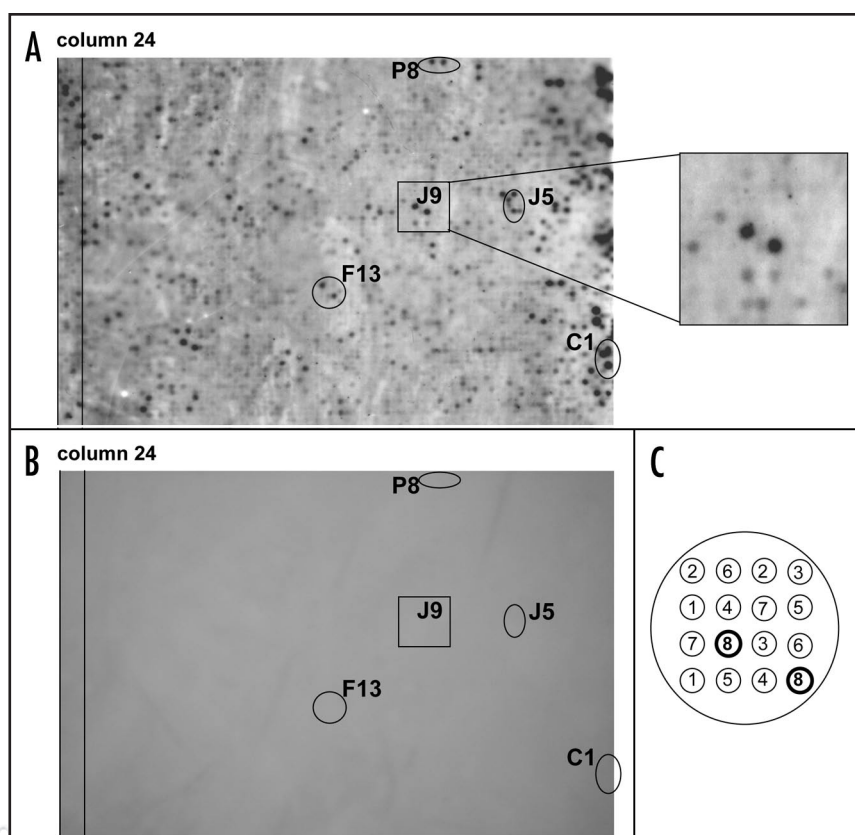


Figure 3. High-density array screening of scFv variants. (A,B) Bacterial clones grown in 384 well plates were double spotted on membranes and probed for binding to NusA-hCXCL9 (A) or to the control protein, NusA (B). Clones from each plate were double spotted in a defined pattern to facilitate identification and retrieval of the hits. (C) Schematic representation of a double spotted pattern corresponding to plate 8. Signals corresponding to clones C1, F13, J5, J9 and P8 are indicated. As control, the parental clone E7 was grown in all the wells in column 24 of plate 8. No spot in orientation 8 could be detected in column 24 thus indicating that under the conditions used E7 did not generate a positive signal, thus facilitating the identification of potentially improved variants for binding to hCXCL9.

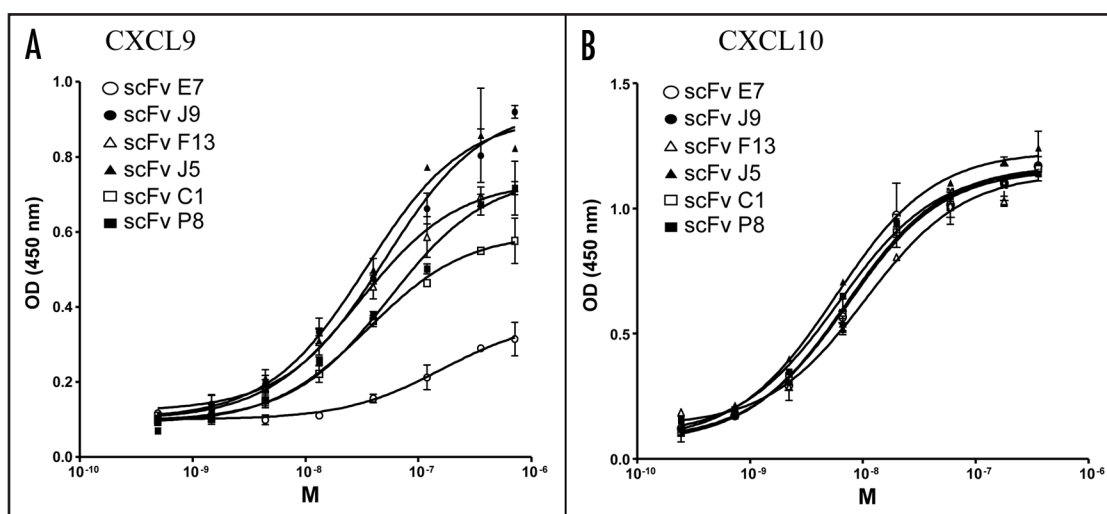


Figure 4. Dose-response of the variants for hCXCL9 and hCXCL10 binding. ELISA were performed using different concentrations of purified scFv against hCXCL10 (A) or hCXCL9 (B). Chemokine target was coated either at 2 μ g/ml for NusA-hCXCL10 or 5 μ g/ml for NusA-hCXCL9 fusion protein. Serial 3-fold dilutions of scFv were performed in 1% milk-PBS buffer and scFv was incubated for one hour at room temperature. Results are expressed as mean $\pm$ S.D. of duplicates.

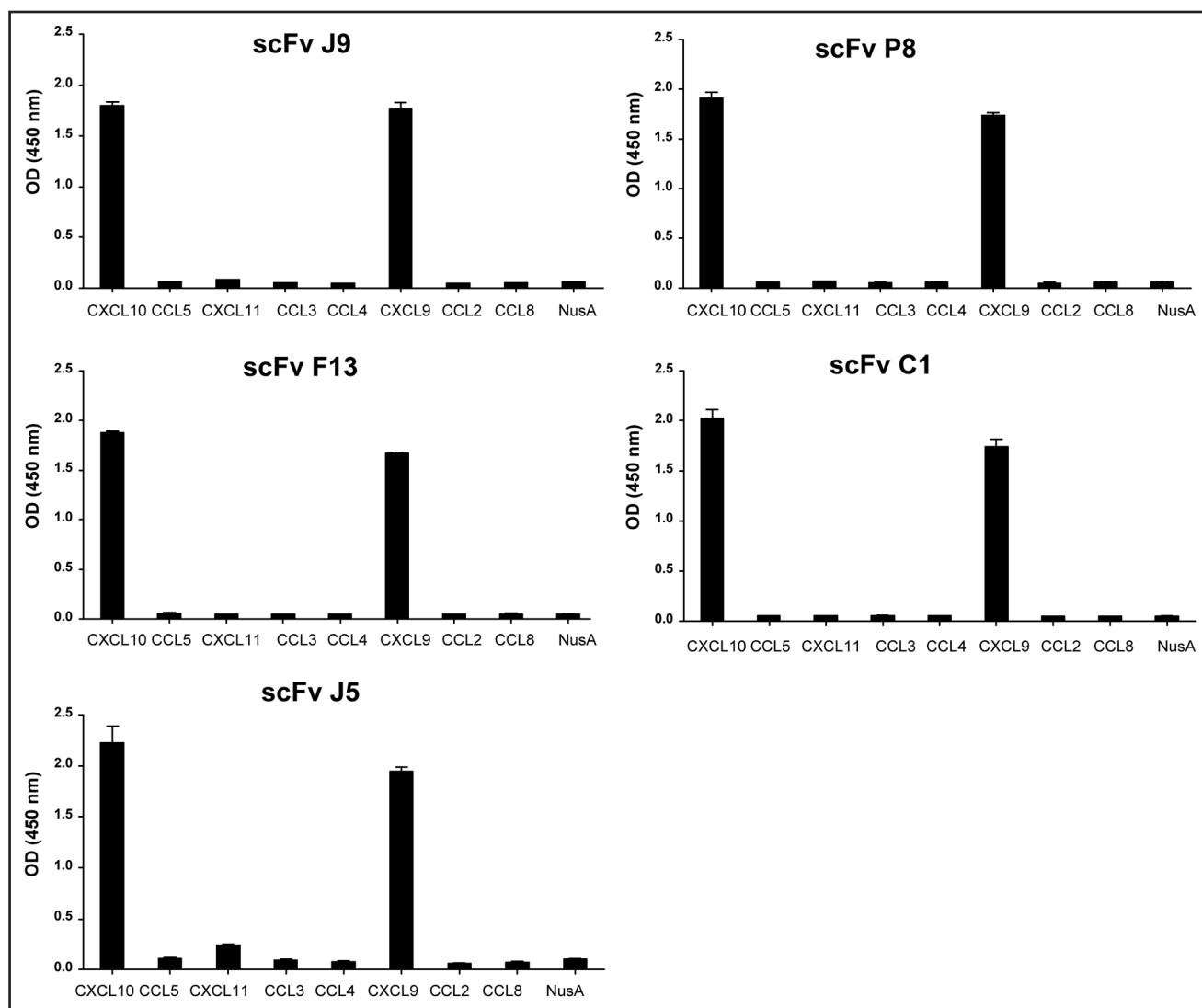


Figure 5. Specificity of improved scFv variants. Binding of the selected scFv candidates was tested in an ELISA against a panel of human chemokines. NusA fusion chemokine was coated at 20 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ variant scFv in 1% milk-PBS buffer was incubated for 1 hour at room temperature. 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 $\pm$ S.D. of duplicates of two representative experiments.

suggests that this region of the VL CDR3 contains critical residues for binding to hCXCL9 or alternatively that these residues might have an indirect effect by modifying the conformation of the VH CDR3. Moreover, for J9 and P8, scFv that share very similar VL CDR3 sequences, a three- to four-fold improvement of the neutralizing capacity against hCXCL10 was also observed. These results indicated that the simultaneous improvement of the neutralization potential of a scFv against two targets was achieved (Figure 6, Table III).

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.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

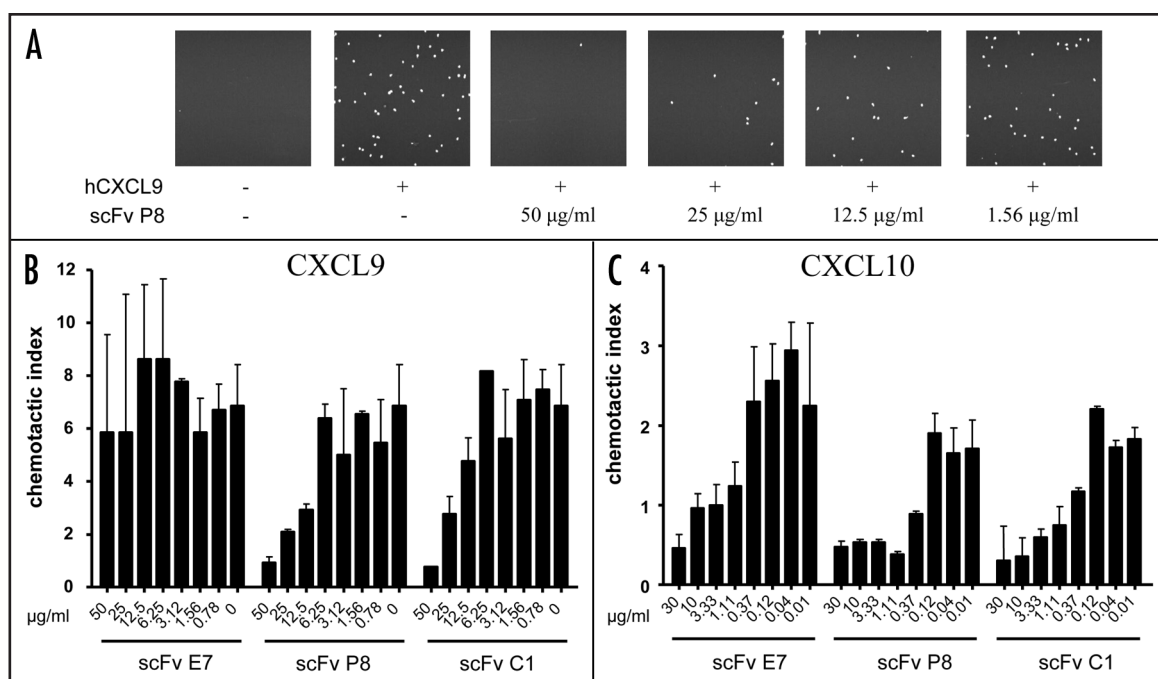


Figure 6. Dose dependant chemotaxis inhibition of improved scFv variants. (A) The assay involves L1.2 cells expressing hCXCR3 which migrate in response to an established chemokine gradient which was visualized and quantified using FMAT. (B and C) Dose response data for the chemokines, CXCL9 (B) or CXCL10 (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 $\pm$ S.D. of duplicates of three representative experiments.

solely against CXCL10 have elicited beneficial effects in animal models, which suggests that the system is not totally redundant. However, the ability to neutralize several ligands of CXCR3 could prove far more effective. We therefore tried to expand the neutralization potential of scFv E7 that was able to block the biological activity hCXCL10 but was only weakly cross-reactive towards hCXCL9 and unable to block its biological activity. Several variants of this scFv were isolated by diversifying the CDR3 of the light chain and combining phage selections against hCXCL10 and high throughput screening of a large number of clones for improved binding against hCXCL9. This approach allowed for clear improvement of the neutralization potential of E7 against hCXCL9 (from no effect to inhibition in the nM range). For two of the scFv, the strategy also lead 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.¹⁹ 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.¹⁹

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-5.²⁰ Interestingly, analysis of the crystal structure of the Fab fragment complexed with hMCP-1 identified key interacting residues, allowing for the generation of variants with improved binding to hMCP-2. In this case, the different MCP chemokines recognized by this polyspecific antibody shared 44%-68% sequence identity. In contrast, hCXCL9 and hCXCL10 are only 37% identical in amino acid sequence thus indicating that engineering the cross-reactivity of antibodies is not restricted to proteins that are highly related in sequence, but is likely to be more easily achieved against structurally conserved proteins (Supplementary Figure 2). Indeed, although chemokines can be very different in linear sequence, they share a conserved three-dimensional fold.² The way by which the scFv described in

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 investigational 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 anti-mouse HRP from Jackson Immunoresearch. Phage display libraries were obtained from MedImmune.^{7,8}

Phage display scFv selection in liquid phase. scFv phage libraries (10^{12} Pfu) were blocked with phosphate buffered saline (PBS) containing 3% (w/v) skimmed milk and then deselected on streptavidin magnetic beads (DynaM-280). Deselected phage was incubated with NusA-hCXCL10 biotinylated fusion protein (10 nM for the first round of selection and 1 nM for the followings). NusA protein added in excess at 1 μ M, for two hours at room temperature. An off-rate, consisting of incubating bound phage with an excess of non biotinylated hCXCL10 in order to eliminate phage with fast off-rate, was also performed in round 3. Beads were captured using a magnetic stand followed by five washes with PBS/0.1% Tween 20 and three washes with PBS. Washing stringency was increased during selection rounds up to ten washes PBS/0.1% Tween 20. Beads were then directly added to 10 ml of exponentially growing TG1 cells and incubated for one hour at 37°C (100 rpm). An aliquot of the infected TG1 was serially diluted to titer the selection output. The remaining infected TG1 were spread on 2xTYAG agar Bioassay plates. After overnight incubation at

30°C, cells were scraped from the agar surface with 2xTYAG (100 μ g/ml ampicillin; 2% glucose) medium and aliquots were stored at -80°C in 17 % glycerol.

Phage rescue. 50 μ l of cell suspension obtained from previous selection rounds were added to 20 ml of 2xTYAG and grown at 37°C (240 rpm) until the OD₆₀₀ reached 0.3 to 0.5. The culture was then super-infected with 3.3×10^{10} MK13K07 helper phage and incubated for one hour at 37°C (100 rpm). Culture medium was then changed for 2xTYAK (100 μ g/ml ampicillin; 50 μ g/ml kanamycin) 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 μ l H₂O and frozen at -80°C. PCR reaction was then performed with 5 μ l of thawed cell suspension and PCR products were purified on PCR μ 96 plate (Millipore). Sequencing reactions were outsourced (Fasteris, Geneva, Switzerland) and the sequences analysed using Sequencher 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 (QPDdisplay, Genetix) into 384-well plates containing 2xTYAG 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 (QPDdisplay, Genetix) onto a nitrocellulose membrane (Protran BA 85 Schleicher & Schuell, 22x22 cm, 0.45 μ m, 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 μ g/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 μ g/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 NusaA-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 4°C 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 to 8.0). The suspension was added to a polyprep column (Biorad). Eight milliliters of cold Wash Buffer 2 (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH to 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 to 8.0). Protein fractions were analyzed by absorption at 280 nm and pooled after buffer exchange on a PD10 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 were cultured in RPMI medium supplemented with 10% FCS. Periplasmic extracts containing the scFv were incubated with 2-10 nM of hIP-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 final concentration of DRAQ-5 (Alexis Corporation) and counted on an FMAT 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

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