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Humanization of the anti-CD18 antibody 6.7: an unexpected effect of a framework residue in binding to antigen

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Abstract

Humanization of monoclonal antibodies by complementary determinant region (CDR)-grafting has become a standard procedure to improve the clinical usage of animal antibodies. However, antibody humanization may result in loss of activity that has been attributed to structural constraints in the framework structure. In this paper, we report the complete humanization of the 6.7 anti-human CD18 monoclonal antibody in a scFv form. We used a germline-based approach to design a humanized VL gene fragment and expressed it together with a previously described humanized VH. The designed humanized VL has only 14 mutations compared to the closest human germline sequence. The resulting humanized scFv maintained the binding capacity and specificity to human CD18 expressed on the cell surface of peripheral blood mononuclear cells (PBMC), and showed the same pattern of staining T-lymphocytes sub-populations, in comparison to the original monoclonal antibody. We observed an unexpected effect of a conserved mouse–human framework position (L37) that hinders the binding of the humanized scFv to antigen. This paper reveals a new framework residue that interferes with paratope and antigen binding and also reinforces the germline approach as a successful strategy to humanize antibodies.

Keywords: Humanization; Framework; CDR-grafting; CD18

1. Introduction

Antibody engineering has become a popular technology for development of a new generation of drugs (Presta, 2002; Maynard and Georgiou, 2000). The amount knowledge available with regards to antibody structure at atomic resolution, allied to its conserved conformation, permits the elaboration of general rules for antigen binding site structural and functional features. To date, the commonest antibody manipulation is the process of humanization. In this process, a murine antibody sequence is replaced by a homologous human sequence yielding a human-like antibody, reducing its immunogenicity, and preserving the binding to the original antigen (Jones et al., 1986; Riechmann et al., 1988;Verhoeyen et al., 1988). This methodology has revolutionized the clinical use of monoclonal antibodies (mAb), since the patients' response to heterologous rodent sequences prevents the full biological effect of the monoclonal antibody (Winter and Harris, 1993). Moreover, heterologous mAbs present a reduced half-life and a diminished capacity to induce effector functions (Isaacs et al., 1992). The limit of this process is in the preservation of the antibody's affinity and specificity. After a decade of research in this process, many rules have been established that help maintain the overall antibody structure while the mouse primary sequence is being saturated with human residues. The use of human germline sequences (Rosok et al., 1996; Caldas et al., 2000) and the maintenance of key framework residues are examples of these rules (Tempest et al., 1991). Many successfully humanized antibodies have been reported, including in this group those that are already being routinely used in therapeutics (Glennie and Johnson, 2000).

Antibody humanization is becoming a trivial methodology, where many different experimental procedures result in an active antibody. In spite of this, many of the designed human antibodies do not fully mimic the behavior of the original murine protein. Loss of affinity, specificity, or stability are the main parameters affected by this experimental

Abbreviations: bp, base-pair; CDR, complementary determinant region; Fab, antigen binding fragment; FR, framework; PCR, polymerase chain reaction; scFv, single chain variable fragment; mAb, monoclonal antibody; VL, variable light chain; VH, variable heavy chain

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procedure. Mild losses are normally associated with this procedure (Carter et al., 1992; Hsiao et al., 1994). A lower affinity is normally tolerated for humanized antibodies.

These effects have been partially remedied by reengineering the framework (Kettleborough et al., 1991; Singer et al., 1993; Nakatani et al., 1994; Zhu and Carter, 1995; Saldanha et al., 1999). Therefore, it is crucial to describe more subtle structural features that allow the preservation of antibody paratope integrity during this process. The rules are far from being completely understood. Long-range effects promoted by certain residues are among those forces responsible for subtle changes in the global structure. Unfortunately these are the most difficult issues to probe, since even small deviations could be responsible for removing residue–residue contact, or reducing surface complementarity. Therefore, even distant residues could interfere with paratope conformation and antigen binding (Chien et al., 1989).

We used an anti-human CD18 as a model antibody for humanization. CD18 is an integrin family membrane protein involved in cell adhesion. It is found in many different cell types and it is always associated with one of the different isoforms of CD11. Antibodies to CD18 may inhibit cell–cell attachment and especially leukocyte-tissue adhesion. Therefore, anti-CD18 antibodies have been proposed as adjuvant in many therapies that involves leukocytes infiltration and inflammation. They have been tested successfully as protective agents in ischemic myocardial injury in animal models (Gao et al., 2002), but much of this enthusiasm was lost after failure of human trials (Dove, 2000). Its potential application is much wider, and it has also been cited as a potential treatment for preventing meningitis sequels or graft rejection (Tuomanen et al., 1989; Isobe et al., 1997). The antibody used in this work, mAb 6.7, binds to CD18 (David et al., 1991) in a unique inhibitory epitope recently mapped to residues 350–432 (Lu et al., 2001).

In this work we describe the complete humanization of murine mAb 6.7 anti-human CD18. In a previous paper we described the successful humanization of the VH domain by means of a germline human VH gene fragment sequence using an expanded CDR1 (CDR1 + H1) graft (Caldas et al., 2000). We apply this same concept to the design of two versions of the humanized VL domain. While testing them, we have shown the effect of a mutation far from the antibody's binding site that resulted in a loss of affinity for the intact CD18 molecule on the cell surface. We propose it could be the result of a new, unexpected effect of a residue that interferes with binding of the completely humanized scFv even though it is located at a distance from the binding site.

2. Materials and methods

2.1. Computational analysis

Similarity analysis was initially performed using FASTA (Pearson, 2000) and the Swiss-prot database (Bairoch and Apweiler, 2000). Blastp (Altschul et al., 1997) was also used either through the IgBlast page (http://www.ncbi.nlm.nih.gov/igblast/) or to analyze the PDB database (Berman et al., 2000). Amino acid residues usage in mouse and human VL was calculated from all-mouse and all-human VL files from Kabat Database (Johnson and Wu, 2000), using perl scripts. Germline VL sequences were obtained from the IgBlast page. Clustal W (Higgins et al., 1996) was used for multisequence alignment that was visualized using BioEdit version 5.0.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Tri-dimensional structure was visualized using RASMOL version 2.6 (Bernstein, 2000). This version of RASMOL permits direct distance calculations, but we also used perl scripts to perform such calculations. Accessibility of each atom in PDB file was calculated using the program Surfrace version 1.1 (Tsodikov et al., 2002). Variable region numbering follows Kabat's convention (Kabat et al., 1991).

2.2. Synthetic oligonucleotides

The overlapping oligonucleotides used for the synthesis of the humanized versions were supplied by DNAgency (Malvern, PA). The oligonucleotides used were: L1 (5′ AGAAGATCTGACGTGGTTATGACCCAAAGCCCCTTGTCCCTGCCAGTCACTCTGGGC3′); L2 (5′GTGCACCAAGCGTTGGCTA GACCTGCAGCTTATAGAGGCAGGCTGGCCCAGAGTGACTGG3′); L3L (5′CAACGCTTGGTGCACACCAACGGTAACACCTACTTCCACTGGTTTC**TT**CAAAGACCAGGACAG3′); L3Q (5′CAACGCTTGGTGCACACCAACGGTAACACCTACTTCCACTGGTTTC**AA**CAAAGACCAGGACAG3′); L4 (5′AAAGAATCTATTGGAAACCTTGTAAATCAACAGACGGGGGCTCTGTCCTGGTCTTTG3′); L5 (5′ TCCAATAGATTCTTTGGAGTCCCAGACAGGTTTTCTGGCTCTGGTAGCGGGACTGATTTC3′); L6 (5′ATACACCCCGACATCCTCAGCTTCTACCCTGGAAATTTTGAGTGTGAAATCAGTCCCGCT3′); L7 (5′GATGTCGGGGTGTATTATTGTTCACAGTCAACACATGTTCCCCGGACTTTCGGTGGTGGC3′); L8 (5′ACCATGGGCTCTCTTGATCTCGAGCTTTGTGCCACCACCGAAAGT3′); EXTL1 (5′GC TAGTAGAAGATCT3′) and EXTL2 (5′CACACCATGGGCTCT3′).

The 5′ L1 oligonucleotide contains a *Bgl*II restriction site and the 3′ L8 carries a *Nco*I and a *Xho*I site (these sites are underlined). The L3L and L3Q contain the codon change between the two VL humanized versions in bold (see results).

2.3. Assembly of the VL humanized versions

The 6.7 VL sequence was previously determined (Caldas et al., 2000) and its amino acid sequence was used for a search for the closest human germline sequence. The closest human germline sequence chosen was used as a framework to graft the murine CDRs. The DNA fragments for two humanized VL versions were generated using eight overlapping oligonucleotides ranging from 45 to 63 bp, with 15 bp of complementarity, in a PCR-based-protocol. Aliquots of

10 pmol of each pair of complementary oligonucleotides were annealed separately in a 50 μl reaction containing 9 mM Tris–HCl (pH 7.6), 13 mM $MgCl_2$, 21 mM DTT and 200 μM dNTPs. The samples were incubated in 400 ml of boiling water for 5 min and left standing until the water reached room temperature. Each pair of primers were elongated by the addition of 24 U DNA polymerase I (Klenow fragment, Biolabs) for 30 min at room temperature. The two pairs of primers coding for the N-terminus were mixed and amplified by PCR, and the same procedure was followed for the two pairs of primers coding for the C-terminus. The DNA fragments for the two N-terminus and C-terminus regions were amplified by 20 thermal cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 2 min and analyzed in agarose gel. Finally, the full-length DNA fragment was amplified using as DNA templates the amplified fragments from the first PCR, extracted directly from the agarose gel with a pipet tip. The 5′and 3′ external primers (EXTL1 and EXTL2) were used in this second PCR; the DNA fragments were PCR-amplified after 25 thermal cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 2 min, purified with Quiaquick gel purification system (QIAGEN), using the manufacturer's recommendation, cloned in the pGEM-T vector (Promega) and sequenced using the T7 Sequencing Kit (Pharmacia).

2.4. Construction of the expression vectors

The constructs that code for the humanized scFvs were assembled based on the pIg17hVH/mVL (Caldas et al., 2000) derived from pIg17Z22 (Brigido et al., 1993). In this system, the proteins can be expressed as a fusion product with a staphylococcal protein A domain, in order to allow the detection of the recombinant protein and also to facilitate the purification in an IgG sepharose chromatography column. The DNA fragments of the humanized VL were cloned in the vector pIg17hVH/mVL, which had its murine VL replaced by the humanized VLs, through digestion with *Bgl*II and *Nco*I. After the construction and verification of the expression cassettes, these were transferred to the *Pichia pastoris* expression vector pPIg16 (Andrade et al., 2000) by replacing the existing scFv, through digestion with the restriction enzymes *Xma*I and *Eco*RI.

2.5. Expression of the humanized scFvs in Pichia pastoris

P. pastoris GS115 cells (Invitrogen, San Diego, CA) were grown in liquid medium and made competent by resuspension in 1 M sorbitol. The cells were eletroporated by pulse discharge (1500 V, 25 μF, 400 Ω; Bio-Rad Gene Pulser) for 5 ms in the presence of 5–10 μg of plasmid DNA linearized with *Sal*I. This enzyme cuts within the plasmid-encoded *HIS4* gene and favors homologous recombination with the endogenous, non-functional *his4* gene of GS115 cells. Therefore, transformants (His+) were screened by their capacity to grow in the absence of histidine as described by the manufacturer (Invitrogen). Protein expression kinetics were determined by growing clones expressing the two humanized scFvs in 25 ml of BMGY medium (1% yeast extract, 2% peptone, 10 mM potassium phosphate, pH 6.0, 1,34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol) at 30 °C in a shaking incubator (250 rpm) until the culture reached $A_{600} = 2.0$–6.0. Cells were then centrifuged and resuspended in 100–200 ml of BMMY medium (which has 0.5% methanol instead of 1% glycerol of the BMGY medium, while the other components are the same) to induce protein expression. Cells were incubated for 4 days at 30 °C in a shaking incubator (250 rpm). Aliquots of culture supernatants were taken daily, and examined by SDS–PAGE and Western blotting. For large scale expression, the clones were grown exactly the same way as above, for 80 h at 30 °C under agitation. The supernatants were harvested following centrifugation and filtration through a 0.45 μm cellulose acetate filter. After the addition of 80 μg of Pepstatin A and 14 μg of PMSF to the supernatants, these were concentrated to about 5 ml using an ultrafiltrating stirred cell (Corning) with a membrane filter with a cut-off of 10,000 Da according to manufacturer's instructions.

2.6. Purification of recombinant scFvs

The concentrated supernatants were run through an IgG Sepharose 6B Fast Flow column (Pharmacia) previously activated by three alternating washes with 0.5 M acetic acid, pH 3.4, and PBST (PBS and Tween 20, 0.1%) and finally equilibrated with PBS. ScFv fragments were eluted with 0.5 M acetic acid, immediately neutralized with 1.5 M Tris–HCl, pH 8.8. The purified proteins were dialyzed against PBS and quantified using the BCA Protein Assay Kit (Pierce).

2.7. Flow cytometric analysis

Peripheral blood mononuclear cells (PBMC) obtained from a normal individual by gradient centrifugation were used for immunofluorescence assays. Antibodies utilized were: recombinant Z22 scFv (Andrade et al., 2000) as a negative control; rabbit anti-human IgG-FITC (Dakopatts, Denmark; used in the second step of the indirect immunofluorescence reaction to bind to the protein A domain present in the recombinant anti-CD18 scFvs, through the Fc fragment); rabbit anti-mouse IgG (Sigma); 6.7 anti-CD18 FITC (Instituto Butantan-InCor, Brazil); anti-CD19PE (Dakopatts, Denmark); anti-CD3 FITC (Dakopatts, Denmark); anti-CD4 FITC (Dakopatts, Denmark); anti-CD8 FITC (Dakopatts, Denmark) and anti-CD45RO PE (Pharmingen). The sample incubated with both anti-CD19PE and rabbit anti-human IgG-FITC was used to evaluate binding of the rabbit antibody to IgG expressed on B cells and exclude any other unspecific binding from the tests with the scFvs. Anti-CD3 was used as a positive control of the assays. 2×10^5 cells were incubated with the different antibodies for 30 min at 4 °C and washed three times. For the samples with the recombinant humanized scFvs, a second incubation was

performed with rabbit anti-human IgG-FITC. All samples were resuspended in 400 μl of FACS buffer (PBS, 2% FCS and 0.01% sodium azide) and analyzed using a FACScan flow cytometer (Becton Dickinson, CA, USA). Ten thousand events were analyzed for each sample, inside the gate of lymphocytes. Recombinant proteins were added in equimolar quantities. Results are expressed as the percentage of stained cells. The antibodies anti-CD4, anti-CD8 and anti-CD45RO were used in order to characterize the T-lymphocyte sub-populations that the humanized scFvs were able to bind.

2.8. Blocking capacity of the humanized scFvs

In order to analyze the binding specificity of the two humanized scFvs, a blocking experiment was performed. The capacity of the scFvs to block the binding of the original 6.7 anti-CD18 FITC to surface CD18 molecules was tested. Cells were initially incubated with the two humanized scFvs, washed, incubated with rabbit anti-mouse IgG (to block the protein A domain present in the recombinant scFvs) and then incubated with 6.7 FITC. The percentage of positive cells and the intensity of immunofluorescence (IF) were compared in samples with 6.7 FITC alone and samples with the different humanized scFvs plus 6.7 FITC. The percentage of inhibition was calculated considering these differences.

3. Results

3.1. Selection of the framework Vκ and Jκ for CDR-grafting

The human framework used to accept the murine CDRs was selected based on the closest germline sequence (Caldas et al., 2000). We used the FASTA program to search for human Vκ sequences deposited in the Swiss-prot database. The closest human sequence found was the germline Vκ fragment KV2F (Klobeck et al., 1985) with 76% identity and 89% similarity to the mouse 6.7 Vκ gene fragment (AF135165). A similar result was obtained using the NCBI IgBlast tool. In this case, two human germline Vκ sequences exhibit good hits with the mouse Vκ. The closest was the A17 (X63403) with 76% identity followed by the A18 (X63396) with 74% identity (Lautner-Rieske et al., 1992). The alignment of the original anti-CD18 Vκ gene segment to the human related sequences is shown in Fig. 1. The A17 coding sequence is identical to the KV2F Swiss-prot record, while A18 is 81% identical (91% similar) to either A17 or KV2F. Either Vκ could be used for grafting the 6.7 Vκ complementary determinant region (CDR), but the KV2F/A17 was chosen due to its closer proximity. The 6.7 Jκ (AF135165) closest human Jκ gene segment is the Jκ4 with only one difference. Thus the human germline Jκ4 sequence was chosen for completing the FR4 of the humanized VL. The conservative V → L codon change observed in the recombinant FR4 (Fig. 1) is due to an *Xho*I site at the end of VL used for the expression vector manipulation.

3.2. Identification of putative constraints

The visual inspection of two of the closest murine (1MRC) and human (1AD9) Fab crystals suggests an overall conserved structure in the VL domain. The systematic survey of the tri-dimensional structure at the replacing residues reveals two positions that could be important for maintaining the framework structure. The murine residue Leu[46] was found to be buried in the VH–VL interface. By using a cut-off distance of 4.0 Å, VL residue 46 makes many contacts

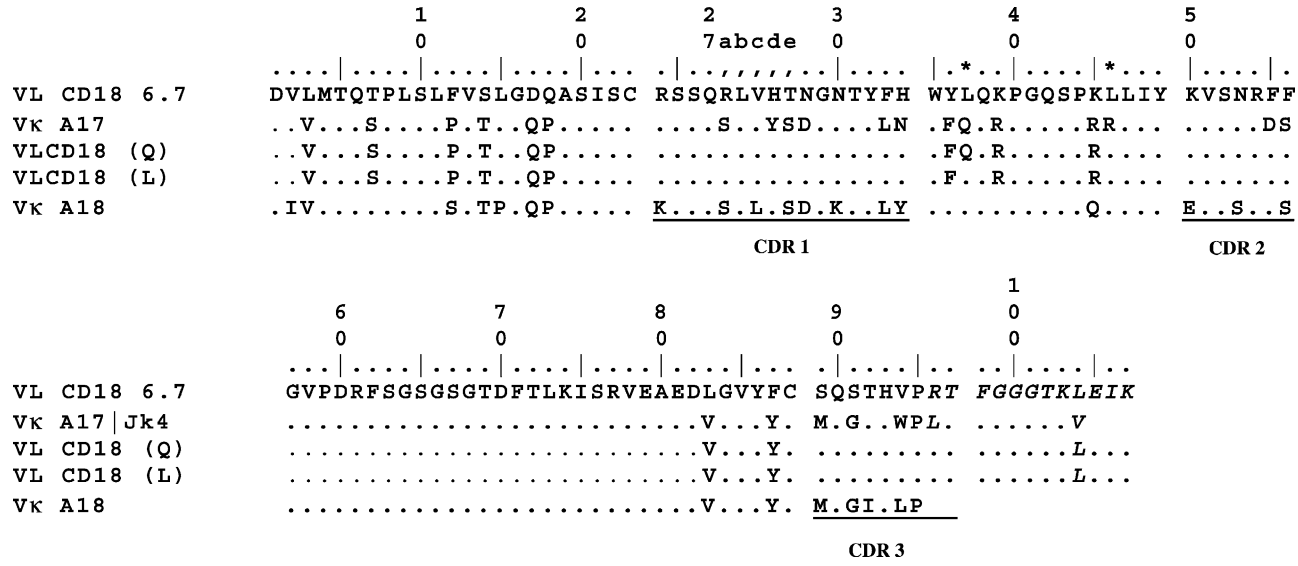

Fig. 1. Design of anti-CD18 VL humanized versions. Amino acid sequence alignments of original murine VL (VL 6.7), closest human germline VL (Vk A17 and Vk A18) and humanized VL versions (VLCD18 (Q) and VLCD18 (L)). CDR residues, according to Kabat et al. (1991) are indicated. Asterisks indicate the residue 37 and 46, maintained in the humanized VL as discussed in the text. Jk residues are shown in italic.

including one to the LCDR 2 residue Phe[55], two "Vernier" zone residues (Tyr[36] and Trp[35]), framework residues Ile[48], Val[58], and the HCDR3 residues Asp[101] e Gly[97]. Therefore, we decided to preserve the original murine Leu[46] due to its array of contacts that includes contacts to VH and VL CDRs.

Murine residue Leu[37] was found to be superficially buried, making many contacts within the VL domain core. Its contact residues in 1MRC structure at 4.0 Å cut-off are: Pro[44], Lys[45], Leu[47] and Tyr[86]. The Lys[45] contact is one of the conserved hydrogen bonds involved in the maintenance of the variable domain structure (Chothia et al., 1985). In the 1AD9 structure, Gln[37] contacts the same subset of residues as above. The only discrepancy is a hypothetical hydrogen bond between the hydroxyl O of the Tyr[86] and the amide N^{ξ} of Gln[37] ($d = 282$ Å). In the 1MRC VL, the residue 37 is partially exposed to the solvent making a contact to a water molecule in the crystal. No water was found close to residue Gln[37] in the VL of 1AD9. A survey of the Kabat database showed that position 37 is filled with either glutamine or leucine. For the mouse light chain, leucine appears in 20% of the antibodies while glutamine responds for 78% of all-mouse antibodies; other amino acid residues make up less than 3%. Similar numbers occur for human light chain. Due to its location in the VL-solvent interface and to its close proximity to the CDR1, we chose this position to construct two humanized versions for the VL domain of the 6.7. The first construction carries the original L[37] residue (named version L) while the other has the human germline Gln[37] residue (version Q). Both constructions retained the murine Leu[46] residue.

3.3. Design of the recombinant humanized VL

The complete humanized VL sequence was obtained by grafting the CDR1, 2 and 3 from the 6.7 VL in the proposed human germline Vκ (KV2F/A17) fused to Jκ4 gene fragment. CDR1 was defined as residues 24 to 34 (24–39 in sequential numbering), CDR2, as residues 50–56 (55–61), and CDR3, residues 89–97 (94–102), following the Kabat definition (Kabat et al., 1991). The grafting process introduced six changes in CDR1, 2 in CDR2 and 5 in CDR3 of the human VL, resulting in 13 differences out of the 32 residues in the CDRs. The complete humanized sequences are shown in Fig. 1. The final proposed humanized sequence had 12 differences compared to the original 6.7 VL (89% identity) and 14 differences to the original $V\kappa_{KV2F/A17}$/Jκ4 (87.6% identity) in the Leu[37] version (Fig. 1). The Jκ fragment was used as is, except for the Leu[104] introduced by an *Xho*I site. The proposed recombinant VL was chemically synthesized for cloning in an scFv expression vector.

3.4. Construction and expression of the humanized scFv

As shown in Fig. 2, a set of ten oligonucleotides was used to generate the humanized VL using recombinant PCR as previously described for the VH domain (Caldas et al., 2000). Briefly, the primers were initially annealed, filled in by Klenow and then amplified as pairs until they reached the full sized VL (Fig. 2). The synthetic DNA fragment was cloned in pGEM-T easy vector. The recombinant clones were initially checked for size of insert prior to being repeatedly sequenced. Several clones with the correct sized insert were tested and one clone (out of six) of the L version and one (out of 8) for Q version had a correct sequence. Correctly synthesized DNA inserts were digested with *Bgl*II and *Xho*I, and isolated from gel for cloning in the pIg17hVH/mVL plasmid cleaved with the same restriction endonucleases. This plasmid already harbored a hemi-humanized version of the 6.7 anti-CD18 scFv composed of a humanized VH fused to the original murine VL (Caldas et al., 2000). The cloning procedure eliminates the original murine VL, replacing it with the synthetically humanized gene fragment. Two new constructions were obtained: pIg17hVH/hVL(L) and pIg17hVH/hVL(Q). For simplicity, these constructions were named pIg17LL and pIg17LQ, respectively. The whole scFv cassette, digested with *Xma*I/*Eco*RI, was used to replace the scFv cassette of pPIg16, a *P. pastoris* expression vector (Andrade et al., 2000). According to the notation used above, the resulting plasmids were named pPIg LL and pPIg LQ.

A protease defective strain of *P. pastoris* was used to receive the expression vectors by electroporation. Both plasmids were used to transform electrocompetent yeast cells. Many clones were obtained and screened for scFv production in the colony filter assay, where scFv producing cells were detected by immunostaining (Andrade et al., 2000). Two clones of each construction, found to be positive in the filter assay, were selected for growth on an analytical scale. In both cases, the detection of recombinant scFv was low. Filter assay positive clones were barely visible and the yield of purified scFv was also limiting. From 200 ml of yeast cultures, we normally obtained about 0.5–1 mg/l. Even so, we were able to purify around 1 mg of recombinant scFv of both L and Q version of the humanized anti-CD18 for further characterization.

3.5. Immunological characterization of recombinant humanized antibodies

The CD18 antigen is expressed at the cell surface of all leucocytes. Therefore, we tested the two variants of recombinant humanized antibody (LL and LQ) directly for binding to peripheral blood mononuclear cells (PBMC) by Flow Cytometry analysis. Our results show that the humanized LL version presents essentially the same binding capacity to lymphocytes as the original anti-CD18 monoclonal antibody, staining 85% of gated cells. In contrast, the LQ version only stained 53% of the cells in the gate of lymphocytes (Fig. 3). The original 6.7 anti-CD18 mAb and an anti-DNA scFv were used as positive and negative controls, respectively. The small population stained with high intensity in

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