Isolation of anti-CD22 Fv with high affinity by Fv display on human cells

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In vitro antibody affinity maturation has generally been achieved by display of mouse or human antibodies on the surface of microorganisms (phage, bacteria, and yeast). However, problems with protein folding, posttranslational modification, and codon usage still limit the number of improved antibodies that can be obtained. An ideal system would select and improve antibodies in a mammalian cell environment where they are naturally made. Here we show that human embryonic kidney 293T cells that are widely used for transient protein expression can be used for cell surface display of single-chain Fv antibodies for affinity maturation. In a proof-of-concept experiment, cells expressing a rare mutant antibody with higher affinity were enriched 240-fold by a single-pass cell sorting from a large excess of cells expressing WT antibody with a slightly lower affinity. Furthermore, we successfully obtained a highly enriched mutant with increased binding affinity for CD22 after a single selection of a combinatorial library randomizing an intrinsic antibody hotspot. Important features are that one display selection cycle requires only 1 week, and transfection of cells in a single 100-mm dish produces 10^7 individual clones so that a repertoire of 10^9 is feasible under current experimental conditions.

antibody engineering | B cell malignancy | FACS | human embryonic kidney cells | receptors

Creating high-affinity antibodies to important biomolecules for clinical use is an important and challenging task. Seventeen therapeutic mAbs have been approved by the Food and Drug Administration, and many more mAbs will be approved in the next few years (reviewed in ref. 1). The levels of antibody affinity obtained from hybridomas are often not sufficient for effective clinical use in part because of the *in vivo* affinity ceiling (2, 3); therefore an improvement of antibody affinity is often required (4). For the past 2 decades phage display was used for *in vitro* antibody affinity maturation (5–10), and more recently, bacterial (11–13) and yeast cell surface display systems have been developed (14–16). A significant advantage of cell surface display over phage display is the use of quantitative flow cytometric sorting and analysis to identify high-affinity interactions and normalize for antibody protein expression. Cell-free systems like ribosome display are useful tools in antibody affinity maturation because of the large size of libraries (10^{13} to 10^{14}) that can be produced and analyzed (17–19). These techniques have all been successful, but problems with protein folding, posttranslational modification, and codon usage limit the number of improved antibodies that can be obtained (20). Many potential therapeutic proteins are highly aggregation-prone upon heterologous expression in bacteria (21). Moreover, serious translational problems can occur when proteins selected in bacteria, yeast, or cell-free systems are transferred into mammalian expression systems to produce therapeutic antibodies.

Mammalian cells such as Chinese hamster ovary cells are the dominant system used for the production of the majority of therapeutic antibodies in the pharmaceutical industry (22). Therefore, mammalian cells should be used in antibody selection to avoid problems caused by selection in microorganisms. To use an animal cell environment for antibody selection and affinity

maturation, a hypermutating chicken B lymphoma line (23) and a retrovirus display system (20) have been developed. Recently, a random peptide library fused to CCR5 and expressed on the mammalian cell surface has been reported (24). The potential of this display system has been demonstrated for small peptides of 9 or 13 aa. A mammalian cell surface-displayed antibody system, which is robust and comparable to existing phage and yeast display technologies used in antibody engineering, has not yet been reported. Here, we show that human cells can be engineered for cell surface display of functional single-chain Fv (scFv) antibodies and is suitable for affinity maturation. For this purpose we have used the anti-CD22 scFv fused to the transmembrane domain of human platelet-derived growth factor receptor (PDGFR) displayed on human embryonic kidney (HEK) 293T cells. We find that the mammalian cell scaffold allows a 240-fold single-pass enrichment of a rare higher-affinity mutant from a large excess of only slightly lower-affinity WT antibody. Furthermore, we show that a mutant with an increased binding affinity for CD22 can be obtained after a single-pass selection of a combinatorial library randomizing an intrinsic antibody hotspot.

Results

Expression and Surface Localization of scFv. We first determined whether HEK 293T cells, widely used for transient protein expression, could be engineered for cell surface display of functional antibody Fvs. Because of our interest in targeting CD22-expressing cancers, we chose the anti-CD22 scFv RFB4 fused to the transmembrane domain of PDGFR as the experimental system. The expression vector contains the cytomegalovirus promoter and the nucleotide sequence encoding the murine Ig κ chain signal peptide (METDTLLWVLLWVPGSTGDJ) and the transmembrane domain (amino acids 514–562) of PDGFR (25). Fusion to the N-terminal portion of PDGFR has been used to anchor other proteins such as a virus hemagglutinin (26), toll-like receptors (27), and a chimeric human IgG-cat allergen fusion protein (28).

We expressed WT anti-CD22 BL22 (RFB4) scFv or its high-affinity derivative, HA22 scFv, on HEK 293T human cells (Fig. 1A). For comparisons, scFvs were also expressed in phage (10) or yeast display systems. Fig. 1B shows a structural model of the BL22 Fv. The protein contains a myc epitope tag at the carboxyl terminal of the scFv that is used to measure the expression level. A time-course experiment indicated that the expression level of transfected scFv–PDGFR is maximized 48–72 h after transfection (data not shown). Surface localization of the scFv–PDGFR fusion was verified by confocal fluorescence microscopy and flow cytometry. Cells labeled simultaneously with biotinylated CD22-Fc proteins and an anti-c-myc mAb were examined by laser scanning confocal microscopy (Fig. 2). Cells bearing the surface display vector expressing the scFv–PDGFR

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Abbreviations: CDR, complementary determining region; HEK, human embryonic kidney; MFI, mean fluorescence intensity; PE, phycoerythrin; PDGFR, platelet-derived growth factor receptor; PT, Pro-91–Thr-92; scFv, single-chain Fv.

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Fig. 3. Flow cytometric analyses of mammalian (A and B) and yeast cells (C and D) displaying anti-CD22 scFv. HEK 293T (A and B) and *S. cerevisiae* (C and D) cells displayed the same anti-CD22 scFv construct (HA22) at the cell surface. (A and B) In mammalian display, HEK 293T cells (A) or transfected cells (B) were labeled with biotinylated CD22-Fc proteins followed by streptavidin-R-PE conjugate and mAb 9E10 (anti-c-myc epitope tag) followed by an anti-mouse IgG-FITC conjugate. (C and D) In yeast display, noninduced (C) and induced (D) yeast cells were labeled with biotinylated CD22-Fc proteins followed by streptavidin-R-PE conjugate and mAb anti-V5 epitope tag followed by an anti-mouse IgG-FITC conjugate. About 40% of yeast cells do not express scFv on their surface, resulting in two histogram peaks.

Construction of a HEK 293T Cell Surface Display Library for *in Vitro* Antibody Affinity Maturation. Finally, we asked whether this system could be suitable for isolating a high binder that could be used to make a more active immunotoxin or for other targeted therapies from a combinatorial library. We constructed a HEK 293T cell surface-displayed combinatorial library randomizing an intrinsic antibody hotspot. There are six complementary determining regions (CDRs), three on antibody heavy chain and three on light chain. CDRs are involved in antigen binding. Previous work has shown that a small number of DNA residues in CDRs called hotspots (30–33) can be used to increase antibody affinity (9, 10, 34, 35). Here we used the same strategy to select a hotspot (Gly-91–Asn-92) in light-chain CDR3 of HA22 to construct an anti-CD22 scFv library.

The scFv library was transduced into *E. coli* cells by electroporation. The library complexity of RFB4LibVL30/31 consisted

of $>1 \times 10^4$ independent clones. Because a library of 1,024 (32×32) independent clones is needed to randomly mutate the hotspot sequence (GGTAAT) to NNSNNS, the size of the current library is 10-fold larger than the one required. Sequence analysis of 10 independent clones demonstrated that the targeted hotspot residues were successfully randomized (data not shown). The library was then transfected into HEK 293T cells with Lipofectamine and the Fv expressed on the cell surface was fused to the PDGFR transmembrane domain (Fig. 1).

Isolation of Mutant scFv With a Higher Affinity from a HEK 293T Cell Surface Display Library. One transfection in a 100-mm Petri dish can produce $\approx 10^7$ transfected HEK 293T cells. Because the required library size is only $\approx 1,000$, the high transfection rate of one dish of HEK 293T cells produced a library with $> 10^4$ copies of each clone. Forty-eight hours after transfection, the mutant

Fig. 4. Enrichment of mammalian cells displaying an improved scFv variant by kinetic selection and flow cytometric sorting. (A and B) The dot plot shows only 50,000 cells of the total cell population (10^7). Each dot represents an individual observed cell. This is the 1:400 mixture of HA22:BL22-displaying cells, labeled with no antigen (A) or 1 nM antigen (CD22-Fc) (B) concentration for separation. FITC fluorescence (binding to the c-myc tag) represents the number of surface expressions on an individual cell. The PE fluorescence represents binding to the antigen (CD22). (C) A sort window was drawn to include the top 0.1% of total cells in terms of ratio of PE/FITC fluorescence (29). Cells that fell within the window were collected.

The high-affinity mutant described here may be useful in the treatment of patients with B cell malignancies. CD22 is a B cell antigen expressed in B cell lymphomas and leukemias and not expressed on stem cells or early in B cell development (39–41). We have shown that more than half of patients with drug-resistant hairy cell leukemia can be brought into complete remission (42) by using the BL22 immunotoxin derived from anti-CD22 antibody (reviewed in ref. 43). The high-affinity mutant described here may expand the use of BL22 mutants in the treatment of patients with chronic lymphocytic leukemia, in which the cells have relatively small amounts of CD22 (44, 45).

Materials and Methods

Construction of Mammalian Cell Surface Display Library. Table 1 outlines the experimental procedure for making an Fv library for affinity maturation by mammalian cell surface display. We displayed mutant library of anti-CD22 scFv on HEK 293T cells. The surface display library was constructed by randomly mutagenizing a hotspot (Gly-91–Asn-92) in light-chain CDR3 of anti-CD22 HA22 scFv. HA22 scFv already contains mutations in the heavy-chain CDR3 hotspot of an anti-CD22 mAb RFB4 (35). The mutagenesis of HA22 scFv was performed as described (10). Degenerate oligomers with NNS were used (N randomizing with all four nucleotides, S introducing C or G) in two-step overlap extension PCR: Dis.SfiIF, 5'-GGGGCCAGCCGGC-CATGGAAGTGCAGCTGGTG-3'; Dis.SacIIR, 5'-CTGC-CGCGGAGCTTTGATTTCAG-3'; RFB4_VL91/92F, 5'-TTTTGCCAACAGNNSNSACGCTTCCGTGG-3'; and RFB4_VL91/92R, 5'-CCACGGAAGCGTSNNSNNCTGTT-GGCAAAA-3'. Phagemid pCANTAB5E-scFv HA22 (clone G3) was used as a template. The PCR product was subcloned into SfiI and SacII sites downstream of the Ig κ chain leader sequence of the pDisplay vector (Invitrogen). The vector encodes a myc epitope tag and a PDGFR transmembrane domain downstream of the scFv. The ligation mix was used to transform electroporation-competent *E. coli* cells. Each transformation produced a cell surface display library containing $\approx 1 \times 10^6$ independent clones. The plasmid library, pDisplay-RFB4VL91/92, contains a mutation targeting G91-N92 residues in light-chain CDR3 of the HA22 scFv. We also made plasmids pDisplay-BL22 (pMH112) and pDisplay-HA22 (pMH113) containing scFv BL22 (RFB4) and HA22, respectively, as controls.

Transfection. The scFv protein was expressed as a fusion to PDGFR transmembrane domain in transfected HEK 293T cells (10). Lipofectamine (Invitrogen) was used for transfections. Five micrograms of plasmid DNA was used for transfection in a 100-mm Petri dish containing 10^7 HEK 293T cells per dish.

Confocal Fluorescence Microscopy. HEK 293T cells transfected with pMH113 were grown on cover slips. After 48 h, cells were washed with PBS and fixed in 1 ml of 4% formaldehyde in PBS for 10 min at 25°C. Cells were blocked with 5% BSA in PBS for 30 min at 25°C and then incubated with 1 μ g/ml of biotinylated CD22-Fc or CD30-Fc proteins and 20 μ g/ml of mAb 9E10 (anti-c-myc) or IgG1 isotype control (Sigma) in PBS containing 5% BSA and 0.1% sodium azide. After incubation for 90 min at 25°C, cover slips were washed, incubated with Alexa Fluor-488-labeled goat anti-mouse IgG (1:500; Molecular Probes) and streptavidin-Alexa Fluor-594 conjugate (1:500; Molecular Probes) for 60 min at 25°C, and washed. To visualize the nucleus, cells were stained with DAPI (Molecular Probes) for 5 min at 25°C. Slides were analyzed in a Zeiss LSM 510 confocal microscope.

Flow Cytometric Analysis. In a typical experiment, 48 h after transfection, 1×10^6 cells ($\approx 10\%$ of total HEK 293T cells collected from a 100-mm Petri dish) were used. The cells were incubated with 0.1–1 $\mu\text{g}/\text{ml}$ of biotinylated CD22-Fc or CD30-Fc proteins and 20 $\mu\text{g}/\text{ml}$ of mAb 9E10 (anti-c-myc) or IgG1 isotype control (Sigma) in 500 μl of PBS containing 5% BSA and 0.1% sodium azide (FACS buffer). After incubation for 1 h at 4°C , the cells were washed with PBS and incubated on ice with 1:200 dilution of FITC-labeled goat anti-mouse FITC (AM10408; BioSource International, Camarillo, CA) and streptavidin-R-PE conjugate (S866; Molecular Probes) for 1 h. After washing, the cells were suspended in 0.5 ml of FACS buffer, and the fluorescence associated with the live cells was measured with a FACS Calibur flow cytometer (BD Biosciences).

In some experiments (Fig. 5A), the MFI of cells was converted to the molecular number of fluorochrome (FITC, PE) on cells using Quantum FITC MESF (Bangs Laboratories, Carmel, IN) or QuantiBrite PE (BD Biosciences) calibration beads.

Kinetic Screen of Library by Sorting. Approximately 1×10^7 HEK 293T cells were incubated with 0.2 nM biotinylated CD22-Fc proteins and 20 $\mu\text{g}/\text{ml}$ of 9E10 mAb in PBS buffer at 25°C for 1 h. Finally, the cells were rinsed with ice-cold PBS buffer and labeled with FITC-antimouse IgG and streptavidin-PE conjugate as described above. Samples were sorted on a FACSVantage SE (BD Biosciences) with a sort window as shown in Fig. 4. During a single-pass sorting, 1×10^7 HEK 293T cells were examined and the window was set to collect 0.1% of the population.

Yeast Display. For a comparison between the mammalian cell surface display and yeast display, we expressed the HA22 scFv on yeast. The pYD1 vector (Invitrogen) and *Saccharomyces cerevisiae* strain EBY100 (14) was used for yeast cell surface display of anti-CD22 scFv HA22. The HA22 scFv was fused to the carboxyl terminus of Aga2p, and the fusion tethered to the yeast cell surface by disulfide bonding to the anchoring subunit Aga1p (Fig. 1). Each scFv contains a carboxyl-terminal V5 epitope tag. The pMH111 plasmid contains the HA22 scFv insert at the EcoRI and XhoI sites. Yeast cells were transformed with pMH111 by using the S.c. EasyComp transformation kit (Invitrogen), and Trp⁺ transformants were selected. Yeast cells containing scFv surface display plasmids were grown in minimal SD-CAA medium (20 g/liter dextrose/6.7 g/liter yeast nitrogen base/5 g/liter casamino acids/10.19 g/liter Na₂HPO₄·7 H₂O/8.56 g/liter NaH₂PO₄·H₂O) (14) overnight in a 30°C shaking incubator.

tor. Cells were resuspended in galactose-containing medium SG-CAA (20 g/liter galactose/6.7 g/liter yeast nitrogen base/5 g/liter casamino acids/10.19 g/liter $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ /8.56 g/liter $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) (14) to induce scFv display. Induction was carried out at 20°C for 48 h.

In a typical protocol for flow cytometric analysis of yeast, cells were incubated on ice with 0.1–1 $\mu\text{g}/\text{ml}$ of biotinylated CD22-Fc and mAb anti-V5 (10 $\mu\text{g}/\text{ml}$; Sigma) for 30 min, followed by anti-mouse FITC conjugate (1:200) and streptavidin-PE conjugate (1:200) for 1 h. FITC and PE fluorescence intensities were quantified with a FACalibur flow cytometer (Becton Dickinson).

Preparation and Purification of CD22. CD22-Fc fusion proteins were prepared as described (10). Biotinylation of CD22-Fc was performed according to the manufacturer's instruction (Pierce).

Determination of Affinity Constants (K_D). Antibody affinity measurements were performed with flow cytometry following protocols as described (16, 29). Equilibrium constants and Scatchard plots were determined by using the Marquardt–Levenberg algorithm for nonlinear regression with PRISM software (version 3.0.2; GraphPad, San Diego).

Rescue of scFv Sequence. Plasmid DNA was isolated by using the QIAprep Spin Miniprep Kit following the manufacturer's instruction (Qiagen, Valencia, CA) from HEK 293T cells sorted on

a FACSVantage SE (BD Biosciences). As few as 100 cells were enough for plasmid isolation. The plasmid DNA was transformed into TOP10F-competent *E. coli* (Invitrogen). Individual clones were used for sequencing and retransfection of HEK 293T cells. Alternatively, the plasmid DNAs recovered from HEK 293T cells were used as templates for a PCR using the Tgo DNA polymerase with 3'–5' exonuclease proofreading activity (Roche Diagnostics).

Sequencing Analysis and Structural Modeling. The scFv fragments isolated from HEK 293T cell surface library were sequenced with primers Dis_SfiIF and Dis_SacIIR (National Cancer Institute DNA Sequencing Core Facility, Bethesda).

Both the heavy- and light-chains variable region sequences were numbered following the Kabat rule (46). Antibody Fv sequence analysis and structural modeling were performed as described (10).

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- Adams, G. P. & Weiner, L. M. (2005) *Nat. Biotechnol.* **23**, 1147–1157.
- Foote, J. & Eisen, H. N. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 1254–1256.
- Batista, F. D. & Neuberger, M. S. (1998) *Immunity* **8**, 751–759.
- Hoogenboom, H. R. (2005) *Nat. Biotechnol.* **23**, 1105–1116.
- Smith, G. P. (1985) *Science* **228**, 1315–1317.
- Winter, G., Griffiths, A. D., Hawkins, R. E. & Hoogenboom, H. R. (1994) *Annu. Rev. Immunol.* **12**, 433–455.
- Low, N. M., Holliger, P. H. & Winter, G. (1996) *J. Mol. Biol.* **260**, 359–368.
- de Bruin, R., Spelt, K., Mol, J., Koes, R. & Quattrocchio, F. (1999) *Nat. Biotechnol.* **17**, 397–399.
- Chowdhury, P. S. & Pastan, I. (1999) *Nat. Biotechnol.* **17**, 568–572.
- Ho, M., Kreitman, R. J., Onda, M. & Pastan, I. (2005) *J. Biol. Chem.* **280**, 607–617.
- Francisco, J. A., Campbell, R., Iverson, B. L. & Georgiou, G. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 10444–10448.
- Francisco, J. A. & Georgiou, G. (1994) *Ann. N. Y. Acad. Sci.* **745**, 372–382.
- Georgiou, G., Stathopoulos, C., Daugherty, P. S., Nayak, A. R., Iverson, B. L. & Curtiss, R., III (1997) *Nat. Biotechnol.* **15**, 29–34.
- Boder, E. T. & Wittrup, K. D. (1997) *Nat. Biotechnol.* **15**, 553–557.
- Boder, E. T., Midelfort, K. S. & Wittrup, K. D. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 10701–10705.
- Feldhaus, M. J., Siegel, R. W., Opreko, L. K., Coleman, J. R., Feldhaus, J. M., Yeung, Y. A., Cochran, J. R., Heinzelman, P., Colby, D., Swers, J., et al. (2003) *Nat. Biotechnol.* **21**, 163–170.
- Hanes, J. & Plückthun, A. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 4937–4942.
- Hanes, J., Jeremius, L., Weber-Bornhauser, S., Bosshard, H. R. & Plückthun, A. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14130–14135.
- Gold, L. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 4825–4826.
- Urban, J. H., Schneider, R. M., Compte, M., Finger, C., Cichutek, K., Alvarez-Vallina, L. & Buchholz, C. J. (2005) *Nucleic Acids Res.* **33**, e35.
- Schimmele, B., Gräfe, N. & Plückthun, A. (2005) *Protein Eng. Des. Sel.* **18**, 285–294.
- Andersen, D. C. & Reilly, D. E. (2004) *Curr. Opin. Biotechnol.* **15**, 456–462.
- Cumbers, S. J., Williams, G. T., Davies, S. L., Grenfell, R. L., Takeda, S., Batista, F. D., Sale, J. E. & Neuberger, M. S. (2002) *Nat. Biotechnol.* **20**, 1129–1134.
- Wolkowicz, R., Jager, G. C. & Nolan, G. P. (2005) *J. Biol. Chem.* **280**, 15195–15201.
- Gronwald, R. G., Grant, F. J., Haldeman, B. A., Hart, C. E., O'Hara, P. J., Hagen, F. S., Ross, R., Bowen-Pope, D. F. & Murray, M. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3435–3439.
- Santiago, C., Bjorling, E., Stehle, T. & Casasnovas, J. M. (2002) *J. Biol. Chem.* **277**, 32294–32301.
- Lee, J. Y., Zhao, L., Youn, H. S., Weatherill, A. R., Tapping, R., Feng, L., Lee, W. H., Fitzgerald, K. A. & Hwang, D. H. (2004) *J. Biol. Chem.* **279**, 16971–16979.
- Zhu, D., Kepley, C. L., Zhang, K., Terada, T., Yamada, T. & Saxon, A. (2005) *Nat. Med.* **11**, 446–449.
- VanAntwerp, J. J. & Wittrup, K. D. (2000) *Biotechnol. Prog.* **16**, 31–37.
- Neuberger, M. S. & Milstein, C. (1995) *Curr. Opin. Immunol.* **7**, 248–254.
- Neuberger, M. S., Ehrenstein, M. R., Klix, N., Jolley, C. J., Rada, C. & Milstein, C. (1998) *Immunol. Rev.* **162**, 107–116.
- Jolly, C. J., Wagner, S. D., Rada, C., Klix, N., Milstein, C. & Neuberger, M. S. (1996) *Semin. Immunol.* **8**, 159–168.
- Rada, C., Ehrenstein, M. R., Neuberger, M. S. & Milstein, C. (1998) *Immunity* **9**, 135–141.
- Beers, R., Chowdhury, P., Bigner, D. & Pastan, I. (2000) *Clin. Cancer Res.* **6**, 2835–2843.
- Salvatore, G., Beers, R., Margulies, I., Kreitman, R. J. & Pastan, I. (2002) *Clin. Cancer Res.* **8**, 995–1002.
- Holler, P. D., Holman, P. O., Shusta, E. V., O'Herrin, S., Wittrup, K. D. & Kranz, D. M. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 5387–5392.
- Blaise, L., Wehnert, A., Steukers, M. P., van den Beucken, T., Hoogenboom, H. R. & Hufton, S. E. (2004) *Gene* **342**, 211–218.
- Jin, M., Song, G., Carman, C. V., Kim, Y. S., Astrof, N. S., Shimaoka, M., Wittrup, D. K. & Springer, T. A. (2006) *Proc. Natl. Acad. Sci. USA* **103**, 5758–5763.
- Tedder, T. F., Tuscano, J., Sato, S. & Kehrl, J. H. (1997) *Annu. Rev. Immunol.* **5**, 481–504.
- Tuscano, J. M., O'Donnell, R. T., Miers, L. A., Kroger, L. A., Kukis, D. L., Lamborn, K. R., Tedder, T. F. & DeNardo, G. L. (2003) *Blood* **101**, 3641–3647.
- Tedder, T. F., Poe, J. C. & Haas, K. M. (2005) *Adv. Immunol.* **88**, 1–50.
- Kreitman, R. J., Wilson, W. H., Bergeron, K., Raggio, M., Stetler-Stevenson, M., Fitzgerald, D. J. & Pastan, I. (2001) *N. Engl. J. Med.* **345**, 241–247.
- Pastan, I. (2003) *Cancer Immunol. Immunother.* **52**, 338–341.
- Kreitman, R. J., Margulies, I., Stetler-Stevenson, M., Wang, Q. C., Fitzgerald, D. J. P. & Pastan, I. (2000) *Clin. Cancer Res.* **6**, 1476–1487.
- Rossmann, E. D., Lundin, J., Lenkei, R., Mellstedt, H. & Osterborg, A. (2001) *Hematol. J.* **2**, 300–306.
- Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M., Gottesman, K. S. & Foeller, C. (1991) *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda), 5th Ed.