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Selection of antibodies to cell surface determinants on mouse thymic epithelial cells using a phage display library

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SUMMARY

The network of thymic epithelium contributes significantly to the thymic stromal cell environment, which plays a vital role in the generation and maturation of thymocytes. Monoclonal antibodies (mAb) have revealed considerable heterogeneity within this epithelial component of the mouse thymic microenvironment, but many of these antibodies recognize epitopes that are located inside the cell and so cannot be used in functional studies. As an alternative approach to isolate antibodies specific to thymic epithelium, we used a phage display library expressing single chain Fv antibodies. For selection, a thymic cell suspension was incubated with the phage display library, and major histocompatibility complex class II positive cells, the majority of which are epithelial, were then specifically selected. Phage bound to these cells were eluted and the selection procedure was repeated for a further five rounds. Immunohistochemical analysis revealed that these phage antibodies show differential staining of thymic epithelial subsets. Flow cytometric analysis of a thymic epithelial cell line using a panel of these antibodies demonstrated that they recognize epitopes on the cell surface. Furthermore, some of these antibodies also labelled human thymic epithelium, suggesting that the epitopes recognized by these antibodies are conserved between human and rodent thymus. Our approach therefore provides a rapid method to select antibodies specific for thymic epithelial cell surface determinants in their native configuration.

INTRODUCTION

T-cell maturation involves a complex series of events that requires direct contact between the developing thymocyte and the network of stromal cells,[1,2] which are mostly epithelial, that make up the thymic microenviroment.[3,4] Although existing panels of monoclonal antibodies (mAb) have revealed considerable heterogeneity within this epithelial component, such that they have been classified into various CTES (clusters of thymic epithelium staining) groups,[5] many antibodies are of immunoglobulin M (IgM) isotype and recognize internal/cytoplasmic determinants.[5–7] Thus, with the exception of molecules such as the major histocompatability complex (MHC),[8] structures present on the cell surface of epithelial cells that might also play a role in T-cell maturation are largely unknown. Therefore, the identification of such molecules and a greater understanding of their role of thymocyte differentiation, requires the generation of novel antibodies against epithelial cell surface determinants.

An alternative strategy to hybridoma technology is the selection of antibody fragments, such as single chain fragments (sFv), displayed on the surface of filamentous phage.[9–12] Single chain Fv consist of the variable domains of the antibody heavy and light chains linked via a flexible polypeptide spacer, consisting of 12–15 amino acids. The selection of phage antibodies with a range of binding activites and specificities has been successfully achieved from various libraries, illustrating that this technique offers a powerful means of generating antibodies without immunization.[9–14]

The selection of antigen-specific phage antibodies largely relies on immobilized antigen, with limited analysis using cells as a target antigen.[15,16] In this study, we describe with the use of freshly isolated class II positive thymic cells (as summarized in Fig. 1), the selection of specific phage antibodies that recognize cell surface determinants on thymic epithelial cells. These antibodies show a range of novel binding patterns and so may allow identification and characterization of proteins involved in thymocyte development.

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Abbreviations: CTES, clusters of thymic epithelium staining; FACS, fluorescence activated cell sorter; mAb, monoclonal antibody; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG, polyethylene glycol; sFv, single chain fragment; TCR, T-cell receptor.

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MATERIALS AND METHODS

Preparation of thymic cell suspension

To obtain a thymic cell suspension, containing stromal cells, thymus was removed from a 1-week-old (B10 × CBA)F1

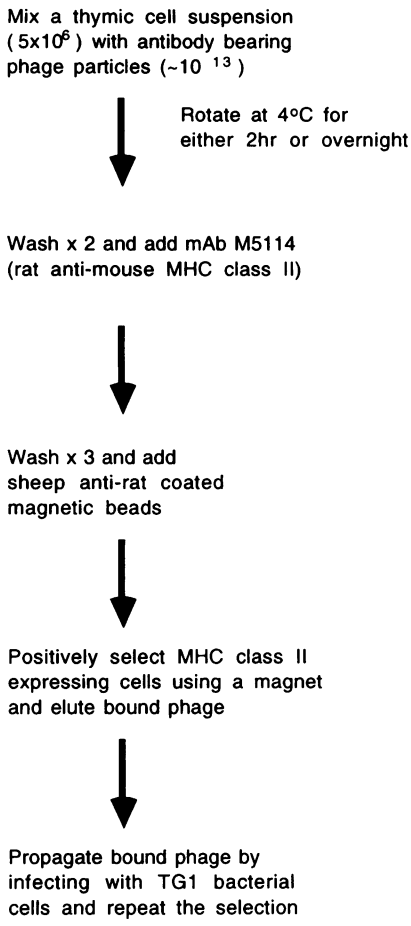

Figure 1. Outline of the protocol used to obtain phage antibodies specific for mouse thymic epithelium by selecting phage that bind to MHC class II positive thymic cells.

mouse, washed in phosphate-buffered saline (PBS) and then teased to remove a substantial number of thymocytes. The remaining thymic fragments were then placed in a petri dish containing enzyme medium (50 ml RPMI-1640 serum free; 50 μl DNase (Sigma, Poole, UK. DN-25, 580 U/ml) and 1 ml collagenase (Sigma C1889; 25 mg/ml, 540 U/mg)), teased and incubated for 1 hr at 37°. The resulting cell suspension, containing both thymocytes and stromal cells ($\approx$ 2% of MHC class II positive cells, see below) was collected in a tube, washed twice in PBS and counted.

Selection of phage antibody

We used the 'single pot' human semi-synthetic phage display library as described by Nissim *et al.*[13] (a kind gift of Dr G. Winter, Cambridge, UK). In this library a collection of 49 human germline V_H gene segments have been cloned into the pHEN1 vector.[13] The sequence corresponding to the complementarity determining region 3 was randomized in both sequence and length (between 4 and 12 amino acids) and paired with a single unmutated Vλ3 light chain gene segment. The library was rescued with VC3M13 helper phage (Stratagene, Cambridge, UK) and phage particles were purified using polyethylene glycol (PEG).[17] Antibody-bearing phage particles were prepared as described previously.[17] For each round of selection, $\approx 10^{13}$ transducing units of phage in 2 ml of PBS were blocked with 2 ml of 5% milk powder (Marvel)/PBS. After 15 min, 1 ml of 5×10^6 thymic cells was added to the blocked phage and the mixture was allowed to slowly rotate at 4° for either 2 hr or overnight. After the appropriate incubation period, cells were washed twice in 50 ml of PBS/1% bovine serum albumin (BSA) at 4° and incubated with 0·5 ml of culture supernatant of the rat anti-mouse MHC class II mAb; M5114 (I-E,^{d,k} I-A$\beta^{b,d,k}$)[18] for 1 hr on ice. After washing three times with PBS/1% BSA the cells were resuspended in 0·5 ml of PBS/1% BSA containing 50 μl of sheep anti-rat IgG coated magnetic beads (Dynal, Wirral, UK) and incubated for 1 hr on ice. Cells coated with magnetic beads were then removed using a magnet and resuspended in 200 μl of PBS. Phage were eluted from the MHC class II positive cells by adding 300 μl of 76 mM citric acid in PBS (pH 2·5), neutralized with 400 μl of 1 M Tris–HCl pH7·4 and amplified by infecting *Escherichia coli* TG1.[17] Phage particles were prepared and the panning procedure was repeated for a further five rounds. Individual phage antibodies isolated from the sixth round of selection were prepared from ampicillin-resistant colonies grown in 10 ml of 2 × TY medium and resuspended in 500 μl of PBS, after PEG purification.

Immunohistochemistry

Five micrometre sections were cut from tissue, air dried, fixed in acetone and stained using the indirect immunoperoxidase method as described.[19] Staining was performed using 100 μl of phage antibody particles mixed with 25 μl of 10% Marvel/PBS. Bound phage were detected using a sheep anti-M13 polyclonal antibody (5 Prime-3 Prime, Hertfordshire, UK) followed by horseradish peroxidase-conjugated rabbit anti-sheep immunoglobulin antibodies (Dako, High Wycombe, UK).

Flow cytometry

The thymic cortical cell line TM25.F1[20] (a kind gift of Dr D. Kioussis, NIMR, UK) was grown as previously described.[21] Approximately 1×10^6 cells were preincubated with 100 μl of phage antibody particles mixed with 25 μl of 10% Marvel/PBS. Bound phage were detected using a sheep anti-M13 polyclonal antibody, followed by fluoroscein isothiocyanate (FITC)-conjugated rabbit anti-sheep immunoglobulin antibody (Dako) as previously described.[21] Labelled cells were analysed using the EPICS Profile flow cytometer (Coulter Electronics, Luton, UK).

RESULTS

Selection for phage antibody that bind preferentially to thymic epithelium

In the mouse thymus, MHC class II expression is limited mainly to cortical epithelium together with a small subset of medullary epithelium, dendritic cells and some macrophages.[22,23] Thus, our strategy to obtain specific anti-thymic epithelium antibody was to elute phage that bound to MHC class II positive cells with the prediction that a majority of those recovered after six rounds of selection would recognize thymic epithelium. Since MHC class II positive cells represented only a minor population[24] (2%, data not shown) of the thymic cell suspension as prepared, we would also predict that the majority of cells, which are MHC class II negative and therefore not immuno-selected, would absorb out a greater

proportion of the non-specific phage, than would the minority MHC class II positive population, together with those that recognize shared epitopes. In addition, by repeating the selection on this mixed population of cells, this would progressively enrich for phage specific for MHC class II positive cells. We incubated the phage library with the thymic cell suspension for either two hours or overnight to determine which approach produced the greater number of phage that would specifically bind to our target cell population.

The number of clones recovered from each round of selection, approximately 10^{10} transducing units in total, did not vary significantly over the six rounds. In the early rounds of selection, phage eluted from MHC class II positive cells exhibited no antithymic epithelium activity as determined by immunohistochemistry using frozen sections of mouse thymus. After the sixth round of selection, over 80% of clones derived from the overnight selection demonstrated specific binding activity to mouse thymic epithelium, whereas this was seen with only 50% of clones following the two hour selection protocol, and these latter clones in general gave weaker staining.

Thymic epithelial staining: reactivity of phage antibodies with mouse thymus

Representative examples of the staining pattern seen with phage antibodies on mouse thymic epithelium are illustrated in Fig. 2.

The phage antibody clones 15 (Fig. 2a) and 65 (Fig. 2b) stained exclusively the network of cortical epithelial cells which are characterized by their long thin processes separated by densely packed thymocytes; these would therefore be defined using the CTES nomenclature as belonging to group III.A.[3] However, clone 65 also stained the subcapsule and the vascular endothelium, demonstrating shared antigenicity present on the surfaces of these cells, a reaction that has not been previously described for CTES III antibodies. While clone 47 (Fig. 2c) showed small, relatively infrequent areas of intense confluent staining in the thymic cortex (one to two discrete areas per thymic lobule). In contrast, clone 83 stained isolated cells or small clusters of thymic epithelial cells located in the medulla (Fig. 2d), that morphogically resemble small Hassall's corpuscles. Godfrey *et al.*[25] have described a similar staining pattern with the monoclonal antibody MTS 20 which they categorized as CTES IV.A. An interesting feature of several of the anticortical phage antibodies, was the varying degree to which they bound to epithelial cells present in the cortex, as illustrated with clones 55 (Fig. 2e) and 85 (Fig. 2f). In particular, clone 85 reacted with only a subset of cortical epithelial cells seen as scattered patches of peroxidase activity. In contrast, clone 11 (Fig. 2g), which reacted weakly with a majority of cortical epithelial cells, showed intense focal staining at the point of contact between thymocytes and epithelial cells.

Reactivity of phage antibodies with human thymus

The reactivity of these antibodies with human thymus was examined. From the panel of phage antibodies illustrated in Fig. 2, clones 65 and 47 reacted with the cortical epithelium of the human thymus (Fig. 3) with a pattern similar to that of mouse thymus, suggesting that the epitopes recognized by these antibodies were also present on human thymus epithelium.

Reactivity of phage antibodies with a thymic epithelial cell line

To confirm the epithelial specificity and cell location of the antigens detected by the phage antibodies, the thymic cortical epithelial cell line TM25.F1[20] was stained with the phage antibody clones 11, 15, 65 and 83 and analysed using flow cytometry. This analysis revealed, with the exception of clone 83, that they were able to detect a cell surface antigen on TM25.F1 cells. Furthermore these clones (again with the exception of clone 83) bound to this thymic epithelial cell line and not to thymocytes as cytospin preparations (data not shown), indicating that they recognize epitopes specific for epithelial cells within the thymus. The epitope recognized by clone 83 is expressed only in the medulla (Fig. 2d) and therefore it was not surprising that it did not stain TM25.F1, which is recognized by conventional mAb to cortical but not medullary epithelium.[21]

DISCUSSION

In this study we have demonstrated that it is possible to isolate phage antibodies from class II positive thymic cells that specifically bind to mouse thymic epithelial cells and which differ significantly from the panels of anti-mouse thymic epithelium mAb derived from hybridoma technology.[5–7,21,25] Our approach used to select these phage antibodies specific for thymic epithelium meant that, unlike several of the existing anti-thymic stroma mAb, many were also able to recognize cell surface determinants expressed on epithelial cells (Fig. 4). In addition, some of these antibodies showed unique thymic epithelial staining patterns (Fig. 2). In particular, the focal staining pattern observed with clone 11 (Fig. 2g), suggests that the target cell surface determinant present on cortical epithelial cells, is in direct contact with thymocytes. A similar localized pattern has been described for TCR expression on the cell surface of cortical, but not medullary thymocytes, at the point of contact with epithelial cell processes,[27] and it is tempting to postulate that the antigen recognize by clone 11 may be important in thymocyte development.

Although we were able to isolate antibody-bearing phage that reacted with cortical epithelium, a subset of medullary epithelial cells, or with both cortical and medullary epithelium, we did not find one that bound exclusively to all medullary epithelium (CTES IV). It is possible that since these cells represent a small population of the total epithelial cell population, then perhaps further round(s) of panning may be required to obtain phage antibodies with this specificity. Alternatively, they could be enriched for selection, by the removal of cortical epithelial cells. Furthermore, it appears that a significant population of medullary epithelium does not express MHC class II antigen[28] and would therefore not be selected using an anti-MHC class II mAb. We were only able to detect a few clones (2%) that appeared to recognize thymic macrophages, it is likely that these cells adhered to the petri dish during the preparation of the thymic cell suspension and so were rare in the selection.

Despite illustrating the heterogenity of the mouse thymic

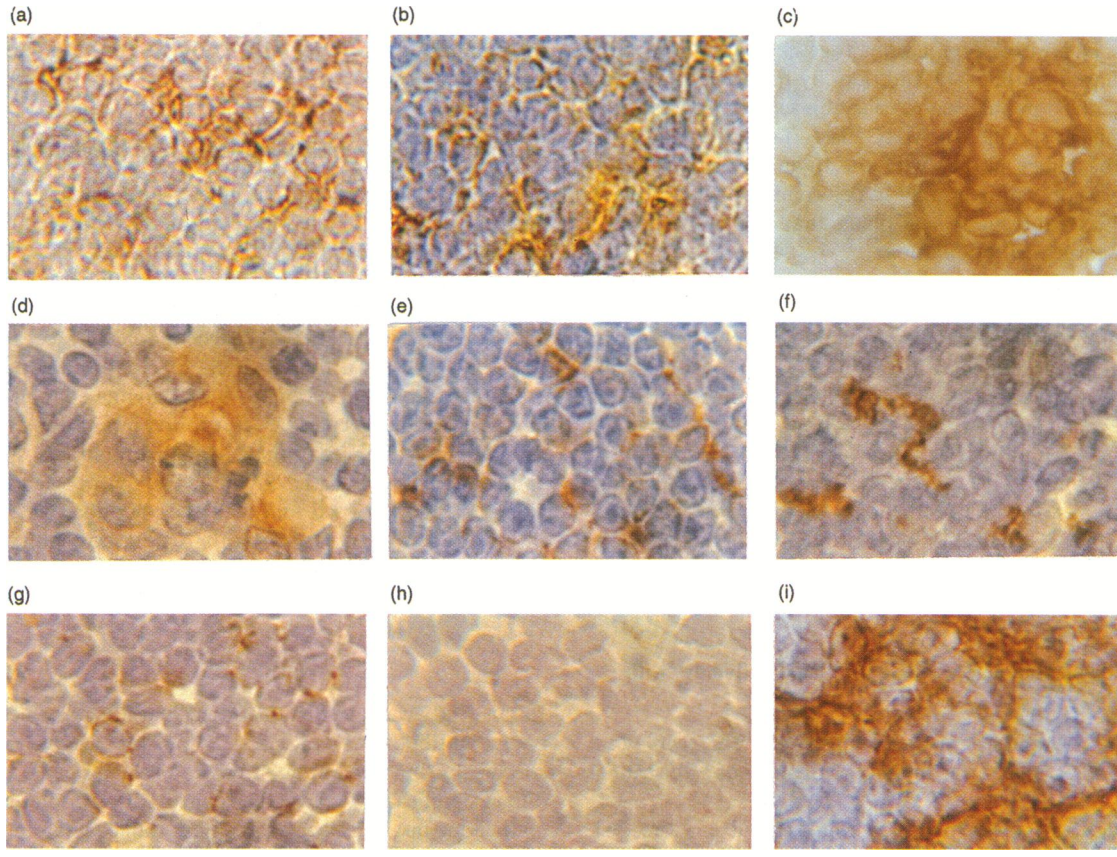

Figure 2. Immunoperoxidase staining of frozen sections of normal mouse (BALB/c) thymus with representative phage antibodies isolated after the sixth round of selection on MHC class II positive mouse thymic cells. Positive binding phage were detected using a sheep anti-M13 antibody, followed by peroxidase-conjugated rabbit anti-sheep immunoglobulin antibody. Phage antibody clones 15 (a), and clone 65 (b), labelled the cortical epithelium, the latter clone also labelled subcapsule and vascular endothelium. Clone 47 (c) showed small relatively infrequent areas of intense confluent staining in the thymic cortex, while Clone 83 (d), only reacted with a subset of medullary epithelial cells. Clones 55 (e), 85 (f), and 11 (g), all labelled the cortical epithelium but demonstrated different staining patterns. An irrelevant phage antibody, U7·6: anti-dinitrophenol[26] was used as a negative control (h). Mouse MHC class II staining with mAb M5114 (I). Magnification × 800.

microenviroment, many of the existing rat mAb raised against thymic stroma are of IgM isotype and recognize epitopes located intracellularly,[5–7] suggesting that the epithelial determinants between mouse and rat are conserved. Furthermore, since human thymocytes can develop to some extent within murine fetal thymic organ cultures,[29,30] it is likely that the structures present within the microenviroment that are recognized by developing thymocytes may also be conserved between rodents and human. This would be in agreement with our observation, since some of the phage antibodies selected on mouse MHC class II positive cells stained human thymus with a similar pattern to that seen with mouse thymus (Fig. 3). In fact, clone 65 also appears to stain thymus from pig, rabbit and chicken, with a similar pattern to mouse (data not shown), indicating that this approach can produce phage antibodies which are able to recognize conserved epitopes present on epthelial cells from different species. Thus, the advantage of our strategy is that it can be used to obtain novel antibodies to cell surface molecules, as well as to detect target antigens that would be impossible to identify using conventional hybridoma methods, because of their evolutionary conservation and, therefore, lack of immunogenicity, and to the rarity of the cell on which they are expressed. We have also produced free sFv from some of our selected phage antibodies and have successfully used those to label thymic epithelial cells (data not shown). Monomeric sFv have been generated that specifically block the interaction between rhinovirus and its receptor on HeLa cells,[31] illustrating that the sFv generated in this study may be useful reagents in blocking the interaction between thymocytes and thymic stroma.

Our observations, and those recently of de Kruif *et al.*,[16] illustrate that it is possible to select phage antibodies using cells as a target antigen, such that they recognize cell surface determinants in their native conformation. This is particularly important where there are limited reagents that have this specificity, such as those against the mouse thymic epithelium. In addition, by using the sFv that we have generated in blocking experiments, their small size and unique range of specificities may now make it possible to more fully address the role of the thymic epithelial cells in thymocyte development.

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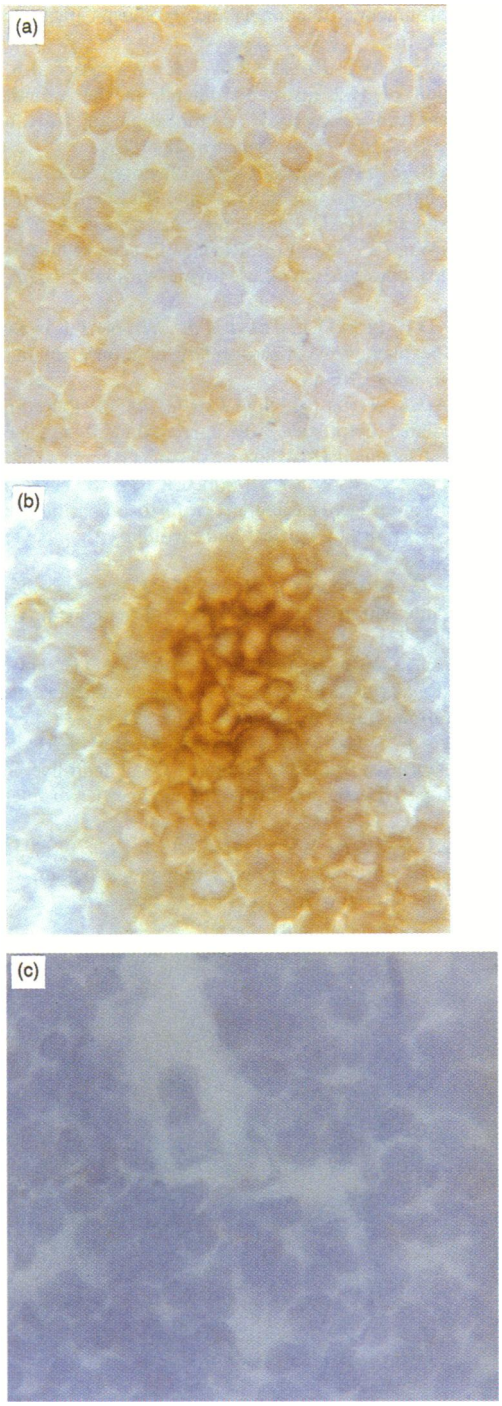

Figure 3. Immunoperoxidase staining of frozen sections of normal human thymus with phage antibodies isolated from MHC class II positive mouse thymic cells. Positive binding phage were detected using a sheep anti-M13 antibody, followed by peroxidase-conjugated rabbit anti-sheep immunoglobulin antibody. Phage antibody clone 65 (a) and clone 47 (b) labelled human cortical epithelium with a similar pattern as seen with mouse thymus (Fig. 2a and c). The irrelevant phage U7·6: anti-dinitrophenol[26] showed no binding to human tissue (c). Magnification ×600 (a,c) and ×240 (b).

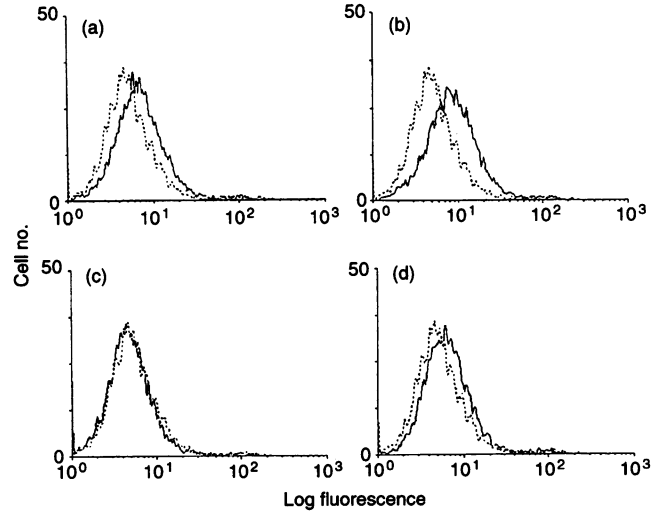

Figure 4. Indirect fluorescence staining of the thymic epithelial cell line TM25.F1 using phage antibodies isolated after the sixth round of selection on MHC class II positive mouse thymic cells. For FACS analysis, 5×10^5 cells were incubated with 100 μl of phage antibody at 4° for 1 hr, followed by a sheep anti-M13 polyclonal antibody and finally with fluorescein-conjugated rabbit anti-sheep immunoglobulin. Labelled cells were analysed using the EPICS Profile flow cytometer. The dotted line in all panels (a–d) is the negative control, the phage antibody, U7·6.[26] (a) clone 15; (b) clone 65; (c) clone 83; (d) clone 11.

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