Electronic Supplementary Information Section

Detailed Materials and Methods

Mouse B-cell preparation

All of the following animal experiment protocols were approved by the Animal Ethics Committee of the University of Toyama. Lymphocytes were prepared from the mashed spleen of C57BL/6 mice or MD4 transgenic mice (C57BL/6-Tg(IghelMD4)4Ccg/J from The Jackson Laboratory, Bar Harbor, ME), of which transgenes encoded mouse antibody (HyHEL10) for hen egg lysozyme (HEL) [1]. B-cells were isolated by removing T-cells from lymphocytes with anti-mouse CD90-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) using automated magnetic cell sorting (AutoMACS, Miltenyi Biotec) according to the manufacturer's instructions.

Vaccination and human B-cell preparation

For human studies, we received approval from the Ethical Committee of the University of Toyama and obtained informed consent from each of the participating subjects. Healthy volunteers were boosted with the influenza vaccine (Biken HA, The Research Foundation for Microbial Diseases of Osaka University, Osaka, Japan). Ten days or one month after the boost, 100 ml of heparinized peripheral blood was collected from four healthy donors, and lymphocytes were prepared by centrifugation at 400 *g* on a Ficoll-Hypaque gradient. Human B-cells were isolated by removing non-B-cells from the lymphocytes with anti-human CD2-, CD14-, CD16-, and glycophorin A microbeads (all from Miltenyi Biotec) using the autoMACS separator according to the manufacturer's instructions.

Cell microarray analysis using the MAC-CCD system and detection of antigen-specific lymphocytes

Microwell-array chips with 45,000 wells were provided from the Toyama Industrial Technology Center (Takaoka, Japan). The lymphocytes were loaded with 0.1 μM fluo-4

(fluorescent Ca^{2+} indicator; Invitrogen, Carlsbad, CA) for 30 min at room temperature. After the cells were washed, the cells were suspended in Hanks' Balanced Salt Solution (HBSS; 130 mM NaCl, 4.4 mM KCl, 0.4 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM $MgSO_4$, 4 mM $NaHCO_3$, and 5 mM glucose in 5 mM HEPES pH 7.45). The cells were then applied onto the microwell-array chips, and the fluo-4 fluorescence intensity of the individual cells was monitored with the (CCD) time-lapse scanner (Nano System Solutions, Tokyo, Japan), which has a fluorescence mirror unit, U-MWB2 (BP460-490, BA520IF, and DM500) (OLYMPUS, Tokyo, Japan) and shows a resolution of up to 3.8 μm. The CCD scanner monitored the fluo-4 fluorescence signals every 10 s and calculated the fluorescence intensities of the individual cells in each of the wells using the Array Scanner Program software (Nano System Solutions). In the initial 100 s, cell fluorescence was monitored in the absence of antigen stimulation (scanning points 1 to 10). Cells were then stimulated with antigen at room temperature in air by exchanging the HBSS buffer on the chip with the HBSS containing antigen, and the cellular fluo-4 fluorescence was monitored with the CCD scanner for 300 s (scanning points 11 to 40). As antigens, HEL (Sigma-Aldrich, St. Louis, MO) and type A influenza nucleoprotein (A-NP, obtained from Kyowa Medex Co., Ltd, Shizuoka, Japan) were used.

Correlation method

Antigen-stimulated B-cells were selected by analyzing the time course of the alteration of fluo-4 fluorescence intensities of individual cells with the correlation method that has been developed for effectively detecting targets in radar or for recognizing very noisy images [2]. The decision for selecting the B-cells that responded to antigen was performed according to a correlation value (*C* value), which scores the degree of correlation between the observed pattern (fluo-4 fluorescence alteration in each cell) and a reference template pattern that represents an average Ca^{2+} alteration of typical B-cells responding to antigen. The *C* value for each cell is calculated by the following equation:

$$C = \frac{\sum_{i=1}^{40} (\sigma_i \cdot T_i)}{\sqrt{\sum_{i=1}^{40} \sigma_i^{\,2}} \cdot \sqrt{\sum_{i=1}^{40} T_i^{\,2}}}$$

where σ_i is the normalized fluo-4 fluorescence intensity at the *i*-th scanning point of a cell of interest and T_i is the average of normalized fluo-4 fluorescence intensity at the *i*-th scanning point of the cells that responded to antigen. In this study, the fluorescence intensity of each cell was sampled every 10 s and normalized as described in the "Results" section. The template was also calculated as described in the "Results" section. The *C* value is ≤ 1. If the *C* value of a cell of interest is above a preset criterion and close to 1, the observed fluorescence alteration of the cell was matched with that of the template and we selected the cell as being a B-cell that responded to antigen.

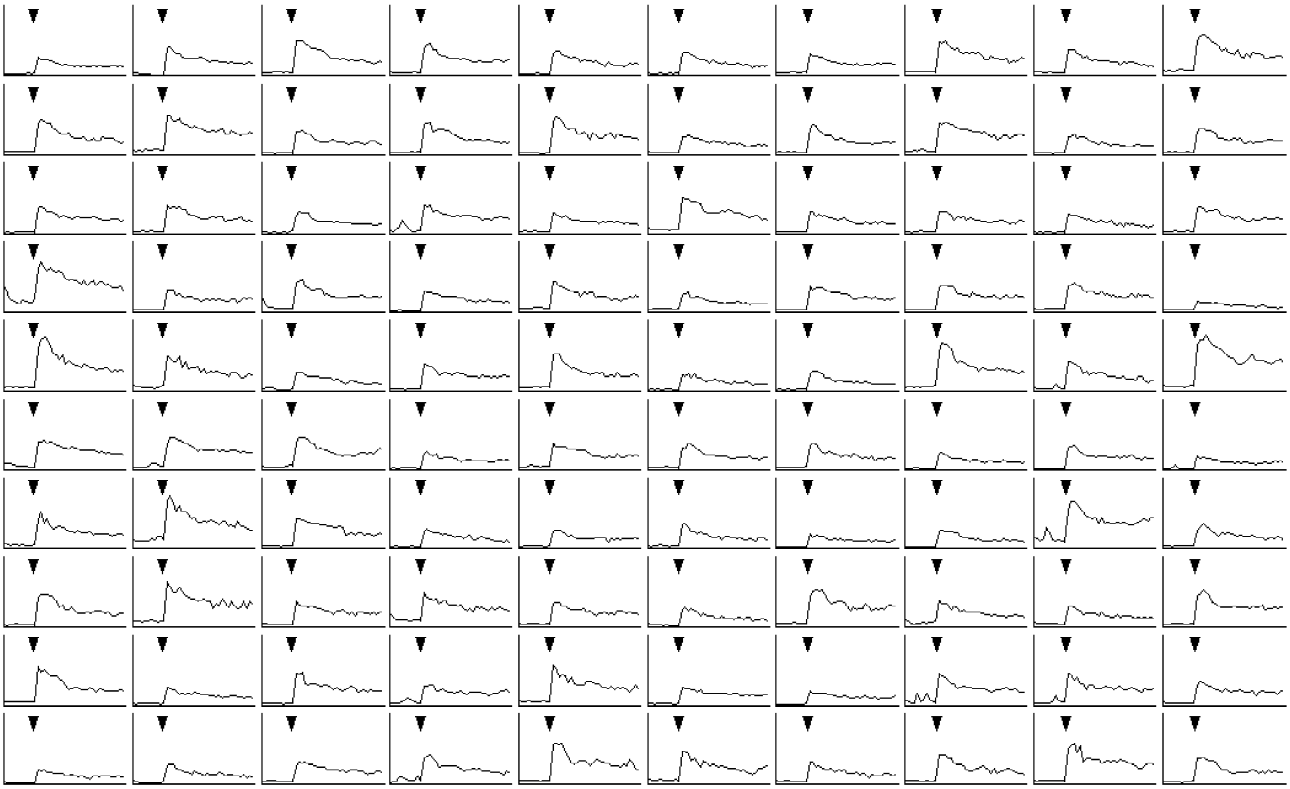
Single-cell retrieval and antibody gene amplification from a cell

Selected cells were retrieved from each well with a micromanipulator, TransferMan NK2 (Eppendorf, Hamburg, Germany) with a glass capillary micropipette (12 μm ID) (Primetech Co., Ltd., Tokyo, Japan) under a fluorescence microscope, BX51WI (Olympus) and then transferred to tubes containing reverse transcription solution as described previously [3,4]. In the study using MD4 mouse B-cells, we amplified cDNAs for the heavy chain of HyHEL10 antibody from a single cell by RT-PCR (primers for the first PCR, 5'-TTTGAAGAAAGGGGTTGTAG-3' and 5'-TGCAGAGACAGTGACCAGAG-3'; primers for the nested PCR, 5'-AGCCTAAAAGATGATGGTGTT-3' and 5'-CAGTAATCACCGTCCCAGTT-3') for confirmation that the retrieved cells were HEL-specific MD4 B-cells. Augmented fragments were analyzed on agarose gel electrophoresis to ascertain the amplification of objective cDNA as described previously [4] . For the human study, the heavy chain and light chain cDNAs of human antibodies were amplified from each retrieved cell using the single-cell 5'-RACE method [35] and inserted into the expression vectors as described previously [3,4]. Antibody cDNAs were transfected

into 293T cells and the antigen specificity of recombinant antibodies in culture supernatants was examined by competitive ELISA.

Competitive ELISA for Detection of A-NP Antibody

Ninety-six-well plates (Nunc, Roskilde, Denmark) were coated with 50 μl/well of 10 μg/ml A-NP in phosphate buffered saline (PBS) and then blocked with 3% bovine serum albumin in PBS. After washing, cell culture supernatant containing the antibody was added to the plates and incubated for 1 hr at room temperature. The binding of human antibody to the coated antigen was detected using horseradish peroxidase-conjugated goat anti-human IgG (Sigma-Aldrich) and *o*-phenylenediamine dihydrochloride (Sigma-Aldrich) according to the manufacturer's instructions. The optical absorbance was measured at 492 nm with an ELISA reader, Multiskan Spectrum (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. To confirm the antigen-specificity of an antibody, a competitive binding assay was performed. Briefly, in the ELISA assay, one or 5 μg/ml A-NP was added together with the culture supernatants that contained the anti-A-NP antibody.



Supplementary Figure 1. High *C* values of fluorescence mobilization of MD4 B-cells

Representative fluo-4 fluorescence mobilization of 100 individual MD4 B-cells in 100% MD4 B-cell preparation, which shows a *C* value of more than 0.99.

Supplementary Table 1.

Number of positively detected cells using correlation methods

% of MD4 B-cells	Number of analyzed cells	Positively detected cells *	% of positively detected cells *
100	21,684 ± 4,118	14,092 ± 3,718	65.08 ± 12.45
3.7	18,141 ± 1,123	661 ± 269	3.63 ± 1.41
1.23	17,318 ± 2316	227 ± 68	1.31 ± 0.38
0.41	18,900 ± 3016	143 ± 68	0.75 ± 0.33
0.14	19,237 ± 2933	143 ± 74	0.75 ± 0.35
0.046	18,539 ± 3063	69 ± 30	0.38 ± 0.16
0	16,911 ± 4311	72 ± 49	0.40 ± 0.17

Mean ± S.D. (n = 4)

* C value > 0.875

References

1. C. C. Goodnow, J. Crosbie, S. Adelstein, T. B. Lavoie, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, K. Raphael and et al., *Nature*, 1988, **334**, 676-682.
2. B. V. K. V. Kumar, A. Mahalanobis and R. D. Juday, *Correlation Pattern Recognition*, 2005, **CAMBRIDGE University press, New York**.
3. Y. Tokimitsu, H. Kishi, S. Kondo, R. Honda, K. Tajiri, K. Motoki, T. Ozawa, S. Kadowaki, T. Obata, S. Fujiki, C. Tateno, H. Takaishi, K. Chayama, K. Yoshizato, E. Tamiya, T. Sugiyama and A. Muraguchi, *Cytometry A*, 2007, **71**, 1003-1010.
4. K. Tajiri, H. Kishi, Y. Tokimitsu, S. Kondo, T. Ozawa, K. Kinoshita, A. Jin, S. Kadowaki, T. Sugiyama and A. Muraguchi, *Cytometry A*, 2007, **71**, 961-967.