SUPPLEMENTAL MATERIAL

Duty et al., http://www.jem.org/cgi/content/full/jem.20080611/DC1

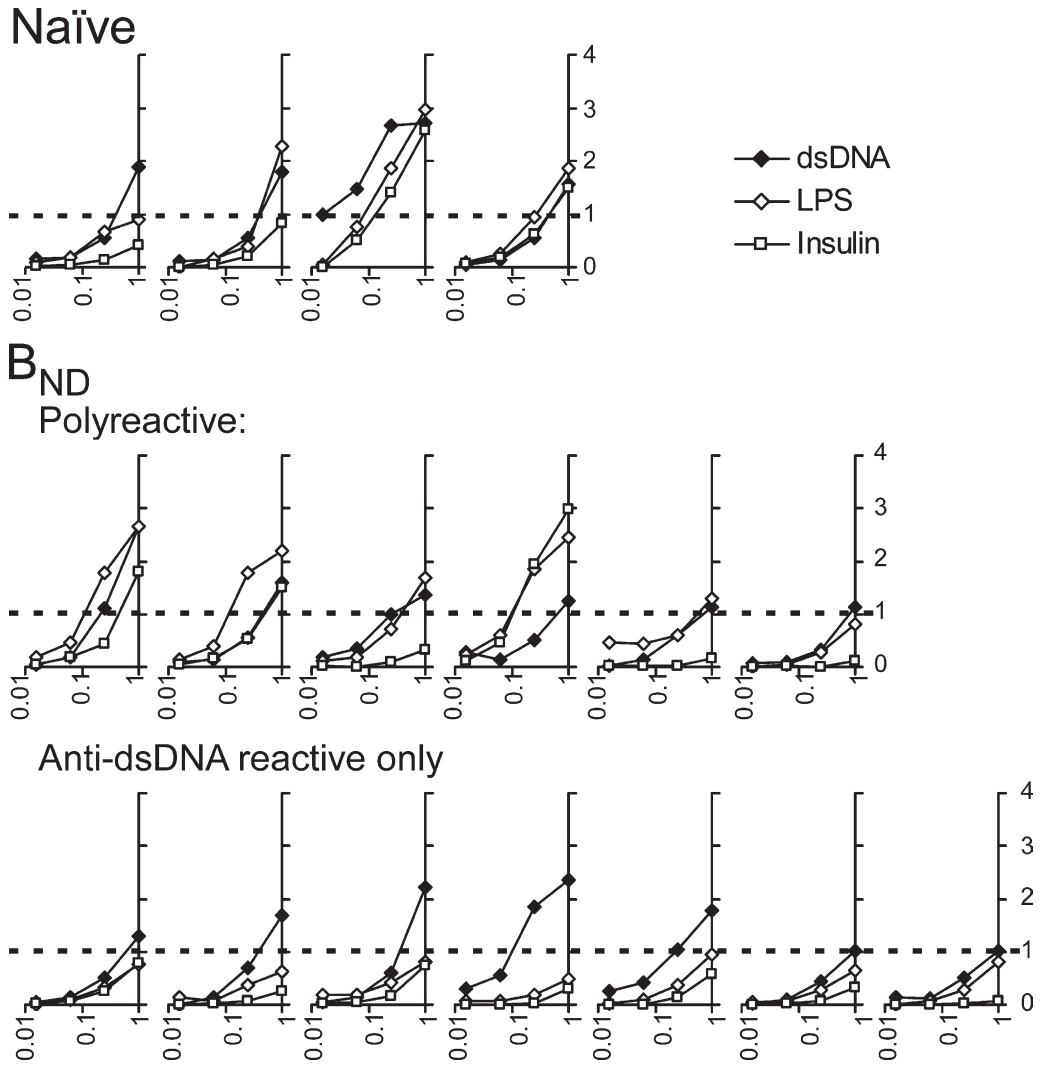

Figure S1. Polyreactivity does not account for all B_{ND} self-reactivity. Of the four naive anti-dsDNA reactive clones, all four are also polyreactive to LPS and insulin, according to antigen-specific ELISAs, and amount positive over threshold (dashed line), as determined in Fig. 2. Absorbancies were measured at OD_{415} (y axis). However, more than half of the antibodies derived from B_{ND} clones are not polyreactive by these tests and are specific for dsDNA only.

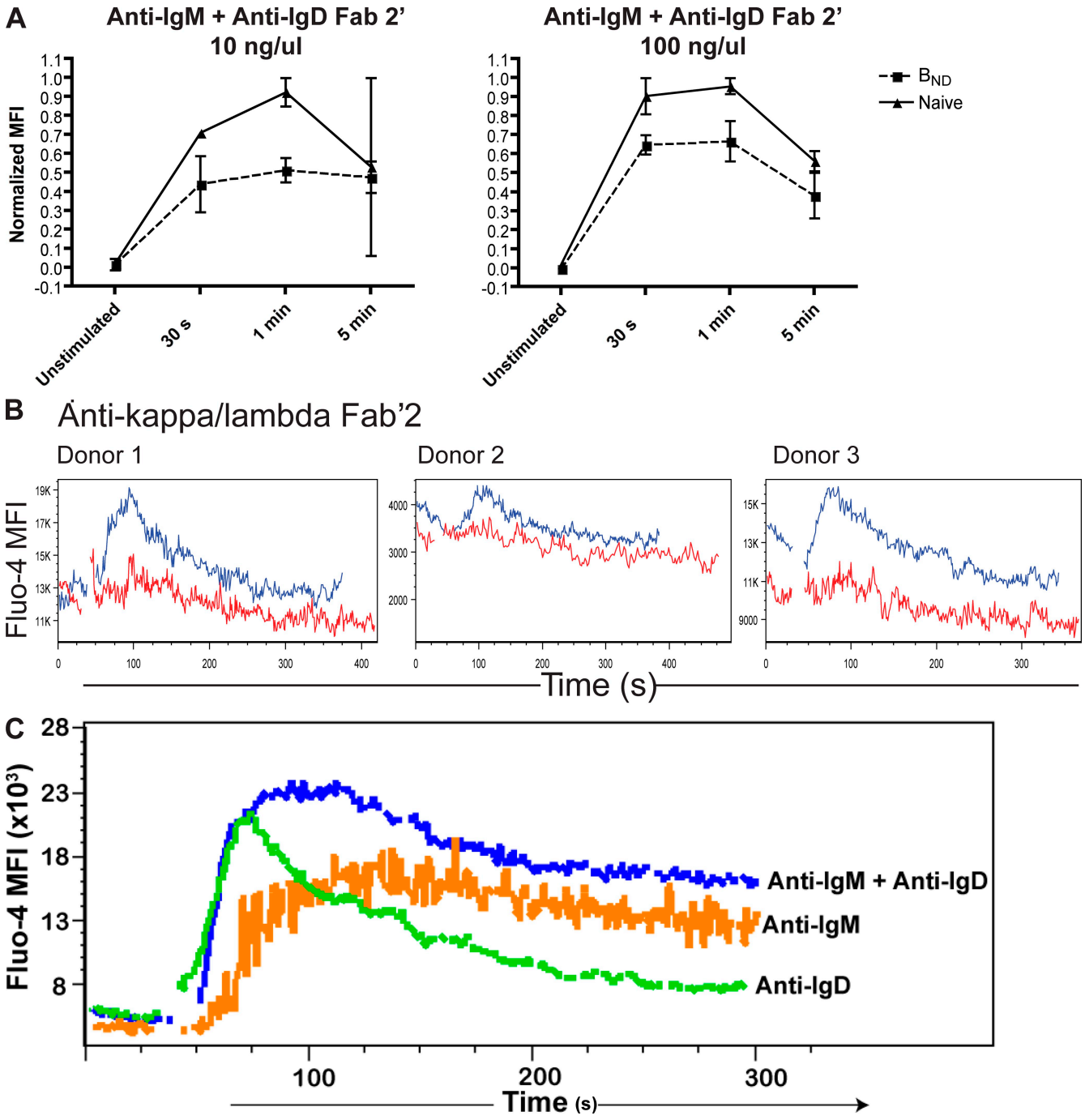

Figure S2. Phosflow was optimized for time of stimulation. (A) 10^5 cell equivalents of least-touch sorted B_{ND} and naive cells were treated with either 10 ng/µl (left) or 100 ng/µl (right) for 0, 0.5, 1, or 5 min, followed by fixation and permeabilization (see Material and methods). Optimal peaks were seen between 0.5 and 1 min of stimulation and, thus, a mean of 45 s was used in subsequent phosflow stimulation assays. (B) Similar differences in flux were also seen when 25 ng/µl of anti-κ/λ Fab'2 was used to cross-link receptors in a similar manner regardless of Ig class. Data shown is representative of two to three donors. (C) Naive cells alone were fluxed with 25 µg/ml of anti-IgM+IgD Fab'2, anti-IgD Fab'2 alone, or anti-IgM Fab'2 alone. Mean florescence intensity for the various responses was calculated over time.

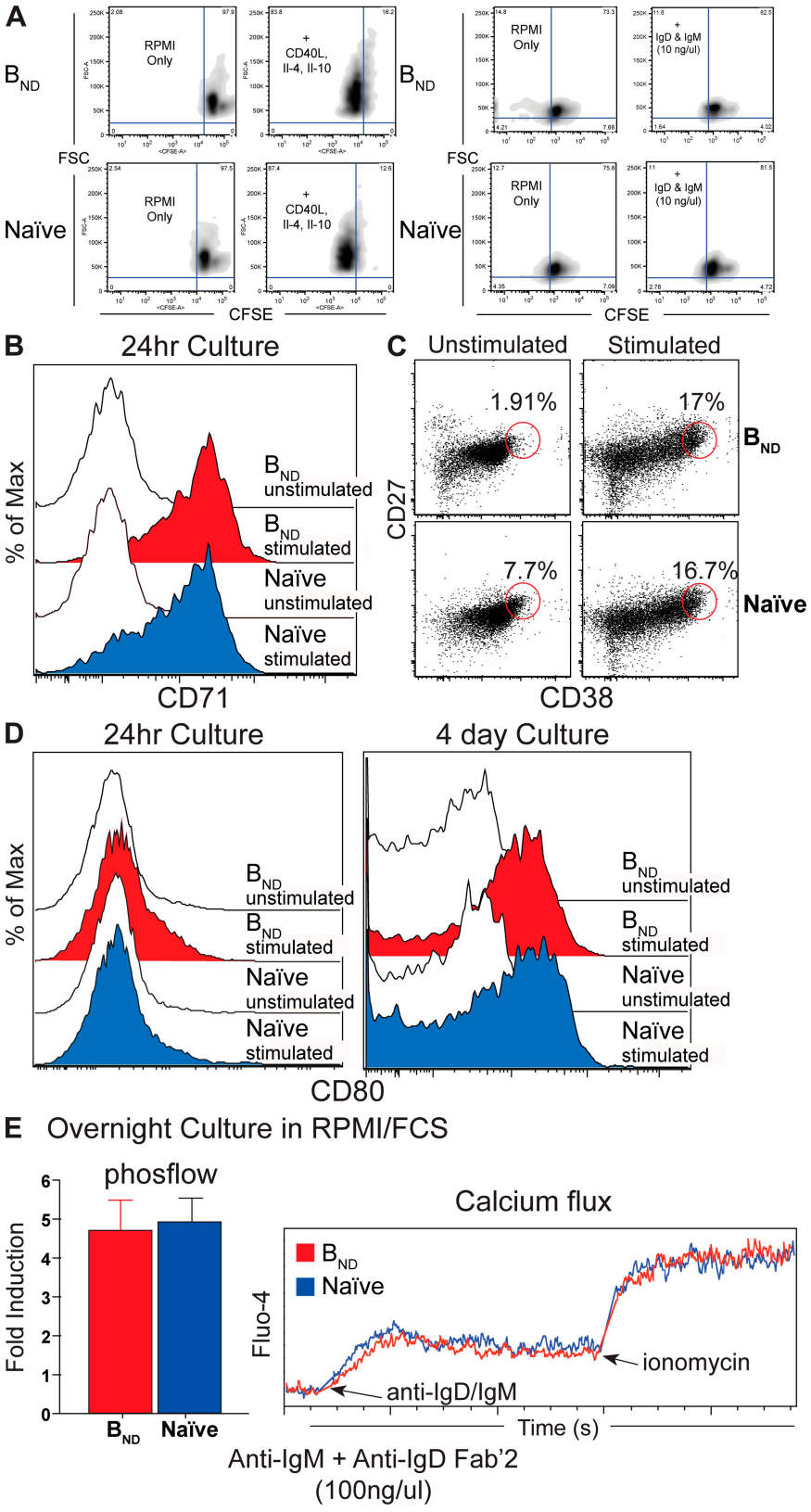

Figure S3. B_{ND} cells eventually become activated ex vivo with T cell–dependent stimulation. B_{ND} and naive cells were sorted as in Fig. 1 and cultured in equal numbers ($5–10 \times 10^5$ cells per well) in a 96-well plate in 100 µl RPMI 1640 liter Glutamine medium (Invitrogen) supplemented with 5% inactivated FBS, L-Glutamine, penicillin/streptomycin, and Na-pyruvate. For stimulation, cells were cultured with irradiated (5,000 rads) L-type mouse fibroblastic tissue culture cells previously transfected with membrane from CD40 ligand (Garrone, P., E.M. Neidhardt, E. Garcia, L. Galibert, C. van Kooten, and Banchereau, J. 1995. *J. Exp. Med.* 182:1265–1273) in a 1:10 ratio (L cell: B cell), 0.5 µg/ml Il-4 (R&D Systems), and 0.02 µg/ml Il-10 (R&D Systems). Before staining, cells were removed from cultures by gentle pipetting, washed twice in PBS supplemented with 2.5% BSA, resuspended in PBS/BSA, and stained with appropriate antibodies. (A) Cells were loaded before culture with CFDA-SE (Invitrogen) and stimulated with CD40L, Il-4 and Il-10 (left), or 10 µg/ml of anti-IgM/IgD Fab'2 (right) or were cultured in media alone. Proliferation was measured on day 4 for CFDA-SE dilution. (B and D) Representative histograms for B_{ND} and naive cells of CD71 and CD80 expression at 24 h or 4 d in the presence or absence of CD40L, Il-4, and Il-10 treatment. (C) CD27 versus CD38 dot plot of B_{ND} and naive cells after 4 d of culture in the presence or absence of stimulation. Circle area highlights double-positive differentiating cells. (E) B_{ND} and naive cells return to similar levels of phosphorylated pTry (left) or calcium flux (right) when rested over night in RPMI/5% FCS and treated with 100 µg/ml of anti-IgM+IgD Fab'$_2$. Plots were generated as in Fig. 6 and are representative of at least two independent trials. Fold induction ± SD represents fold induction of treated over untreated cells for phosflow.