

CD53, a Protein with Four Membrane-Spanning Domains, Mediates Signal Transduction in Human Monocytes and B Cells¹

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ABSTRACT. CD53 is a member of a novel family of molecules with four presumably membrane-spanning domains. The structure and functional characteristics of these molecules indicate that they may play an important role in transmembrane communication. We therefore investigated whether CD53 is involved in activation of human leukocytes. Cross-linking of cell-bound F(ab')₂ fragments of two different anti-CD53 mAb with F(ab')₂ anti-mouse Ig led to cytoplasmic calcium fluxes in B cells, monocytes, and granulocytes and activation of the monocyte oxidative burst. These responses were specific for CD53, as cross-linking of CD11a, CD18, CD35, CD43, CD44, CD45, or CDw50 did not induce leukocyte activation. Low concentrations of staurosporine (10 to 20 nM) completely inhibited PMA-mediated activation, but had no effect on CD53-mediated calcium fluxes and inhibited only partially CD53-mediated oxidative burst. This suggests that CD53-mediated signaling is largely independent of protein kinase C. CD53-mediated calcium fluxes were inhibited by high concentrations of staurosporine (300 to 500 nM) but not by ADP-ribosylating toxins, suggesting dependence on tyrosine kinases rather than GTP-binding proteins. The results indicate that CD53, like several other leukocyte Ag with four membrane-spanning regions, has the ability to mediate cell activation, and support the view that these molecules are involved in transmembrane communication. *Journal of Immunology*, 1993, 151: 707.

The CD53 leukocyte Ag belongs to a novel family of cell membrane molecules consisting of a single polypeptide with a large extracellular loop and four presumably membrane-spanning regions (1–3). The characteristic topology of these proteins seems to be highly conserved during evolution and resembles that of certain receptors and transport molecules (1, 3). CD53-like molecules have therefore been suggested to play important roles in cell biology, possibly those of transmembrane communication (3, 4). This hypothesis has been strengthened

by results showing that mAb to members of this family, including CD9, CD37, CD63 (ME491), and TAPA-1, have effects on activation of platelets, leukocytes, and melanoma cells (5–11). Coupling of the CD53 homologue, OX44, to signal transduction pathways in rat T cells and NK cells may further indicate that CD53 is involved in cell activation (12).

The present study investigated the possibility that the CD53 molecule on human leukocytes may have receptor-like characteristics in functional assays. F(ab')₂ fragments of anti-CD53 mAb were used in combination with F(ab')₂ anti-mouse Ig to induce specific cross-linking of CD53 in leukocyte plasma membranes. Anti-CD53-induced cell activation was monitored by multiparameter measurements of cytoplasmic calcium levels and activation of oxidative burst in monocytes, granulocytes, and lymphocyte subsets. The activation pathway of CD53-mediated responses was compared with those of anti-CD3, anti-IgM, FMLP, and PMA by measuring cellular responses in the presence of inhibitors of signal transduction.

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Materials and Methods

Reagents

PBS-Ca³ or PBS, FCS, pertussis toxin, cholera toxin, and FMLP were from Sigma Chemicals (St. Louis, MO). Pertussis toxin was dissolved in 50% glycerol and dialyzed against PBS with 5 mM glucose before use. Cholera toxin was dissolved in sterile PBS and stored at 4°C. Dihydrorhodamine 123 and Fura red-AM were from Molecular Probes (Eugene, OR). EGTA, PMA, and CytB were from Fluka (Buchs, Switzerland). Dispace, DNase I, and staurosporine were from Boehringer Mannheim (Mannheim, Germany). Genistein was from ICN Biochemicals (Costa Mesa, CA). Staurosporine (100 μ M), CytB (10 mM), FMLP (20 mM), dihydrorhodamine 123 (30 mM), Fura red-AM (2 mM), and genistein (20 mg/ml) were dissolved in dry DMSO (Merck, Darmstadt, Germany) and stored in aliquots at -20°C (or -70°C for dihydrorhodamine 123). On the day of each experiment DMSO containing genistein was diluted 1:20 in FCS and immediately further in PBS 1:20 to obtain maximal solubility. The solvent concentrations were less than 0.5% in final dilutions. All salts used in laboratory-made solutions, were analytical grade from Merck.

Polyclonal secondary antibodies

Fluorochrome-conjugated and unconjugated GAM-M, GAM-HL, and GAM-Fc were generously provided by Jackson ImmunoResearch (West Grove, PA). All had been adsorbed against human, bovine, horse, and rabbit serum proteins. Fluorochrome-conjugated antibodies specific for mouse Ig subclasses were from Caltag (San Francisco, CA). Unconjugated secondary antibodies were used in final concentrations of 50 to 100 μ g/ml, whereas conjugated antibodies were used at 20 μ g/ml in PBS containing 2% FCS and 5 mM glucose (solution referred to as PBS-FCS) and 0.1% sodium azide. Polyclonal anti-human IgM was from DAKO (Copenhagen, Denmark).

mAb

Two CD53 mAb were used, HD77 (kindly supplied by Dr. G. Moldenhauer, German Cancer Research Center, Heidelberg, Germany) and MEM-53 (1). mAb to other Ag were MEM-92 (IgM, CD3), MEM-25 (IgG1, CD11a), MEM-48 (IgG1, CD18), MEM-59 (IgG1, CD43), MEM-85 (IgG2b, CD44), MEM-28 (IgG1, CD45), 68-5A5 (IgG1, CD18), 33-3B3 (IgG1, CD43), and 140-11 (IgG2b, CDw50) were generously provided by Dr. R. Vilella (Clinic i Provincial,

Barcelona, Spain). ML-2 (IgG2a, CD24) was a gift from Dr. S. Funderud (The Norwegian Radium Hospital, Oslo, Norway). KB61 (IgG1, CDw32) was kindly provided by Dr. K. Pulford (John Radcliffe Hospital, Oxford, UK). FITC-conjugated and unconjugated 3g8 (IgG1, CD16), ION31 (IgG1, CD31), 2E1 (IgG2a, CDw32), J3D3 (IgG1, CD35) were from Immunotech (Marseilles, France). IV.3. (IgG2b, CDw32) was from Meda Rex (West Lebanon, NH). Leu-4-FITC (IgG1, CD3) was from Becton Dickinson (San Jose, CA). All mAb were diluted in PBS-Ca containing 5 mM glucose, 2% FCS (PBS-FCS-Ca), and 0.1% sodium azide and used at concentrations giving the maximal fluorescent staining and minimal cell aggregation as determined by flow cytometry.

Preparation of F(ab')₂ fragments of IgG mAb

IgG (ascites 1:10 in 0.02 M phosphate buffer, pH 7.4) was purified on a protein A-Sepharose 4 fast flow column (Pharmacia, Stockholm, Sweden). F(ab')₂ fragments were prepared as described by Parham (13). The purity of F(ab')₂ fragments was demonstrated by measuring a >99% reduction in cell staining with phycoerythrin-conjugated antibodies to the Fc part of mouse IgG, as compared with the intact IgG mAb (data not shown).

Isolation of leukocytes

Heparin-anticoagulated venous blood was diluted 1:10 with a solution containing 0.8% NH₄Cl, 0.08% NaHCO₃, and 0.08% EDTA, pH 6.8, at 15 to 20°C. Following lysis of red cells, leukocytes were sedimented by centrifugation at 160 \times g for 5 min. The pellet was resuspended in 10 ml of the lysing solution, the cells centrifuged again, and finally washed once in PBS-FCS.

Isolation of tonsillar lymphocytes

Human tonsils were obtained from patients undergoing surgical tonsillectomy. The tissue was cut in small pieces and incubated in PBS containing 1.5 mg/ml dispace for 30 min at 37°C. After incubation the supernatant containing lymphocytes was removed. This procedure was repeated up to five times. Isolated cells were treated with DNase I at 0.1 mg/ml for 5 min at 37°C before remaining red cells were lysed as described above. The cells were then washed once in PBS-FCS and filtered through a 40- μ M filter (Nytal Seidengaze Fabrik, Switzerland).

Cell lines

The U698 B cell line was kindly provided by Drs. Steinar Funderud and Erlend Smeland, The Norwegian Radium Hospital, Oslo.

³ Abbreviations used in this paper: PBS-Ca, PBS with calcium and magnesium; CytB, cytochalasin B; GAM-M, F(ab')₂ fragments of goat antibodies to mouse IgM, μ -chain specific; GAM-HL, F(ab')₂ fragments of goat antibodies to mouse IgG H and L chains; GAM-Fc, F(ab')₂ fragments of goat antibodies to mouse IgG, Fc-specific; PBS-FCS, PBS with 2% FCS and 5 mM glucose; PBS-FCS-Ca, PBS with calcium, magnesium, 2% FCS and 5 mM glucose.

Immunofluorescence

Leukocyte pellets were incubated with mAb at 20°C for 45 min, washed twice, and stained with 10 μ l of fluorochrome-conjugated secondary antibodies for 30 min on ice. Ig-subtype-specific secondary antibodies conjugated to FITC or phycoerythrin were used to allow two-color immunofluorescence. The antibody-labeled cells were washed, resuspended in ice-cold PBS-FCS-Ca, and analyzed by flow cytometry.

Measurement of cytoplasmic calcium fluxes and immunofluorescence

Isolated leukocytes were loaded with PBS-FCS-Ca containing 1 μ M Fura red at 37°C for 25 min, washed once in PBS-FCS-Ca, transferred to microwell plates, and pelleted (14). The cells were then stained indirectly with anti-CD43 and GAM-Fc-FITC. Immunostained leukocytes were washed and resuspended in 20 μ l PBS-FCS-Ca containing F(ab')₂ fragments of various mAb. The cells were then kept at 22°C for a minimum of 30 min before flow cytometric measurement. After this time interval, samples could be kept for at least 3 h without changes in FITC fluorescence or cellular responsiveness to any given stimulus (data not shown). Three minutes before measurement, the antibody/cell suspension was transferred to a microcytometry tube (Robbins Scientific, Mountain View, CA) containing 200 μ l of prewarmed PBS-FCS-Ca and incubated at 37°C. The sample was then placed in a laboratory-made flow cytometry chamber holding 37°C (14). B lymphocytes and non-B-lymphocytes were identified as CD43_{weak/negative} and CD43_{bright} respectively (Fig. 2, *top*) (15). Twenty μ l of PBS containing secondary antibodies or other stimuli were added after 30 to 40 s of measurement (Fig. 2). Calcium fluxes were measured by gated analysis of Fura red fluorescence from lymphocyte subsets (Fig. 2). Separate experiments with two-color immunofluorescence showed that >90% of the CD43-weak/negative lymphocyte population expressed high levels of the B cell Ag CD24, whereas <1% of the CD43 bright population were positive for CD24 ($n = 3$, data not shown). The anti-CD43 mAb did not induce cytoplasmic calcium fluxes when cross-linked with GAM-HL or change lymphocyte responsiveness to other stimuli such as anti IgM or anti-CD3 (Fig. 2 and data not shown). Measurements of cytoplasmic calcium fluxes in tonsillar lymphocytes or U698 cells were performed as for blood leukocytes, except that these cells were not stained with anti-CD43 (data not shown). Amplitudes of the calcium fluxes were calculated by dividing the lowest post-stimulated Fura red fluorescence by the mean prestimulated value (14). Based on experiments with positive and negative controls, a rapid decrease of 15% or more in the Fura red fluorescence after addition of stimuli was considered significant of a cytoplasmic calcium flux.

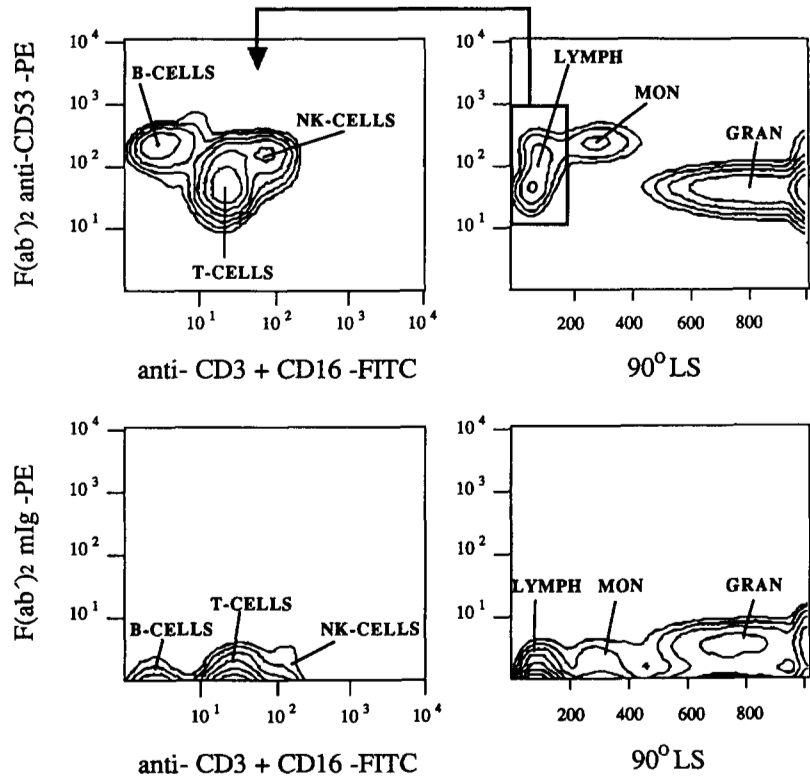
Measurement of cytoplasmic calcium fluxes and oxidative burst

Simultaneous flow cytometric measurement of monocyte and granulocyte cytoplasmic calcium fluxes and activation of the oxidative burst was performed by using Fura red and dihydrorhodamine 123 (14, 16). Fura red-loaded leukocytes were washed once in PBS-FCS-Ca, transferred to microwell plates, and pelleted. Twenty μ l of mAb solution was added to each pellet, the cells resuspended by whirlmixing, and kept at 22°C for a minimum of 30 min before flow cytometric measurement. The cells could be kept in this 20- μ l suspension for at least 4 h without changes in the responsiveness to positive and negative controls (data not shown). Before measurement the samples were preincubated with 100 μ l of prewarmed PBS-FCS-Ca at 37°C for 2 to 3 min. One hundred μ l prewarmed PBS-FCS-Ca containing 20 μ g/ml dihydrorhodamine 123 was then added and the tube was immediately placed in the thermostat-controlled flow cytometry sample chamber. During flow cytometric analysis, monocytes and granulocytes were identified by measurements of light scatter (14), and Fura red- and rhodamine 123 fluorescence gated vs time to separate cytograms (Fig. 4 and data not shown). Twenty μ l of PBS containing secondary antibodies or other stimuli were added after 30 to 40 s of measurement (Fig. 4). The calcium fluxes were analyzed as described above. The amplitudes of the oxidative bursts were calculated by the following formula: Δ rhodamine 123 fluorescence = (F stim - F pbs)/F t₀, where F stim = rhodamine 123 fluorescence 7.5 min after addition of stimulus to mAb-labeled/unlabeled cells; F pbs = rhodamine 123 fluorescence 7.5 min after addition of PBS to unlabeled cells and F t₀ = rhodamine 123 fluorescence at start of measurement. Based on experiments with positive and negative controls, an increase in rhodamine 123 fluorescence that was more than twofold higher than the increase seen in unstimulated cells, was considered significant of an oxidative burst.

Inhibition of signal transduction

For inhibition of calcium influx, leukocytes were suspended in PBS-FCS-Ca and diluted 1:2 with PBS-FCS containing 4 mM EGTA immediately before flow cytometric measurement. For inhibition of G-protein function, leukocytes were preincubated at 37°C for 2.5 h in PBS-FCS containing 2 μ g/ml pertussis toxin or 100 ng/ml cholera toxin, the last 30 min with 1 μ M Fura red-AM. For inhibition of protein kinases, leukocytes were preincubated with prewarmed PBS-FCS-Ca containing various concentrations of staurosporine or genistein at 37°C before addition of stimulus. Initial experiments showed a 3-min preincubation period was sufficient to obtain maximal inhibition at the concentrations of protein kinase inhibitors used in this study (data not shown). Control experiments

FIGURE 1. Flow cytometric measurement of CD53 expression on leukocyte subsets. Lymphocytes (lymph), monocytes (mon), and granulocytes (gran) were recognized by combined measurement of 90° light scatter and anti-CD53-PE (MEM-53 F(ab')₂) (top right). The top left diagram shows anti-CD53-PE vs CD3+CD16-FITC for the lymphocyte population. The bottom diagrams show staining of the same cell populations with F(ab')₂ fragments of polyclonal normal mouse IgG (mIgG). The results are representative of three experiments.



with vehicle only showed no inhibition of calcium fluxes and less than 10% inhibition of oxidative burst at the maximal vehicle concentrations (data not shown).

Flow cytometry

Measurements of calcium fluxes and oxidative burst were performed with a Coulter Epics V flow cytometer (Coulter Electronics, Luton, UK) interfaced to a CICERO PC-based data acquisition and analysis system (Cytomation, Englewood, CO). The excitation source was a 488-nm argon laser. A 530 nm band-pass filter was used for detection of rhodamine 123 or FITC fluorescence, and a 630 long-pass filter for Fura red. Measurements of immunofluorescence were performed with a FacSCAN flow cytometer with standard filter setup (Becton Dickinson, San Jose, CA).

Statistical evaluation

Significance of difference was determined by paired Students *t*-tests.

Results

Expression of CD53 on peripheral blood leukocytes

B cells, T cells, NK cells, monocytes, and granulocytes were identified by combined measurement of light scatter and CD3 + CD16 FITC (Fig. 1 and data not shown). Monocytes and B cells stained most intensively with anti-CD53 F(ab')₂ (MEM-53). The weakest staining was observed for

granulocytes and T cells, whereas NK cells were CD53_{intermediate} (Fig. 1). A similar differential staining of leukocytes was observed for another anti-CD53 mAb, HD77 (data not shown).

Cross-linking of CD53 induces a cytoplasmic calcium flux in B lymphocytes

Initial studies showed that about 10 to 15% of peripheral blood lymphocytes responded with a calcium flux after cross-linking of cell-bound F(ab')₂ fragments of anti-CD53 mAb (data not shown). To characterize this subset, combined measurements of immunofluorescence and cytoplasmic calcium levels were performed. After staining of leukocytes with anti-CD43/GAM-Fc-FITC, B cells were identified as CD43_{weak/negative} lymphocytes. The majority of cells in this population responded with a calcium flux to anti-human IgM, but not to anti-CD3, whereas the opposite was observed for the majority of CD43_{bright} lymphocytes (Fig. 2). When anti-CD43/GAM-Fc-FITC-stained leukocytes were labeled with F(ab')₂ anti-CD53 (MEM-53), the CD43_{weak/negative} lymphocyte population responded uniformly with a calcium flux after cross-linking with GAM-HL, whereas no response was seen among CD43_{bright} lymphocytes. No response was seen if PBS was added instead of GAM-HL or when GAM-HL was added in the absence of anti-CD53 mAb (Fig. 2 and data not shown). This indicates that cross-linking of the Ag was necessary for activation. The decrease in Fura red fluorescence after cross-

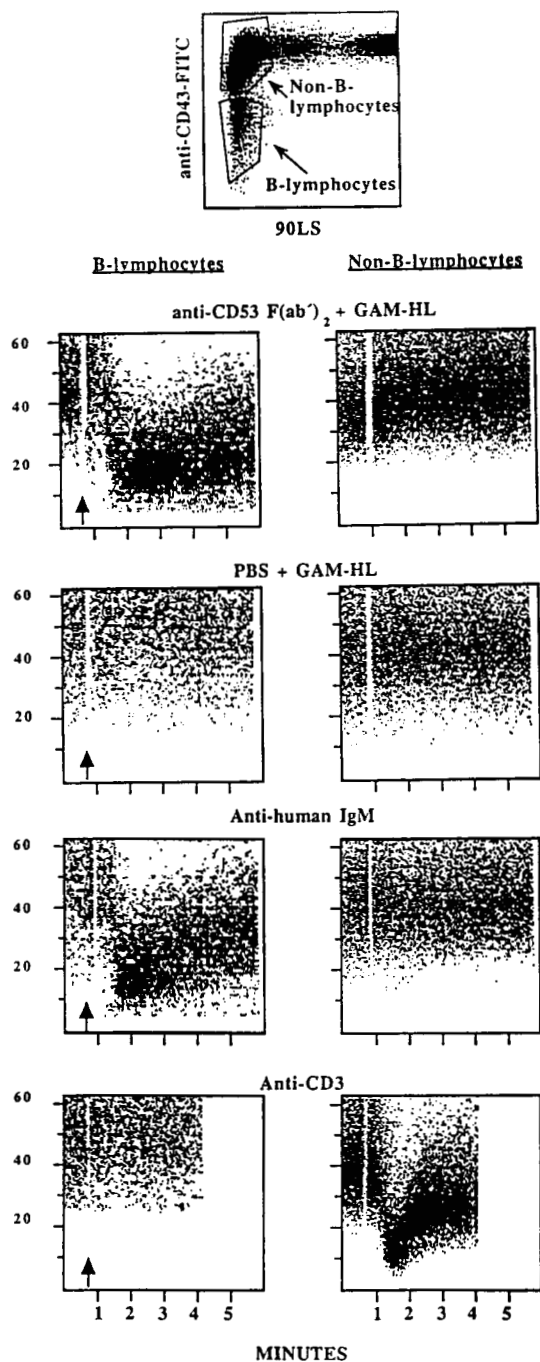


FIGURE 2. Flow cytometric measurement of cytoplasmic calcium fluxes in human lymphocyte subsets. *Top*, recognition of lymphocyte subsets by combined measurement of 90° light scatter and CD43-FITC fluorescence. *Bottom*, Fura red fluorescence gated vs time from B lymphocytes (*left*) and non-B lymphocytes (*right*). GAM-HL, anti-human IgM, or anti-CD3 was added at indicated time points (*arrows*). The diagrams are taken from a single experiment and are representative of four performed.

linking of CD53 was $50 \pm 4\%$ and similar to the $45 \pm 5\%$ decrease observed after addition of anti-IgM (mean \pm SEM, $n = 4$). This demonstrates that the response to cross-

linking of CD53 leads to a calcium flux comparable to that induced by cross-linking of well characterized receptor molecules. The duration of the response was typically longer in anti-CD53 stimulated cells than anti-IgM stimulated cells (Fig. 2). Calcium fluxes induced by anti-human IgM or anti-CD3 were not altered when cells were labeled with anti-CD53 mAb (data not shown). Results similar to those observed for MEM-53 were also seen when $F(ab')_2$ fragments of another mAb to CD53, HD77, were used, and intact mAb (ascites and purified mAb) gave the same results as $F(ab')_2$ fragments (data not shown).

No calcium fluxes were observed after cross-linking of cell-bound mAb to several other Ag expressed by B cells, including two mAb to the $Fc\gamma RII$ (CDw32) (KB61 or 2E1) and CD11a, CD18, CD31, CD35, CD44, CD45, or CDw50 ($n = 3$, data not shown). These results suggest that induction of calcium fluxes was a specific characteristic of mAb to CD53 and further document that the responses are independent of the B cell $Fc\gamma R$.

Results similar to those obtained with CD43^{weak/negative} peripheral blood lymphocytes were observed for tonsillar lymphocytes and U698 B cells (Fig. 3 and data not shown).

Cross-linking of CD53 induces a cytoplasmic calcium flux and activation of the oxidative burst in monocytes

Addition of GAM-HL to leukocytes preincubated with anti-CD53 $F(ab')_2$ (MEM-53) induced a rapid decrease in monocyte Fura red fluorescence of $61 \pm 4\%$ indicating a cytoplasmic calcium flux (Fig. 4) (mean \pm SEM, $n = 8$). The cytoplasmic calcium flux was followed by an increase in monocyte R123 fluorescence relative to unstimulated cells, indicating activation of the oxidative burst (Figs. 4 and 5). If PBS was added instead of GAM-HL, or if GAM-HL was added to unlabeled cells, no responses were seen suggesting that cross-linking of the Ag was necessary for activation (Fig. 4 and data not shown). Stimulation of MEM-53-labeled cells with $4 \mu M$ FMLP instead of GAM-HL led to a $65 \pm 4\%$ decrease in monocyte Fura red fluorescence, indicating a calcium flux comparable to that seen with cross-linking of CD53 (Fig. 4) (mean \pm SEM, $n = 8$). However, the FMLP-induced calcium flux was associated with only low activation of oxidative burst compared with the response seen with GAM-HL (Figs. 4 and 5) ($p < 0.01$, $n = 8$). The amplitude of the anti-CD53-induced oxidative burst was higher than seen after cross-linking of CDw32 ($Fc\gamma RII$) with mAb IV.3 and GAM-HL (Fig. 5) ($p < 0.05$, $n = 8$). Addition of $4 \mu M$ FMLP/CytB induced a response comparable to the CD53 mediated burst, whereas 100 ng/ml PMA induced a more extensive oxidative burst in monocytes (Fig. 5). Labeling with anti-CD53 $F(ab')_2$ did not change monocyte responsiveness to FMLP, FMLP/CytB, or PMA (data not shown).

FIGURE 3. Flow cytometric measurement of cytoplasmic calcium fluxes (Fura red) in human tonsillar lymphocytes. Cells were preincubated with F(ab')₂ anti-CD53 (MEM-53) or PBS. GAM-HL, anti-human IgM, or anti-CD3 was added at indicated time points (arrows). The diagrams are taken from a single experiment and are representative of four performed.

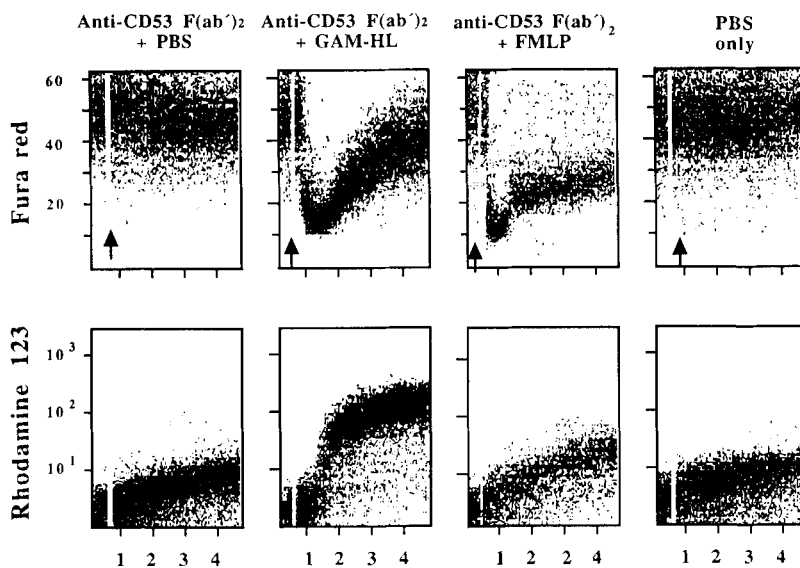
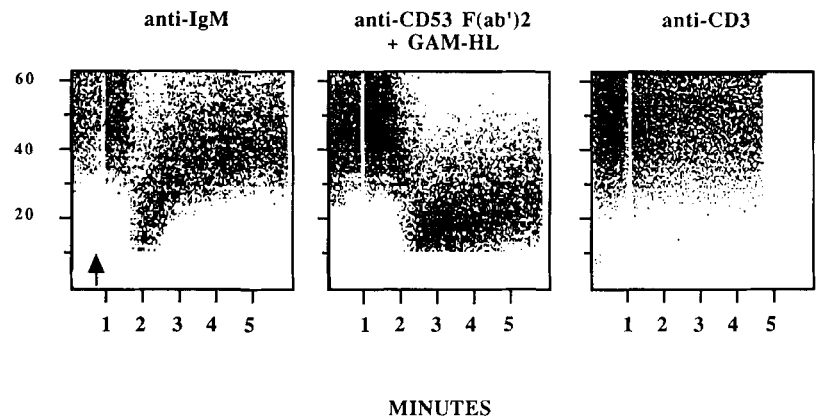


FIGURE 4. Flow cytometric measurement of cytoplasmic calcium flux (Fura red) and oxidative burst (rhodamine 123) in monocytes after cross-linking of CD53 or stimulation with 4 μ M FMLP. Monocytes were recognized by light scatter measurements (data not shown). Fura red-loaded leukocytes were incubated with anti-CD53 or PBS (right), resuspended in prewarmed PBS-FCS-Ca containing 20 μ g/ml dihydrorhodamine 123, and analyzed at 37°C. GAM-HL, 4 μ M FMLP, or PBS was added at indicated time points (arrows). The results are from a single experiment and are representative of five performed.

Results similar to those observed with MEM-53 F(ab')₂ were obtained when using F(ab')₂ fragments of HD 77 (anti-CD53) (data not shown). Both of these mAb also induced calcium fluxes and oxidative bursts in granulocytes when cross-linked, but the responses in granulocytes were considerably weaker than in monocytes and weaker than after stimulation with FMLP or anti-CDw32 mAb (data not shown). No calcium fluxes or oxidative bursts were observed in monocytes or granulocytes after addition of GAM-HL to leukocytes prelabeled with F(ab')₂ fragments of mAb to CD11a, CD18, CD31, CD35, CD43, or CD45 (data not shown).

The CD53-mediated calcium flux in monocytes involves release of calcium from intracellular stores and is inhibited by staurosporine but not by ADP-ribosylating toxins

Chelation of extracellular calcium ions by 2 mM EGTA led only to a partial reduction in the amplitude of the MEM 53-/GAM-HL-induced calcium flux in monocytes (Fig. 6). This indicates that a substantial contribution to the response

comes from intracellular release of calcium ions. A similar sensitivity to EGTA was seen for the FMLP/CytB-induced calcium response (Fig. 6). In contrast, there was a difference in the sensitivity of CD53- and FMLP/CytB-mediated calcium fluxes to the phosphorylation inhibitor staurosporine (Fig. 6). At concentrations above 100 nM, staurosporine inhibits several protein kinases including C kinases and tyrosine kinases (17–19). In the presence of 500 nM staurosporine the CD53-mediated calcium flux was completely blocked, whereas the FMLP/CytB-induced response was unaffected (Fig. 6). This selective inhibition of CD53-mediated calcium fluxes was not likely to be due to non-specific toxic effects, as no inhibition of calcium fluxes or oxidative bursts was observed when leukocytes were preincubated for 25 min with 500 nM staurosporine, washed twice, labeled with mAb, and stimulated with GAM-HL (data not shown). Preincubation with the G-protein inhibitor pertussis toxin revealed further differences between CD53- and FMLP/CytB-mediated calcium fluxes. Doses of pertussis toxin that led to a 99% and 50% inhibition of the FMLP/CytB-induced calcium response in granulocytes and

FIGURE 5. Oxidative burst (Δ rhodamine 123 fluorescence) in monocytes after activation with anti-CD53 (MEM-53) F(ab')₂/GAM-HL, anti-CDw32 (IV.3)/GAM-HL, FMLP, FMLP/CytB, or PMA. The cells were activated as described in the legend to Figure 3. Δ rhodamine 123 fluorescence was determined as described in *Materials and Methods*. Error bars indicate SEM of five experiments.

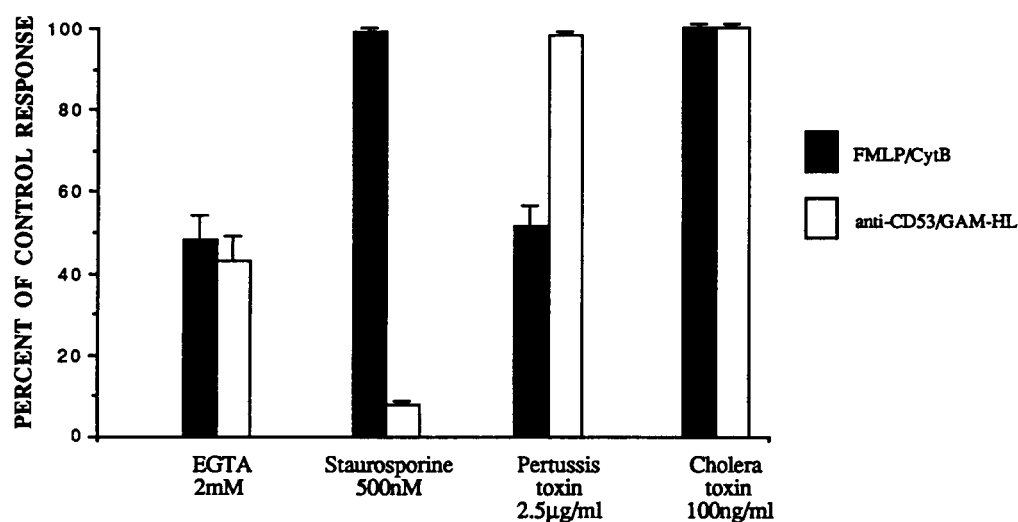
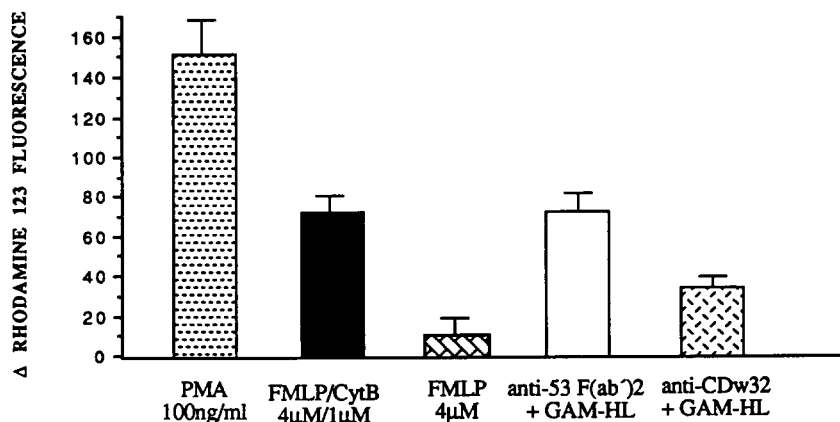


FIGURE 6. Inhibition of calcium fluxes induced in monocytes by stimulation with anti-CD53/GAM-HL or FMLP/CytB. The cells were treated with inhibitors as described in *Materials and Methods* and stimulated as described in the legend to Figure 4. The results represent the amplitudes of the calcium fluxes in inhibitor-treated cells in percentage of the amplitude of untreated cells in the same experiments. The amplitudes were calculated as described in *Materials and Methods*. Error bars indicate SEM of five experiments. The mean decrease in Fura red fluorescence of monocytes in the absence of inhibitors was $60 \pm 5\%$ and $65 \pm 4\%$ for anti-CD53 and FMLP/CytB, respectively (mean \pm SEM, $n = 5$).

monocytes respectively, had no effects on the CD53-mediated calcium flux in either cell type (Fig. 6 and data not shown). Preincubation with cholera toxin had no effects on calcium fluxes induced by either stimulus, although the toxin was found to cause elevation of cAMP in leukocytes (Fig. 6 and data not shown).

The CD53-mediated calcium flux in tonsillar B cells was abolished in the presence of 300 nM staurosporine (data not shown). The same concentration was needed for complete inhibition of the anti-IgM-induced calcium flux, whereas the anti-CD3-mediated calcium flux in peripheral blood T cells was abolished in the presence of 100 nM staurosporine ($n = 3$, data not shown). Genistein, another tyrosine kinase inhibitor, was less efficient than staurosporine in inhibiting anti-CD53, -IgM, or -CD3-induced calcium fluxes. When used in concentrations up to 50 μ g/ml, there was only inhibition of the late phase of the response, even when cells

were preincubated with the inhibitor for 20 min (data not shown).

Cross-linking of CD53 induces an oxidative burst in monocytes at concentrations of staurosporine that abolish the PMA-mediated response

At concentrations between 1 and 20 nM, staurosporine has selectivity for protein kinase C (14, 20). We therefore compared the inhibitory effect of staurosporine on CD53- and PMA-mediated responses to evaluate the importance of protein kinase C for activation of the oxidative burst through CD53. Results from these experiments showed only 50% inhibition of the CD53-induced oxidative burst at concentrations of staurosporine that abolished the PMA-mediated oxidative burst (Fig. 7). In contrast, the CD53-mediated oxidative burst was more sensitive to genistein,

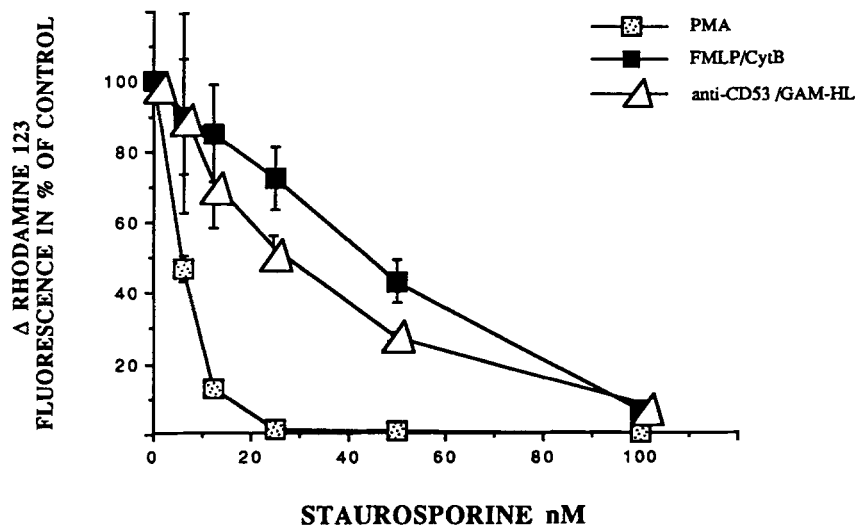


FIGURE 7. Inhibitory effect of staurosporine on oxidative burst induced in monocytes by anti-CD53/GAM-HL, FMLP/CytB, or PMA. The cells were treated with the inhibitor as described in *Materials and Methods* and stimulated as described in the legend to Figure 4. The results represent the Δ rhodamine 123 fluorescence (see *Materials and Methods*) in inhibitor-treated cells in percent of the amplitude in untreated cells in the same experiments. Error bars indicate SEM of five experiments. The mean Δ rhodamine 123 fluorescence of monocytes in the absence of inhibitors was 67 ± 5 , 72 ± 4 , and 157 ± 12 for anti-CD53, FMLP/CytB, and PMA, respectively (mean \pm SEM, $n = 5$). The mean baseline rhodamine fluorescence was 2.0 ± 0.1 at t_0 for all samples, and 8.2 ± 0.3 at 5 min in samples stimulated with PBS only (mean \pm SEM, $n = 5$).

an inhibitor with selectivity for tyrosine kinases (Fig. 8). The oxidative burst induced by FMLP/CytB in monocytes showed similar sensitivity to staurosporine and genistein as the CD53-mediated response (Figs. 7 and 8).

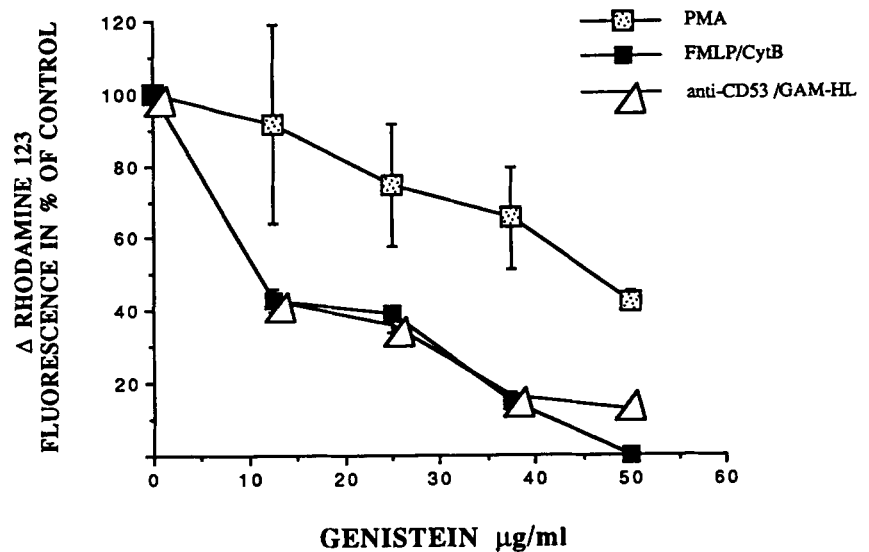
Discussion

The present study shows that cross-linking of CD53, a member of a family of molecules with four membrane-spanning domains, induces activation of human monocytes and B cells. Several lines of evidence suggest that the observed responses are secondary to the specific cross-linking of CD53. 1) Cell activation induced by antibody cross-linking was not likely to be due to Fc γ R interactions as purified F(ab')₂ fragments of antibodies were used in all experiments. Furthermore, cross-linking of B cell Fc γ R did not induce cytoplasmic calcium fluxes in this or previous studies (21). 2) Artifacts due to potential contaminations in the mAb solutions are unlikely from the results showing lack of responses in the absence of a secondary antibody and by the weak oxidative burst when anti-CD53-labeled cells were stimulated by FMLP instead of GAM-HL. 3) Cell activation could be induced by two distinct mAb to CD53, but not with mAb specific for a number of other Ag present in similar density on B cells and monocytes. This suggests that cell activation was not due to nonspecific coaggregation of surface molecules. As the responses seen after cross-linking of CD53 had similar or higher amplitudes than those induced by FMLP or cross-linking of surface IgM or monocyte Fc γ R2, the results collectively

suggest that CD53 is capable of mediating cell activation of similar magnitude as known receptor molecules.

The mAb to CD53 did not induce activation of all cells expressing the Ag. Monocytes responded strongly and uniformly with a calcium flux and an oxidative burst, whereas granulocytes responded only with a weak calcium flux. Although our data do not exclude the possibility that minor subsets of non-B-lymphocytes are present in the responding population, a clear difference was observed between B cells and T cells with regard to anti-CD53 responsiveness. An explanation for the cell type-dependent activating effects of anti-CD53 mAb may be the higher expression of CD53 on B cells and monocytes as compared with the majority of non-B-lymphocytes and granulocytes (Fig. 1). Alternatively, the anti-CD53-induced responses could be dependent on the association between CD53 and other cell membrane components specifically expressed by the responding cell types. Associations with receptors or receptor-like membrane components have been shown for other human leukocyte Ag with four membrane-spanning domains, including CD9 and TAPA-1 (11, 22). In rat NK and T cells, the CD53 homologue, OX44, is noncovalently coupled to the CD2 Ag, and this association may be important for the functional effects of anti-OX44 mAb (12). As human B cells, monocytes, and granulocytes do not express the CD2 Ag, it is likely that CD53-mediated signaling in these cells involves a CD2-independent mechanism. Receptor molecules that may associate with CD53 in human leukocytes should, however, be sought in membranes of B cells and monocytes.

FIGURE 8. Inhibitory effect of genistein on oxidative burst induced in monocytes by anti-CD53/GAM-HL, FMLP/CytB, or PMA. The mean Δ rhodamine 123 fluorescence of monocytes in the absence of inhibitors was 71 ± 6 , 74 ± 5 , and 165 ± 14 for anti-CD53, FMLP/CytB, and PMA, respectively (mean \pm SEM, $n = 5$). The mean baseline rhodamine fluorescence was 1.8 ± 0.1 at t_0 for all samples, and 7.8 ± 0.2 at 5 min in samples stimulated with PBS only (mean \pm SEM, $n = 5$). See legend to Figure 7.



Although the mechanism that couples CD53 to cell activation remains to be determined, the results from our experiments with inhibitors of signal transduction give important indications. The ability of anti-CD53/GAM-HL and FMLP/CytB to induce cytoplasmic calcium fluxes in monocytes in the presence of EGTA suggests involvement of phospholipase C and inositoltrisphosphate in both signaling pathways (23). However, whereas FMLP/CytB-induced calcium fluxes were sensitive to the G-protein inhibitor pertussis toxin and unaffected by the phosphorylation inhibitor staurosporine, the opposite was observed for the response to anti-CD53/GAM-HL. A possible explanation for these results is that a protein kinase is involved in the activation of phospholipase C through CD53, whereas the monocyte FMLP receptor activates the phospholipase independently of phosphorylation through a pertussis toxin-sensitive G-protein. As the concentrations of staurosporine that blocked CD53-mediated signaling were 5- to 10-fold higher than those that completely blocked PMA-induced activation, it is likely that CD53-mediated signaling is largely independent of protein kinase C (Figs. 6 and 7). Involvement of tyrosine kinases is possible as earlier studies show that staurosporine inhibits tyrosine phosphorylation of phospholipase C γ 1 when used at 300 nM (19), i.e., the same concentration that blocked CD53-mediated calcium fluxes in B cells in the present study. This concentration of staurosporine was also found to block calcium fluxes induced by anti-IgM, which is known to be dependent on tyrosine phosphorylation (24). The fact that a higher concentration of staurosporine was necessary to block CD53-mediated calcium fluxes in monocytes may reflect cell type-dependent differences in the tyrosine kinases that are involved in early signaling. The difference in the inhibitory concentrations of staurosporine for CD3- and IgM-mediated calcium fluxes in T cells and B cells, which both depend on tyrosine kinases, supports this explanation

(24). The data from the inhibitor studies therefore suggest that CD53 shares early signaling pathways with surface IgM and indicate that tyrosine kinase activity may be an early event after cross-linking of the Ag.

The present study on CD53-mediated signaling extends previously published results on the function of molecules with four membrane-spanning domains. Anti-CD9 mAb have long been known to affect platelet activation (5, 6, 22). mAb to CD9 and CD37 have been shown to influence human B cell aggregation and proliferation, respectively (7, 8). Another B cell molecule in the same family, TAPA-1, was identified by screening of mAb for inhibitory effects on proliferation of B-lymphoma cells (11). The mAb IA4, which recognizes a molecule with four membrane-spanning domains on B cell lines and monocytes, has also been shown to have effects on cell activation (25). Results showing mAb-induced nuclear uptake of CD63 (ME491) and inhibition of transcription in melanoma cells have led to the hypothesis that this Ag may represent a receptor for a growth factor not yet described (9). A CD63-like molecule on rat basophilic leukemia cells may be functionally linked to the IgE-FcR (26). Particularly interesting for the present study are the results described in a recent article in which a large panel of mAb were screened for their ability to induce activation of phosphatidylinositol signaling in rat NK cells (12). Only the rat CD53 homologue, OX44, and two other Ag were identified as signal transducing molecules (12). Our results therefore support the hypothesis of a possible receptor function for CD53, based on studies of its structure and reported functions of similar molecules, including the CD53 homologue on rat leukocytes.

In conclusion, the present study shows that cross-linking of CD53 induces activation of human B cells and monocytes. The results support the view that leukocyte membrane Ag with four membrane-spanning regions are involved in transmembrane communication.

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