Biochem. J. (1992) 288, 465–473 (Printed in Great Britain)

Regulated Ca^{2+} signalling through leukocyte CD11b/CD18 integrin

Dario C. ALTIERI,* Susan J. STAMNES* and Carl G. GAHMBERG†

*Department of Immunology, Committee on Vascular Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, U.S.A., and †Department of Biochemistry, University of Helsinki, Unioninkatu 35 SF-00170, Helsinki, Finland

General mechanisms of adhesion in the immune response are coordinated by the leukocyte integrins CD11/CD18. The possible participation of these differentiation molecules in early events of transmembrane signalling was investigated. Monoclonal antibody (mAb) cross-linking of CD18, the integrin β subunit ubiquitously expressed by all leukocytes, increased the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by 2–3-fold in monocyte THP-1 cells. Digitalized imaging in single adherent cells showed that this Ca^{2+} response is temporally biphasic, involves both release of Ca^{2+} from the intracellular stores as well as Ca^{2+} influx from the external compartment, and is dramatically down-modulated by terminal differentiation of THP-1 cells to a mature monocyte phenotype. Similarly, only a minor subset of 20–30% of peripheral blood monocytes heterogeneously maintain the CD18-mediated Ca^{2+}-signalling properties. Cross-linking of CD18 also increased cytosolic free $[Ca^{2+}]_i$ in a subset of approx. 15–20% of resting T lymphocytes, in a Ca^{2+} response that was completely abrogated during T-cell mitogenic activation with lectins or alloreactive antigen. While cross-linking of CD11a or CD11c was without effect, occupancy of CD11b increased cytosolic free $[Ca^{2+}]_i$ in monocytic cells. This response was functionally coupled with a transient activation state of CD11b/CD18, which was reflected in its increased avidity to bind the complementary ligand fibrinogen. These results suggest that occupancy of CD18 transduces a stimulatory Ca^{2+} signal that is critically regulated by the state of cell activation/differentiation and by the association with the unique α-subunit CD11b. These intrinsic signalling properties may directly participate in regulating the oligospecific ligand recognition of leukocyte integrins.

INTRODUCTION

The three heterodimers CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), and CD11c/CD18 (p150, 95), comprise the leukocyte-restricted members of the integrin gene superfamily of adhesion receptors [1–3]. Broadly distributed on cells of myelo-monocytic and lymphoid lineage, these molecules play a crucial role in a variety of cell-adhesion reactions during immune–inflammatory mechanisms [1–3]. This is dynamically accomplished through a regulated process of oligospecific ligand binding, which is governed by disparate stimuli including protein kinase C activators [4], extracellular nucleotides [5], and chemo-attractants [6].

By analogy with other adhesion receptors in the immune system [7], recent studies have also raised the possibility that CD11/CD18 may be capable *per se* of transducing intracellular signals, thus directly modulating disparate cellular responses. In this context, occupancy of CD11/CD18 with the complementary ligands or with specific monoclonal antibodies (mAbs) has been shown to modulate neutrophil oxidative bursts [8], to deliver mitogenic or co-mitogenic signals during B-cell [9], or T-cell activation [10–13], to generate second intracellular messengers [14], and to directly activate specific ligand-binding properties [15].

In this study, we sought to investigate the ability of CD18, the β subunit shared by all leukocyte integrins [1–3], to release intracellular free Ca^{2+} upon engagement with a panel of specific mAbs. Using real-time fluorescence measurements in single adherent cells, we show that mAb cross-linking of CD18 heterogeneously increases cytosolic free $[Ca^{2+}]_i$ in cells of monocytic and lymphoid lineage. This Ca^{2+} response, that is functionally coupled to transient receptor activation and increased avidity for ligand binding, is crucially regulated by the state of cell differentiation/activation and by the preferential assembly of CD18 with the complementary α subunit CD11b.

MATERIALS AND METHODS

Cells and cell culture

The monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD, U.S.A.) was maintained in continuous culture of RPMI 1640 medium (M. A. Whittaker Bioproducts, Walkersville, MD, U.S.A.) supplemented with 7% (v/v) fetal calf serum (Whittaker), 2 mM-L-glutamine (Irvine Scientific, Santa Ana, CA, U.S.A.), 10 mM-Hepes, and 10^{-5} M-2-mercaptoethanol (Eastman Kodak Co., Rochester, NY, U.S.A.). Terminal differentiation of THP-1 cells to a mature monocyte/macrophage phenotype was induced according to published methods [16], by cultivating aliquots of the cell suspension at 1×10^6 cells/ml in complete growth medium supplemented with 114 nM-phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 24–48 h at 37 °C in a 5% humidified incubator. The T-cell line, MOLT13, was generously provided by Dr. D. P. Dialynas (The Scripps Research Institute, CA, U.S.A.) and maintained in continuous culture in complete RPMI 1640 growth medium supplemented with 10% (v/v) fetal calf serum, 2 mM-L-glutamine, and 10 mM-Hepes. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll–Hypaque differential centrifugation. Monocytes were separated from unfractionated PBMC by adherence on to autologous serum-coated plastic Petri dishes (Costar Co., Cambridge, MA, U.S.A.) for 1 h at 37 °C. T

Abbreviations used: mAb, monoclonal antibody; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PMA, phorbol 12-myristate 13-acetate; PBMC, peripheral blood mononuclear cells; ConA, concanavalin A; PHA, phytohaemagglutinin; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PMT, photomultiplier; LPS, lipopolysaccharide.

cells were purified from PBMC using a nylon-wool fractionation method. Briefly, 10 ml aliquots of PBMC at 1×10^7 cells/ml suspended in complete RPMI 1640 medium plus 10% (v/v) fetal calf serum, were applied to a nylon-wool column (2.5 g/15 cm column) and equilibrated for 1 h at 37 °C. Non-adherent (T-cell-enriched) cells were eluted with 35 ml of prewarmed RPMI 1640 medium at a flow rate of 1 drop/s. Purity of the monocyte or T-cell preparations was always >95% as assessed by flow cytometry using mAbs OKM1 or OKT3, directed to CD11b and CD3 respectively [17,18]. In some experiments, suspensions of freshly isolated, resting T cells were polyclonally activated by a 5-day culture in complete RPMI 1640 growth medium supplemented with 1 μg of concanavalin A (ConA; Calbiochem Boehring, La Jolla, CA, U.S.A.)/ml or 5 μg of phytohaemagglutinin (PHA; Calbiochem)/ml. To generate long-term alloreactive-stimulated T cells, responder cells were cultivated in 24-well plates at 2×10^5 cells/well in complete RPMI 1640 growth medium plus 10 units of recombinant interleukin 2 (IL-2)/ml (Genzyme Corp., Cambridge, MA, U.S.A.) in the presence of 7×10^6 alloreactive PBMC/well, preparatively irradiated with 3000 Rad. Responder T cells were recovered by Ficoll–Hypaque differential centrifugation, and re-stimulated at weekly intervals with irradiated alloreactive PBMC as described above. Phenotypical characterization of the responder-T-cell population was carried out by flow cytometry using a panel of specific mAbs (see below).

mAbs

The mAbs raised against CD18 used in this study were 60.3 [19], TS1/18 [20], IB4 [17], 7E4, 2E7, and 1D10 [21]. Anti-CD11a mAb was TS1/22 [20]. Anti-CD11b mAbs were OKM1, M1/70 and 60.1 [17,19]. Anti-CD11c mAb was 3.9 [22]. Anti-CD14 mAbs 3C10 and 63D3 were generously provided by Dr. R. Ulevitch (The Scripps Research Institute, CA, U.S.A.). Purified IgG fractions of the various mAbs were prepared by chromatography on Affigel MAPS II columns (Bio-Rad, Richmond, CA, U.S.A.) according to the manufacturer's instructions. $F(ab')_2$ fragments of mAb IB4 were the generous gift of Dr. K. Gelsen (La Jolla Institute for Experimental Medicine, La Jolla, CA, U.S.A.). The reactivity of anti-CD11/CD18 mAbs with various cells was assessed by flow cytometry. Briefly, aliquots of undifferentiated or PMA-differentiated THP-1 cells, or resting or activated T cells, were preparatively incubated at 1×10^7 cells/ml in 20% (v/v) human serum for 30 min at 4 °C to saturate Fc binding sites. After washes, cells were incubated with 25 μg/ml aliquots of the various anti-CD11/CD18 mAbs or control mAb 6B4 for an additional 30 min at 4 °C, washed and mixed with aliquots (1:20 dilution) of fluorescein isothiocyanate (FITC)-conjugated affinity-purified $F(ab')_2$ goat anti-(mouse IgG) antibody (H+L) (Tago Inc., Burlingame, CA, U.S.A.) for 30 min at 4 °C. At the end of the incubation, cells were washed, and immediately analysed on a Becton Dickinson Facscan.

Single-cell analysis of cytosolic free $[Ca^{2+}]_i$

Dynamic fluorescence measurements of cytosolic free $[Ca^{2+}]_i$ in single adherent cells were carried out as described [5]. Briefly, suspensions of THP-1 cells or other blood-isolated leukocyte subpopulations were washed twice in phosphate-buffered saline (PBS) containing 5 mM-EDTA, pH 7.2, and suspended at 1×10^7 cells/ml in complete RPMI 1640 medium. Two million cells were plated and allowed to adhere for 2 h at 37 °C on to 22 mm^2 optical-grade glass coverslips (< 16 μm thickness) preparatively coated with either 10 μg of Cell-Tak (BioPolymers, Farmington, CT, U.S.A.) in 0.1 M-$NaHCO_3$, pH 9.2, or with 5 μg of human fibronectin/ml (generously provided by Dr. E. F. Plow, The Scripps Research Institute, CA, U.S.A.). At the end of the incubation, the monolayer of attached cells was gently washed four times to remove non-adherent or loosely adherent cells, and further incubated with 1 μM-Indo-1/AM (Calbiochem) in a loading buffer containing 145 mM-glucose, 5 mM-KCl, 1 mM-Na_2HPO_4, 2.5 mM-$CaCl_2$, 0.5 mM-$MgSO_4$, 10 mM-glucose, 25 mM-Hepes, pH 7.45, for an additional 45 min at 37 °C. After washing, cells were covered with 200 μl of Indo-1 loading buffer, protected in the dark from photobleaching, and analysed within 2 h of the intracellular dye loading procedure. Real-time fluorescence measurements of cytosolic free $[Ca^{2+}]_i$ in single adherent cells were carried out with the interactive laser cytometer ACAS 470 (Anchored Cell Analysis and Sorting, Meridian Instruments Inc., Okemos, MI, U.S.A.). The instrument is equipped with an Olympus IMT2 inverted microscope (Olympus, Tokyo, Japan) with 10×, 20× and 40× long-working-distance objectives. Illumination and fluorescence are provided by a 5 W argon laser for u.v./visible excitation, coupled to two photomultipliers (PMT) with a computer-controlled high-voltage power supply for simultaneous dual-wavelength analysis. Fluorescence emission for detector 1 (unbound Indo-1) or detector 2 (Indo-1–Ca^{2+} complex) were adjusted to 485 and 405 nm respectively. Before each experiment, a two-dimensional microprocessor-controlled scanning stage of a selected area of adherent cells was carried out. Cells were selected for further analysis only if they showed < 10% photobleaching after 200 left to right scans across the cell centre. In a typical experiment groups of 25–40 adherent cells were analysed utilizing an Image-Scan option with the following parameters: PMT1 (detector 1) 60–70%, PMT2 (detector 2) 35–55%, step size 1 μm, laser power 200 mW, scanning strength 35–50%, stage speed 20 mm/s. Unless otherwise specified, 14 scans were carried out with a scan delay of 25 s and an elapsed time between each scan of 2–4 s. Analysis of cytosolic free $[Ca^{2+}]_i$ upon CD11/18 cross-linking was carried out by substituting the Indo-1 loading buffer covering the monolayer of adherent cells with an equal volume of buffer containing 25 μg of control mAbs/ml, or of the various anti-CD11/CD18 mAbs. Additions were made after the first scan of each experiment. After the fifth scan (125 s), 70 μg of affinity purified $F(ab')_2$ goat anti-(mouse IgG) antibody (Tago) was added as a cross-linking reagent. In separate experiments, the addition of 7 μM-ionomycin (Calbiochem) served to monitor the maximal Ca^{2+} response of the various cell populations examined. To investigate the effect of removal of extracellular Ca^{2+} ions, monolayers of adherent THP-1 cells were rapidly washed twice in buffer containing 3 mM-EGTA and no Ca^{2+} ions immediately before the first scan, and further incubated with the various anti-CD18 mAbs appropriately diluted in the same EGTA-containing buffer.

Analysis of results

Fluorescence measurements of cytosolic free $[Ca^{2+}]_i$ in single adherent cells were electronically integrated as the ratio of detector 1/detector 2, or quantitatively calculated using a standard curve constructed by adding increasing concentrations of $CaCl_2$ to 1 μM-Indo-1 free acid (Calbiochem) diluted in 10 mM-3-*N*-morpholinepropanesulphonic acid (Sigma), 115 mM-KCl, 20 mM-NaCl, 1 mM-$MgSO_4$, 1 mM-EGTA, pH 7.4.

Binding studies

The experimental procedures for the purification and ^{125}I-labelling of plasma fibrinogen have been described previously in detail [23]. The biochemical characterization of the interaction of fibrinogen with stimulated cells of myelo-monocytic lineage and the identification of CD11b/CD18 as the inducible and high-affinity fibrinogen receptor on these cells have also been reported

[23]. The role of the mAb-stimulated increase in cytosolic free $[Ca^{2+}]_i$ on CD11b/CD18 recognition of fibrinogen was investigated as follows. Serum-free suspensions of THP-1 cells, at 2×10^7 cells/ml containing 2.5 mM-$CaCl_2$, were incubated for 5 min at room temperature with 25 μg/ml of anti-CD11b mAbs 60.1, or with a diluted culture supernatant (1:4) of anti-CD11b mAb M1/70 respectively. Cells were further incubated with increasing concentrations of ^{125}I-fibrinogen (0.017–0.14 μM) for an additional 20 min at room temperature before separation of free radiolabel from cell-associated radioactivity by centrifugation of aliquots of the cell suspensions through a mixture of silicone oils at 12000 *g*. Control cells incubated in the absence of anti-CD11b mAbs were stimulated with 10 μM-ADP in the presence of 2.5 mM-$CaCl_2$ before the addition of increasing doses of ^{125}I-fibrinogen [23]. Non-specific binding was quantitated in the presence of a 100-fold molar excess of competing unlabelled fibrinogen, and was subtracted from the total to calculate net specific binding [23].

RESULTS

CD18 expression on THP-1 cells

Preliminary experiments were carried out to assess the reactivity of an established anti-CD18 mAb panel with the monocytic cell line THP-1. As reported in previous studies, THP-1 cells provide a reliable and homogeneous *in vitro* model for a monocyte-like phenotype, expressing quantitatively abundant levels of all three leukocyte integrins CD11a–c/CD18 (see below), functionally responsive to intracellular signalling pathways of activation and ligand binding [5,23]. When analysed by flow cytometry, the various anti-CD18 mAbs tested showed a unique pattern of reactivity with THP-1 cells (Fig. 1), consistent with their postulated recognition of spatially distant and non-overlapping epitopes on CD18 [17,19–21].

Cross-linking of CD18 increases cytosolic free $[Ca^{2+}]_i$ in THP-1 cells

The ability of the various anti-CD18 mAbs to increase cytosolic free $[Ca^{2+}]_i$ in THP-1 cells was investigated in real-time fluorescence at the single-cell level. When assessed in the presence of millimolar concentrations of extracellular Ca^{2+} ions, the Ca^{2+} ionophore ionomycin immediately produced an homogeneous and large increase in cytosolic free $[Ca^{2+}]_i$ in the entire population of THP-1 cells analysed per field (Fig. 2*d*). Cross-linking of CD14 using mAb 63D3 also heterogeneously increased cytosolic free $[Ca^{2+}]_i$ in adherent monocytes (Fig. 2*b*), in agreement with previous observations [24]. Under these experimental conditions, anti-CD18 mAbs 60.3 and TS1/18 consistently increased cytosolic free $[Ca^{2+}]_i$ in virtually all adherent THP-1 cells analysed per field (Figs. 2*a* and 2*c*). This Ca^{2+} response, that was quantitatively larger than the Ca^{2+} signal elicited after CD14 cross-linking (Fig. 2*b*), was composed of two chronologically distinct phases, with a first wave observed immediately after the addition of the primary mAb, and a second, more prolonged and sustained increase in cytosolic free $[Ca^{2+}]_i$, after the addition of the $F(ab')_2$ cross-linking reagent (Figs. 2*a* and 2*c*). Although coordinately observed in the entire group of THP-1 cells analysed, the CD18-mediated Ca^{2+} transient was characterized by qualitative differences in the kinetics of onset and decay in individual cells, especially upon occupancy of distinct epitopes on CD18 (Figs. 2*a* and 2*c*). A similar pattern of Ca^{2+} response was also observed after CD18 cross-linking with mAbs 7E4, 2E7, and with $F(ab')_2$ fragments of mAb IB4 (see below), while anti-CD18 mAb 1D10, detecting a low-density epitope on THP-1 cells (Fig. 1), was ineffective. Similarly, mAbs of matched isotype directed against high-density surface molecules on THP-1 cells (see below), or $F(ab')_2$ cross-linking reagent alone failed to increase cytosolic free $[Ca^{2+}]_i$ under the same experimental conditions (results not shown).

The temporal kinetics of the CD18-mediated Ca^{2+} transient in THP-1 cells was further analysed in digitalized imaging. The Ca^{2+}-concentration map of a monolayer of resting, unstimulated THP-1 cells during a typical experiment is shown in Fig. 3(*a*). Integration of fluorescence measurements showed that the majority of THP-1 cells had a basal value for $[Ca^{2+}]_i$ of approx. 120 nM, consistent with earlier spectrofluorimetric determinations [5]. The $[Ca^{2+}]_i$ in single cells was, however, quantitatively

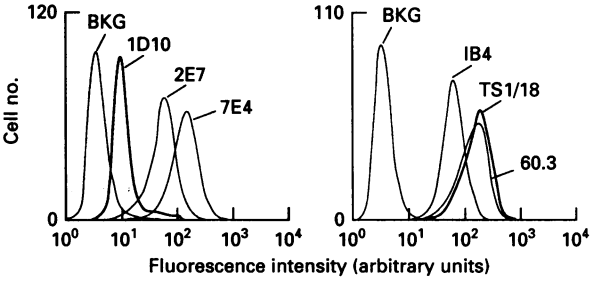

Fig. 1. Expression of CD18 on THP-1 cells

Suspensions of THP-1 cells (1×10^7 cells/ml) were incubated with 20% (v/v) human serum in complete RPMI 1640 medium for 30 min at 4 °C, washed, and mixed with saturating concentrations of the various anti-CD18 mAbs, or with the control mAb (6B4) for 30 min at 4 °C. After washes, binding of the various anti-CD18 mAbs was revealed by the addition of a 1:20 dilution of affinity-purified FITC-conjugated $F(ab')_2$ goat anti-(mouse IgG) antibody for an additional 30 min at 4 °C. Each histogram shows the reactivity of the indicated anti-CD18 mAb. Abbreviation: BKG, background fluorescence in the presence of the mAb 6B4.

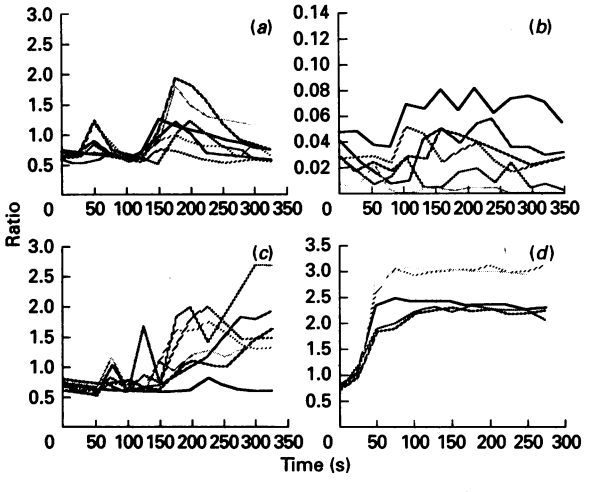

Fig. 2. Cross-linking of CD18 elevates cytosolic free $[Ca^{2+}]_i$ in THP-1 cells

Monolayers of adherent THP-1 cells were loaded intracellularly with 1 μM-Indo-1/AM for 45 min at 37 °C, washed, and analysed by real-time fluorescence measurements of cytosolic free $[Ca^{2+}]_i$ during continuous transectional laser scanning [5]. mAbs were diluted in Indo-1 loading buffer containing 2.5 mM-$CaCl_2$ and were added at a concentration of 25 μg/ml after the first scan of the experiment (25 s). $F(ab')_2$ goat anti-(mouse IgG)antibody (70 μg) was added as a cross-linking reagent after the fifth scan (125 s). CD18 cross-linking was carried out using mAb 60.3 (*a*), or TS1/18 (*c*). (*b*) CD14 cross-linking with mAb 63D3. (*d*) Ca^{2+} response elicited by addition of 7 μM-ionomycin after the first scan of the experiment. Each line represents the Ca^{2+} response of an individual cell. Results are expressed as changes in the ratio detector 1 (unbound Indo-1)/detector 2 (Indo-1–Ca^{2+} complex).

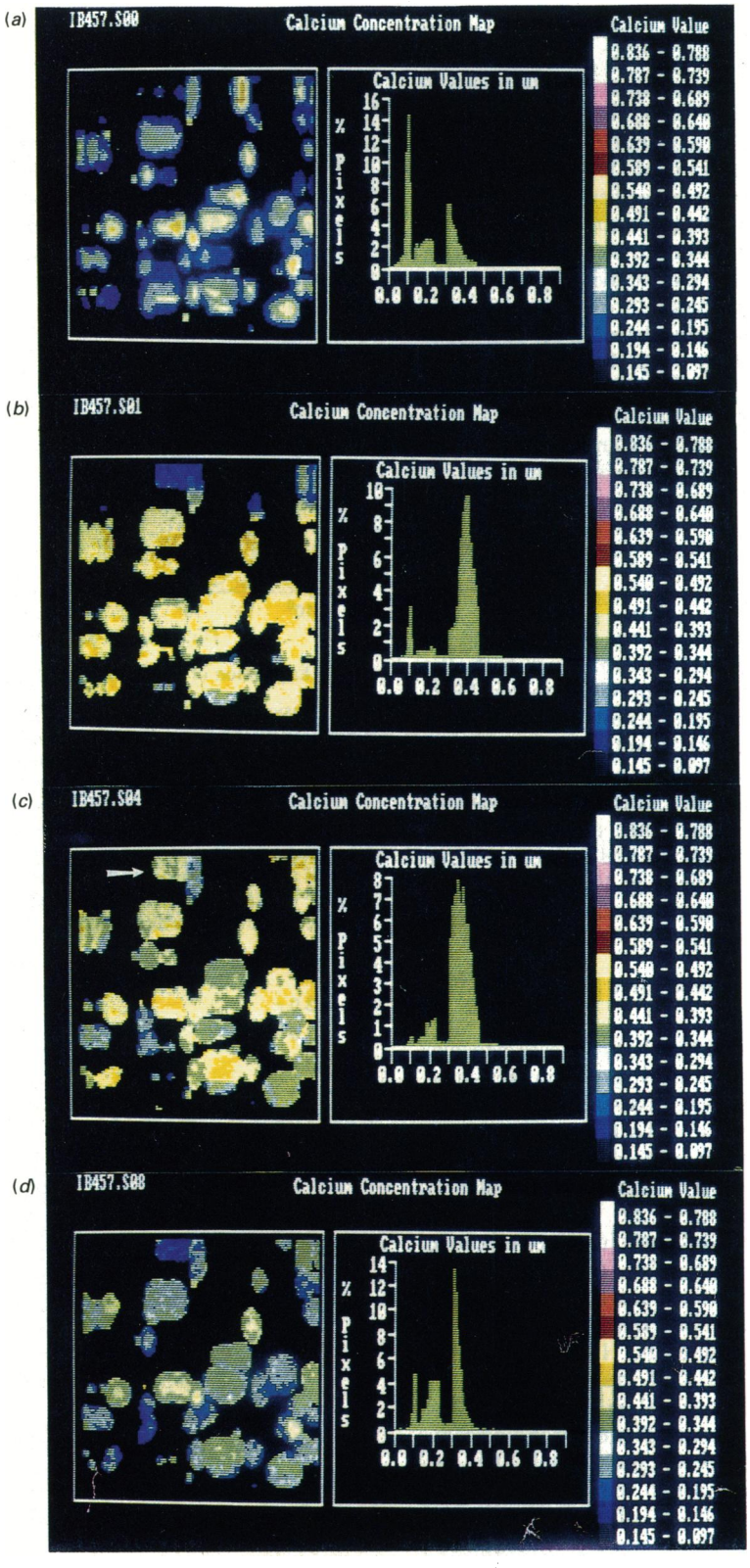

FIGURE 3

Fig. 3. Temporal analysis of CD18-mediated Ca^{2+} response in THP-1 cells

The experimental conditions for measurement of cytosolic free $[Ca^{2+}]_i$ are the same as in Fig. 2. (*a*) Basal level of $[Ca^{2+}]_i$ in adherent THP-1 cells measured after the first scan of the analysis (25 s). (*a*, right) Integration of fluorescence measurements from the analysed field pixels (%) and quantitation of $[Ca^{2+}]_i$ from a standard curve shown on the right. (*b*) Effects of $F(ab')_2$ fragments of anti-CD18 mAb, IB4, on cytosolic free $[Ca^{2+}]_i$ (50 s). (*c*) CD18 cross-linking with $F(ab')_2$ goat anti-(mouse IgG) prolongs the Ca^{2+} response in THP-1 cells (125 s). The arrow indicates a delayed Ca^{2+} response in a previously negative cell. (*d*) Reversibility of the CD18-mediated Ca^{2+} transient (225 s).

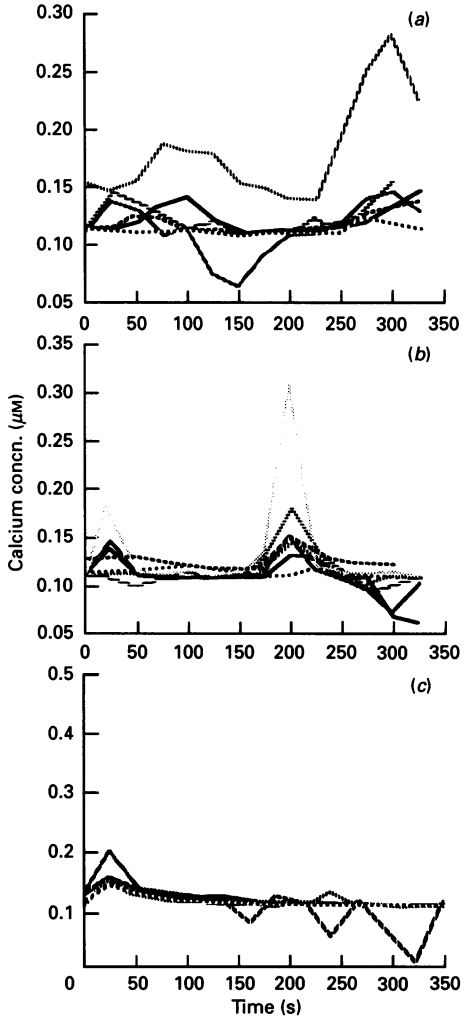

Fig. 4. CD18-mediated Ca^{2+} response in the absence of external Ca^{2+} ions

Adherent THP-1 cells were rapidly washed twice in a 3 mM-EGTA-containing buffer, pH 7.4, immediately before the experiment. Anti-CD18 mAbs, 60.3, (*a*), or TS1/18 (*b*), diluted at 25 μg/ml in the same EGTA-containing buffer were added as described in Fig. 2. $F(ab')_2$ goat anti-(mouse Ig) cross-linking reagent was added after 125 s (*c*) Addition of 7 μM-ionomycin in 3 mM-EGTA.

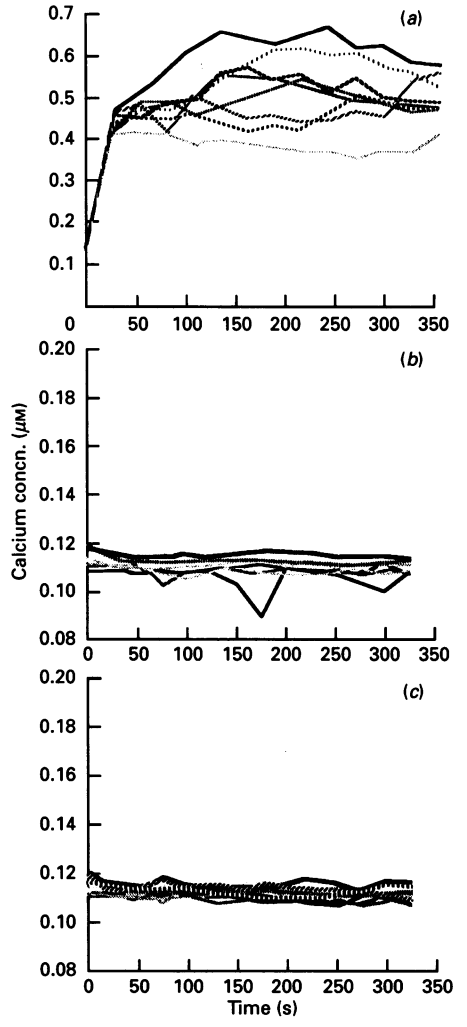

Fig. 5. Effect of cell differentiation on CD18-mediated Ca^{2+} response

Aliquots of THP-1 cells (1×10^6 cells/ml) were terminally differentiated after 1–2 days of culture in the presence of 114 nM-PMA. Changes in cytosolic free $[Ca^{2+}]_i$ in differentiated THP-1 cells were quantitated as described in Fig. 2. (*a*) Stimulation with 7 μM-ionomycin. Cross-linking of CD18 with mAb 60.3 was carried out after 24 (*b*) or 48 h (*c*) culture with PMA. Indistinguishable results were also obtained after CD18 cross-linking with mAbs IB4 and TS1/18.

heterogeneous with individual values reaching up to 300 nM in certain areas of selected cells (Fig. 3*a*, right). Upon addition of 25 μg of $F(ab')_2$ anti-CD18 mAb IB4/ml, the vast majority of THP-1 cells immediately responded with a co-ordinated 3–4-fold increase in cytosolic free $[Ca^{2+}]_i$ (Fig. 3*b*). Addition of the $F(ab')_2$ cross-linking reagent 100 s after addition of mAb IB4 prolonged the Ca^{2+} transient, eliciting a delayed Ca^{2+} response in some individual cells where cytosolic free $[Ca^{2+}]_i$ was not initially increased by mAb IB4 (Fig. 3*c*, arrow). At variance with the sustained Ca^{2+} response elicited by ionomycin under these experimental conditions (results not shown and Fig. 2*d*) the $[Ca^{2+}]_i$ of the majority of responding THP-1 cells initiated a return to baseline values 200 s after the addition of mAb IB4 (Fig. 3*d*).

In the absence of extracellular Ca^{2+} ions (3 mM-EGTA-containing buffer), cross-linking of CD18 with mAbs 60.3 or TS1/18 typically increased cytosolic free $[Ca^{2+}]_i$ in approx. 20–30% of adherent THP-1 cells (Figs. 4*a* and 4*b*). This Ca^{2+} transient was chronologically biphasic and quantitatively similar to the response observed in the presence of extracellular Ca^{2+} ions (Figs. 2*c* and 2*d*). Consistent with a complete removal of extracellular Ca^{2+} ions, 7 μM-ionomycin failed to increase cytosolic free $[Ca^{2+}]_i$ in THP-1 cells under these experimental conditions (Fig. 4*c*).

Regulation of CD18-mediated Ca^{2+} signalling

Suspensions of THP-1 cells were terminally differentiated *in vitro* to a mature monocyte/macrophage phenotype [16]. While these cells expressed abundant levels of CD11/CD18 (see below), and responded to ionomycin with an immediate and large increase in cytosolic free $[Ca^{2+}]_i$ (Fig. 5*a*), CD18 cross-linking with mAb 60.3 failed to elicit any Ca^{2+} response (Figs. 5*b* and 5*c*). Similarly, anti-CD18 mAbs IB4, 60.3, and 2E7, produced an heterogeneous Ca^{2+} response in only a minor subset of approx. 20–30% of freshly isolated peripheral blood monocytes (Fig. 6). This was generally composed of only the delayed $[Ca^{2+}]_i$ rise after the addition of the $F(ab')_2$ cross-linking reagent (Figs. 6*a* and 6*c*). However, occupancy of certain CD18 epitopes elicited an immediate and large increase in cytosolic free $[Ca^{2+}]_i$, which was rapidly reversible, and not further modulated by secondary-

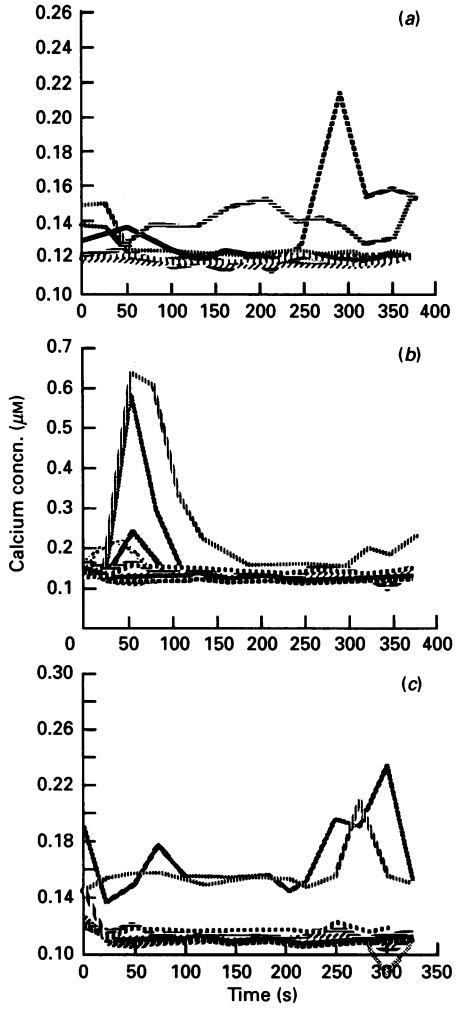

Fig. 6. CD18 cross-linking heterogeneously increases cytosolic free $[Ca^{2+}]_i$ in a subset of peripheral blood monocytes

Peripheral blood monocytes were purified from PBMC by adherence on to autologous serum-coated dishes, loaded intracellularly with 1 μM-Indo-1/AM, and analysed for variations in cytosolic free $[Ca^{2+}]_i$ after CD18 cross-linking. Anti-CD18 mAbs IB4 [$F(ab')_2$; (*a*) 15 scans], 60.3 (*b*; 15 scans), and 2E7 (*c*) were added at a concentration of 25 μg/ml after the first scan of the experiment (25 s). The $F(ab')_2$ goat anti-(mouse Ig) cross-linking reagent (70 μg) was added after the fifth scan of the experiment (125 s).

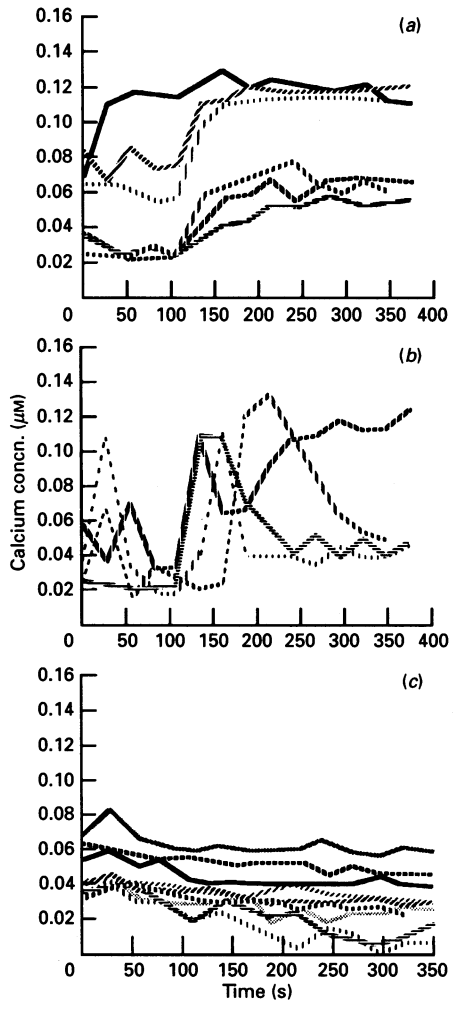

Fig. 7. CD18-mediated Ca^{2+} response in T cells

Resting peripheral blood T cells were prepared from PBMC by nylon-wool fractionation, loaded with 1 μM-Indo-1/AM, and analysed for variations in cytosolic free $[Ca^{2+}]_i$ as described in Fig. 2. (*a*) CD3 cross-linking with mAb OKT3 (25 μg/ml) (15 scans). (*b*) CD18 cross-linking with mAb 60.3 (15 scans). Shown is the typical Ca^{2+} response of four out of 18 cells analysed per field. (*c*) CD18 cross-linking with mAb 60.3 on alloreactive-stimulated T cells. Similar results were also obtained after activation of resting T cells by a 5-day-old culture with PHA or ConA.

receptor cross-linking (Fig. 6*b*). Monocyte pre-incubation with lipopolysaccharide (LPS) (1 μg of LPS/ml for 4 h at 37 °C) did not modify the CD18-mediated Ca^{2+} transient, thus excluding the possibility that the observed Ca^{2+} response was mediated by contaminating endotoxin (results not shown).

In freshly isolated T lymphocytes, CD3 cross-linking with mAb OKT3 homogeneously increased by 2–3-fold cytosolic free $[Ca^{2+}]_i$ in all cells examined (Fig. 7*a*), in agreement with previous observations [25]. Similarly, cross-linking of CD18 with mAb 60.3 also elicited a Ca^{2+} response in the T-cell population (Fig. 7*b*). Although typically observed in only one out of four/six cells analysed, the magnitude of this Ca^{2+} transient was quantitatively similar to that elicited after engagement of CD3 (Fig. 7). Similar results were also observed using anti-CD18 mAbs, 7E4 or TS1/18 (results not shown). However, T-cell mitogenic activation during culture *in vitro* with alloreactive antigen or lectins PHA and ConA, completely abrogated with CD18-mediated Ca^{2+} response of this functional subset (Fig. 7*c*, and results not shown).

The distribution and surface expression of CD11/CD18 during macrophage differentiation or T-cell activation were investigated by flow cytometry. CD11b/CD18 surface expression, as detected by mAb OKM1, was markedly increased during PMA-differentiation of THP-1 cells, or PHA-mediated T-cell activation, as compared with untreated control cultures (Fig. 8). This was also associated with a slight down-modulation of CD11a/CD18 (mAb TS1/22), and a significant increase in CD11c/CD18 surface expression (mAb 3.9) in activated T cells (Fig. 8).

To investigate the possibility that the specific assembly of CD18 with a complementary α subunit might affect its Ca^{2+}-signalling properties, two series of experiments were carried out. First, three specific mAbs directed to CD11a, CD11b, and CD11c were initially tested for their ability to induce a Ca^{2+} response in adherent THP-1 cells. Although all these mAbs reacted strongly with THP-1 cells by flow cytometry (Fig. 8), only the anti-CD11b mAb, OKM1, elicited an homogeneous Ca^{2+} response in all cells examined (Fig. 9*a*). While a similar Ca^{2+}

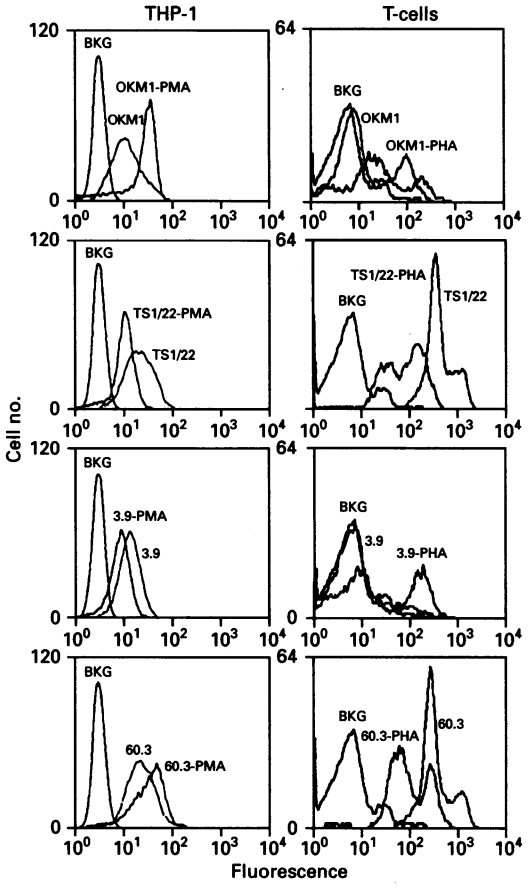

Fig. 8. Surface expression of CD11/CD18 integrins during differentiation/activation

Suspensions of non-induced, or PMA-induced terminally differentiated THP-1 cells, or resting or PHA-activated T cells were analysed by flow cytometry with the indicated anti-CD11/CD18 mAbs as described in Fig. 1. Anti-CD11b mAb was OKM1, anti-CD11a mAb TS1/22, anti-CD11c mAb 3.9, anti-CD18 mAb 60.3. Abbreviation: BKG, background fluorescence in the presence of the mAb 6B4.

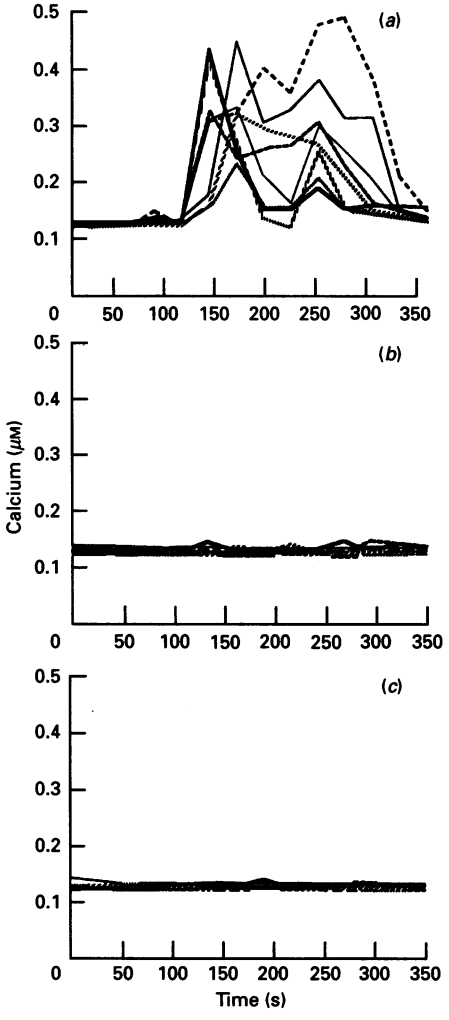

Fig. 9. Effect of anti-α-subunit-specific mAbs on cytosolic free $[Ca^{2+}]_i$ in THP-1 cells

The experimental procedures for mAb cross-linking are the same as in Fig. 2. Anti-CD11b mAb was OKM1 (*a*), anti-CD11a mAb was TS1/22 (*b*), anti-CD11c mAb was 3.9 (*c*). Indistinguishable results were obtained after CD11b cross-linking with mAb OKM1 (*a*) or 60.1.

response was obtained with anti-CD11b mAb 60.1 (results not shown), CD11a or CD11c cross-linking with mAbs TS1/22 or 3.9 did not increase cytosolic free $[Ca^{2+}]_i$ in adherent THP-1 cells (Figs. 9*b* and 9*c*), or resting T cells (results not shown). Secondly, CD18 cross-linking with $F(ab')_2$ fragments of mAb IB4 increased cytosolic free $[Ca^{2+}]_i$ in a long-term cultured T-cell line expressing high levels of CD11b/CD18, by flow cytometry (Fig. 10*a*). In contrast, cross-linking of CD18 with mAb IB4 failed to produce any Ca^{2+} response in the T-cell line MOLT13, which completely lacked CD11b/CD18 surface expression (Fig. 10*b*). In control experiments, mAb IB4 reacted with 63.4% of MOLT13 cells by flow cytometry (results not shown).

Regulation of CD11b/CD18 ligand recognition

Previous studies have shown that the oligospecific ligand recognition of CD11b/CD18 is regulated by an initial agonist-induced increase in cytosolic free $[Ca^{2+}]_i$ in myelo-monocytic cells [23]. Additional experiments analysed the effect of the mAb-mediated Ca^{2+} response described above on the inducible recognition of CD11b/CD18 for fibrinogen [23]. For these studies, THP-1 cells were separately equilibrated with saturating doses of anti-CD11b mAbs 60.1 or M1/70 in the presence of 2.5 mM-$CaCl_2$. As shown in Table 1, exposure of THP-1 cells to the non-blocking mAb 60.1 stimulated the specific association of ^{125}I-fibrinogen with CD11b/CD18 to an even greater extent than control cells stimulated with a previously described Ca^{2+} mobilizing agent [5] such as ADP (Table 1). In contrast, the functionally inhibitory anti-CD11b mAb M1/70 [23] prevented any specific association of fibrinogen with CD11b/CD18, under the same experimental conditions (Table 1).

DISCUSSION

In this study we have shown that mAb cross-linking of CD18 integrin transduces a stimulatory Ca^{2+} signal in selected leukocyte subpopulations. Dissected in real-time fluorescence measurements in single adherent cells, and initially characterized in clonally homogeneous monocytic THP-1 cells, occupancy of CD18 elevates cytosolic free $[Ca^{2+}]_i$ in a temporally biphasic reaction, involving both release of Ca^{2+} from intracellular stores and an active Ca^{2+} influx from the extracellular compartment. Although receptor cross-linking prolongs the duration of the Ca^{2+} transient, and induces delayed responses in individual cells, addition of the primary anti-CD18 mAb is sufficient per se to elevate cytosolic free $[Ca^{2+}]_i$ in most THP-1 cells. This response

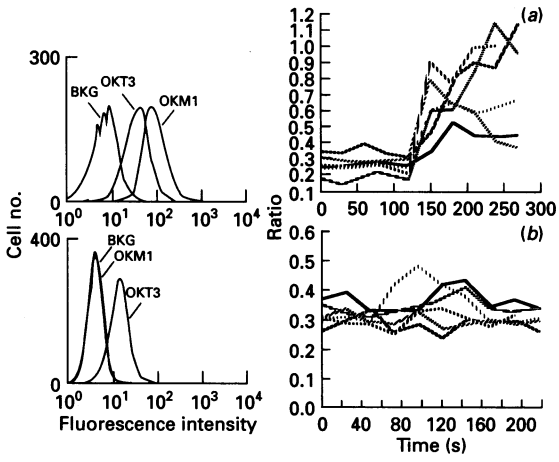

Fig. 10. Requirement of CD11b for CD18-mediated Ca^{2+} response in T cells

The experimental conditions for measurement of cytsolic free $[Ca^{2+}]_i$ in adherent T cells are the same as in Fig. 2. CD18 cross-linking with $F(ab')_2$ fragments of mAb IB4 was carried out on a T-cell line expressing high levels of CD11b [(*a*); 13 scans], or on MOLT13 T cells [(*b*); 10 scans]. Surface expression of CD11b on both cell types was quantitatively assessed by flow cytometry using mAb OKM1 (*a* and *b* left). Controls for flow cytometry experiments were anti-CD3 mAb OKT3, and the mAb 6B4. (BKG, background fluorescence).

Table 1. Effect of anti-CD11b mAbs on ^{125}I-labelled fibrinogen binding to monocyte THP-1 cells

Serum-free suspensions of THP-1 cells (2×10^7 cells/ml) were incubated with either aliquots of 25 μg of anti-CD11b mAb 60.1/ml, or 4-fold diluted culture supernatant of the anti-CD11b mAb, M1/70, in the presence of 2.5 mM-$CaCl_2$ for 5 min at room temperature. At the end of the incubation, cells were immediately mixed with increasing concentrations of ^{125}I-fibrinogen (0.017–0.14 μM) for 20 min at room temperature before separation of free radiolabel from cell-associated radioactivity by centrifugation through a mixture of silicone oils at 12000 *g* for 5 min. Non-specific binding calculated in the presence of a 50-fold molar excess of unlabelled fibrinogen was subtracted from the total to calculate the net specific binding. Control cells incubated in the absence of anti-CD11b mAbs (control), were stimulated with 10 μM-ADP in the presence of 2.5 mM-$CaCl_2$ and mixed with increasing concentrations of ^{125}I-fibrinogen as described in [5].

^{125}I-fibrinogen added (μM)	^{125}I-fibrinogen bound (molecules/cell)		
	Control	60.1	M 1/70
0.017	1680±250	3980±1590	210±240
0.035	2920±680	8100±2400	620±400
0.073	5234±647	14160±3500	1080±700
0.14	11313±1770	34080±11900	2200±1300

was specifically recapitulated by $F(ab')_2$ fragments of anti-CD18 mAb IB4, while mAbs to other high-density surface molecules on THP-1 cells (i.e. CD11a, CD11c) were ineffective. It is unlikely that the process of cell adherence used in this study contributes to the observed Ca^{2+} signal. First, indistinguishable Ca^{2+} responses were obtained regardless of the substrate used, i.e. Cell-Tak, fibronectin, autologous serum, or no substrate at all. Furthermore, tightly adherent THP-1 cells after PMA differentiation, failed to increase cytosolic free $[Ca^{2+}]_i$ upon CD18 engagement, while fully responding to ionomycin stimulation.

The Ca^{2+} response elicited through engagement of CD18 is a highly dynamic mechanism, critically regulated by the state of cell differentiation/activation, and by the structural assembly of CD18 with the unique α subunit, CD11b. Despite the high surface expression of the three CD11/CD18 molecules, occupancy of CD18 completely failed to increase cytosolic free $[Ca^{2+}]_i$ in terminally differentiated THP-1 cells, and produced an heterogeneous Ca^{2+} response in only a minor subset of approx. 20–30% of peripheral blood monocytes. Similarly, T-cell mitogenic activation during short-term culture with alloreactive antigen or polyclonal lectins, abolished the CD18-mediated Ca^{2+} response observed in a small subset of 15–20% of freshly isolated resting T lymphocytes. These responder cells preferentially expressed CD11b/CD18 [26], and similarly, mAb cross-linking of the α subunit, CD11b, produced a large increase in cytosolic free $[Ca^{2+}]_i$ in THP-1 cells, while occupancy of CD11a or CD11c was without effect.

Our studies may help reconcile previous work on the possible role of leukocyte integrins in the early events of transmembrane signalling. While there is general consensus that these molecules participate in mechanisms of T- and B-cell activation and proliferation [10–13], the role of intracellular Ca^{2+} release has been controversially debated. The results presented here at the single-cell level dramatically emphasize the profound heterogeneity of this process. The CD18-mediated Ca^{2+} response of a minor T-cell subset may have escaped detection in previous studies that analysed changes in a whole-cell population [14]. Similarly, the down-modulation of the CD18-mediated Ca^{2+} response in activated T cells is consistent with the inability of long-term cultured T-cell clones to transduce Ca^{2+} signals through CD18 in the absence of other activating stimuli, i.e. CD3 cross-linking [13]. Our results are also in agreement with earlier findings of Ledbetter *et al.* [27], where CD18 cross-linking increased cytosolic free $[Ca^{2+}]_i$ in only a minor subset of PBMC, and with the more recent studies of Ng-Sikorski *et al.* on CD11b/CD18 cross-linking in suspensions of polymorphonuclear leukocytes [28].

Transients in cytosolic free $[Ca^{2+}]_i$ as co-ordinated by leukocyte adherence receptors play a crucial signalling role in physiological mechanisms of host defence during immune/inflammatory reactions. Chemotactic migration of polymorphonuclear leukocytes, and phagocytosis of opsonized particles are associated with polarized increases in cytsolic free $[Ca^{2+}]_i$ in these cells [29]. This is particularly relevant to the studies presented here, in view of the prominent role of CD11b/CD18, in mechanisms of both leukocyte migration and phagocytosis, specifically established through its receptor recognition of the complement fragment iC3b [2,3].

Similarly, the proposed role of CD18 as a lymphocyte activation molecule [13] may also have its biochemical prerequisite in these regulated Ca^{2+}-signalling properties. It is conceivable that on both cells of monocyte and lymphoid lineage, the CD18-mediated Ca^{2+} signals regulate functionally distinct pathways of ligand recognition modulated by the state of cell activation/differentiation. The Ca^{2+} response elicited through monocyte or polymorphonuclear leukocyte CD11b/CD18 may regulate binding of soluble ligands iC3b or fibrinogen to these cells, co-ordinating cellular motility, phagocytosis [29], or adherence to matrix substrate (this study). On lymphoid cells, the nature of integrin-mediated Ca^{2+} signals may reflect the unique CD11a/CD18 recognition of cell-associated counter-receptors, i.e. intercellular adhesion molecules [2,3]. In this context, the lack of Ca^{2+} signalling through CD18 in actively proliferating T cells might function as an homoeostatic regulatory mechanism removing an accessory mitogenic signal [10,12], or alternatively, as a switch to a different inhibitory signalling pathway, as has been

demonstrated for another β integrin [30], and postulated for CD18 itself [31].

The ability of CD11b/CD18 and CD11a/CD18 simultaneously to recognize multiple and unrelated ligands (i.e. oligospecificity) is regulated by a transient activation state, typically recapitulated by agonists, or cell–cell interactions that elevate cytosolic free $[Ca^{2+}]_i$ in target cells [4,23]. In this context, occupancy of the CD11b epitope defined by the functionally non-blocking mAb, 60.1 [23,32], was found to stimulate directly the specific adhesive properties of CD11b/CD18, as reflected in its inducible and high-affinity recognition of fibrinogen [23]. Given the role of CD18 in intracellular signalling initiated by cell adherence [33], it is conceivable that cross-linking of CD11b/CD18 during leukocyte adhesion to immobilized fibrinogen/fibrin substrates might participate in regulating receptor–ligand binding, through its intrinsic Ca^{2+}-signalling properties.

In summary, these studies propose an unprecedented role for leukocyte CD11/CD18 integrins as Ca^{2+}-signalling molecules, dynamically linked to the state of cell differentiation/activation, and participating in a fine self-regulation of ligand-binding specificity.

The authors wish to thank Dr. K. Gelsen for the generous gift of $F(ab')_2$ fragments of anti-CD18 mAb IB4, and Dr. R. Ulevitch for anti-CD14 mAbs, 63D3 and 3C10. This work was supported by the National Institutes of Health grant HL-43773. This is publication no. 6965-IMM from the Department of Immunology, The Scripps Research Institute.

REFERENCES

1. Ruoslahti, E. (1991) J. Clin. Invest. **87**, 1–5
2. Arnaout, M. A. (1990) Blood **75**, 1037–1050
3. Springer, T. A. (1990) Nature (London) **346**, 425–434
4. Dustin, M. L. & Springer, T. A. (1989) Nature (London) **341**, 619–624
5. Altieri, D. C., Wiltse, W. L. & Edgington, T. S. (1990) J. Immunol. **145**, 662–670
6. Schleiffenbaum, B., Moser, R., Patarroyo, M. & Fehr, J. (1989) J. Immunol. **142**, 3537–3545
7. Barber, E. K., Dasgupta, J. D., Schlossman, S. F., Trevillyan, J. M. & Rudd, C. E. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3277–3281
8. Nathan, C., Srimal, S., Farber, C., Sanchez, E., Kabbash, L., Asch, A., Gailit, J. & Wright, S. D. (1989) J. Cell Biol. **109**, 1342–1349
9. Mishra, G. C., Berton, M. T., Oliver, K. G., Krammer, P. H., Uhr, J. W. & Vitetta, K. S. (1986) J. Immunol. **137**, 1590–1598
10. Carrera, A. C., Rincón, M., Sánchez-Madrid, F., López-Botet, M. & de Landaźuri, M. O. (1988) J. Immunol. **141**, 1919–1924
11. Cerdan, C., Lipcey, C., Lopez, M., Nunes, J., Pierres, A., Mawas, C. & Olive, D. (1989) Cell. Immunol. **123**, 344–353
12. van Seventer, G. A., Shimizu, Y., Horgan, K. J. & Shaw, S. (1990) J. Immunol. **144**, 4579–4586
13. Wacholtz, M. C., Patel, S. S. & Lipsky, P. E. (1989) J. Exp. Med. **170**, 431–448
14. Pardi, R., Bender, J. R., Dettori, C., Giannazza, E. & Engleman, E. G. (1989) J. Immunol. **143**, 3157–3166
15. Keizer, G. D., Visser, W., Vliem, M. & Figdor, C. G. (1988) J. Immunol. **140**, 1393–1400
16. Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T. & Tada, K. (1982) Cancer Res. **42**, 1530–1536
17. Wright, S. D., Rao, P. E., Van Voorhis, W. C., Craigmyle, L. S., Iida, K., Talle, M. A., Westberg, E. F., Goldstein, G. & Silverstein, S. C. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 5699–5703
18. Geppert, T. D. & Lipsky, P. E. (1988) J. Clin. Invest. **81**, 1497–1505
19. Wallis, W. J., Hickstein, D. D., Schwartz, B. R., June, C. H., Ochs, H. D., Beatty, P. G., Klebanoff, S. J. & Harlan, J. M. (1986) Blood **67**, 1007–1013
20. Sanchez-Madrid, F., Nagy, J. A., Robbins, E., Simon, P. & Springer, T. A. (1983) J. Exp. Med. **158**, 1785–1803
21. Nortamo, P., Patarroyo, M., Kantor, C., Suopanki, J. & Gahmberg, C. G. (1988) Scand. J. Immunol. **28**, 537–546
22. Malhotra, V., Hogg, N. & Sim, R. B. (1986) Eur. J. Immunol. **16**, 1117–1123
23. Altieri, D. C., Bader, R., Mannucci, P. M. & Edgington, T. S. (1988) J. Cell. Biol. **107**, 1893–1900
24. Lund-Johansen, F., Olweus, J., Aarli, A. & Bjerknes, R. (1990) FEBS Lett. **273**, 55–58
25. Gardner, P. (1989) Cell (Cambridge, Mass.) **59**, 15–20
26. Dianzani, Y., Zarcone, D., Pistoia, V., Grossi, C. E., Pileri, A., Massaia, M. & Ferrarini, M. (1989) Eur. J. Immunol. **19**, 1037–1044
27. Ledbetter, J. A., June, C. H., Grosmaire, L. S. & Rabinovitch, P. S. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1384–1388
28. Ng-Sikorski, J., Andersson, R., Patarroyo, M. & Andersson, T. (1991) Exp. Cell Res. **195**, 504–508
29. Sawyer, D. W., Sullivan, J. A. & Mandell, G. L. (1985) Science **230**, 663–666
30. Groux, H., Huet, S., Valentin, H., Pham, D. & Bernard, A. (1989) Nature (London) **339**, 152–154
31. van Noesel, C., Miedema, F., Brouwer, M., de Rie, M. A., Aarden, L. A. & van Lier, R. A. W. (1988) Nature (London) **333**, 850–852
32. Altieri, D. C. & Edgington, T. S. (1988) J. Biol. Chem. **263**, 7007–7015
33. Jaconi, M. E. E., Theler, J. M., Schlegel, W., Appel, R. D., Wright, S. D. & Lew, P. D. (1991) J. Cell Biol. **112**, 1249–1257

Received 16 April 1992/25 June 1992; accepted 3 July 1992