Selective Activation of $\gamma/\delta^+$ T Cell Clones by Single Anti-CD2 Antibodies

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Summary

The CD2 antigen is the target for an "alternative" T cell activation pathway. Numerous studies have demonstrated that pairs of monoclonal antibodies (mAbs) directed toward two different epitopes are required for activation of T cell receptor (TCR)-$\alpha/\beta^+$ T cells via CD2. We have now explored the activation of human TCR-$\gamma/\delta^+$ T cell clones by a panel of anti-CD2 mAbs directed against the sheep erythrocyte-binding (T11.1) epitope of CD2. Seven of seven $\gamma/\delta^+$ clones expressing different molecular forms of the TCR-$\gamma/\delta$ responded to stimulation by a single anti-CD2 mAb (OKT11, 9E8, BW0110, M-T910) with IL-2 secretion and/or proliferation. Immobilization of anti-CD2 mAbs in microculture plates was essential for activation of $\gamma/\delta^+$ clones, which occurred in the absence of feeder cells. In addition to interleukin 2 (IL-2) production and proliferation, anti-CD2 mAbs also triggered cytotoxic effector activity in $\gamma/\delta^+$ clones as measured against FcR$^+$ P815 target cells. In contrast to $\gamma/\delta^+$ clones (but in line with established data), none of five CD4$^+$ or CD8$^+$ TCR-$\alpha/\beta^+$ clones were activated by any of the tested individual anti-CD2 mAbs. Taken together, our results reveal a striking difference between cloned $\gamma/\delta^+$ and $\alpha/\beta^+$ T cells in that $\gamma/\delta^+$ T cells are selectively activated by a single anti-CD2 (T11.1) mAb, without need for the simultaneous signal of a second anti-CD2 mAb directed against another (T11.2 or T11.3) CD2 epitope.

$T$ cells are activated by the antigen-specific TCR/CD3 molecular complex following interaction with foreign antigenic peptides in association with self MHC proteins. In addition, T cells can be activated via an "alternative" pathway through the CD2 cell surface antigen (1). CD2 is a 50-55-kD single-chain glycoprotein expressed on the vast majority of peripheral blood T cells and thymocytes (2, 3). Three epitopes have been defined on the CD2 molecule, of which T11.1 and T11.2 are expressed on resting T cells, while T11.3 (now designated CD2R) is a conformational epitope that only appears after activation (1). Soluble mAbs directed against T11.1, the sheep erythrocyte-binding epitope of CD2, inhibit certain T cell functions such as IL-2 production, IL-2-dependent proliferation, and cytotoxic effector activity (4–8). Pairs of mAbs directed against the T11.2 and T11.3 epitopes in combination activate resting T cells (1, 9, 10). One of the two anti-CD2 mAbs required for T cell activation may be replaced by the natural ligand for CD2, LFA-3, by sheep erythrocytes, or by PMA (11-13). Moreover, cytolytic activity can be triggered in CD2$^+$ CD3$^-$ TCR$^-$ NK cells by a mitogenic combination of two anti-CD2 mAbs, or by a single anti-CD2 mAb (5, 8, 14). In the latter case, however, CD2 must be cross-linked with CD16, the low affinity Fcγ receptor (14). Thus, it is obvious that activation of TCR-$\alpha/\beta^+$ T cells and TCR$^-$ NK cells via CD2 requires two signals.

A minor fraction of peripheral blood T cells (1–10%) express a TCR-$\gamma/\delta$ heterodimer noncovalently associated with the CD3 complex (15). The biological significance, including the antigen specificity of $\gamma/\delta^+$ T cells, is still a matter of debate (15, 16). A striking feature of $\gamma/\delta^+$ T cells is their frequent reactivity towards mycobacterial antigens (17–20). Activation requirements of $\gamma/\delta^+$ T cells have not been thoroughly investigated so far. Established $\gamma/\delta^+$ clones can be stimulated to secrete IL-2 and other cytokines (21), but freshly isolated $\gamma/\delta^+$ T cells produce less IL-2 upon PMA stimulation than do $\alpha/\beta^+$ T cells (22).

In the present study we have analyzed the "alternative" CD2 activation pathway in cloned $\gamma/\delta^+$ T cells. We found that single anti-CD2 (T11.1) mAbs strongly triggered the activation of seven of seven TCR-$\gamma/\delta^+$ clones, while none out of five TCR-$\alpha/\beta^+$ clones were stimulated under these conditions. These results reveal a striking difference between cloned $\gamma/\delta^+$ and $\alpha/\beta^+$ T cells regarding the CD2-dependent activation pathway.

Materials and Methods

Establishment of IL2-dependent $\gamma/\delta^+$ and $\alpha/\beta^+$ T Cell Clones. PBMC from normal donors were isolated by Ficoll-Hypaque density gradient centrifugation. After depletion of plastic-adherent cells,
PBMC were separated into T and non-T cells by the E rosette procedure using neuraminidase-treated sheep E. γ/δ+ T cells were enriched from E-rosetting T cells as described (22). Briefly, T cells were incubated with supernatants of OKT4 (anti-CD4) and OKT8 (anti-CD8) hybridomas (both from American Type Culture Collection, Rockville, MD). After being washed, the cells were incubated with nontoxic rabbit complement (Cedarlane Laboratories, Hornby, Ontario) for 1 h at 37°C. Dead cells were removed by Ficoll-Hypaque density gradient centrifugation. Viable cells were cultured at 0.3 cells per well in 96-well round-bottomed culture plates (Nunc, Roskilde, Denmark) in the presence of 10⁵ irradiated PBMC feeder cells and 0.5 μg/ml PHA-P (Wellcome, Burgwedel, FRG). The culture medium was RPMI 1640 (Biochrom KG, Berlin, FRG) supplemented with 10% heat-inactivated FCS (batch 4MO2; Biochrom KG), antibiotics, 2 mM L-glutamine, and 10 mM Hepes. After 2 d, rIL2 (EuroCetus, Amsterdam, The Netherlands) was added at 2 ng/ml. Growing clones were expanded in 24-well culture plates (Greiner, Unterschleissheim, FRG). mAbs used in this study are listed in Table 1. mAb 9E8 was obtained in our laboratory from a fusion of spleen cells from a female Balb/c mouse immunized with a CD2+γ/δ+ human T cell clone. 9E8 blocks rosette formation between human T cells and sheep E, and identifies by Western blot analysis a single band of ~50 kD mol mass on resting T cells and established T cell clones (our unpublished results). Based on these criteria, mAb 9E8 is classified as anti-CD2. Like 9E8, all other anti-CD2 mAbs listed in Table 1 strongly react with resting T cells and block E rosette formation. Therefore, they are classified as anti-CD2 (anti-T11.1) and not anti-CD2R mAbs (27). Hybridoma cells producing OKT11 mAb (IgG1) were obtained from ATCC. IOT11 and IOT11a were from Dionova (Hamburg, FRG). mAb M-T910 (28) was kindly donated by Dr. E.F. Rieber (University of Munich, FRG), and mAbs BW0110, BMA030 (anti-CD3), and BMA031 were generously provided by Drs. K.H. Enssle and R. Kurrle (Behringwerke, Marburg, FRG). mAbs OKT11 (ATCC), 9E8, OKT4 (ATCC), and 7A5 were purified from hybridoma culture supernatants on protein G columns (Pharmacia, Uppsala, Sweden).

Table 1. mAbs Used in this Study

| Antibody | Specificity | Ig subclass | Source (reference) |
|----------|-------------|-------------|--------------------|
| OKT3     | CD3         | IgG2a       | Cilag (Sulzbach, FRG) |
| BMA030   | CD3         | IgG2a       | Dr. Kurrle (Behringwerke) |
| BMA031   | TCR-α/β     | IgG2b       | Dr. Kurrle (42) |
| TCRδ-1   | TCR Cδ     | IgG1        | T Cell Sciences (43, 44) |
| 7A5      | TCR Vγ9    | IgG1        | This laboratory |
| TγA      | TCR Vγ9    | IgG2a       | Dr. Hercend/Dr. Triebel (25) |
| TiVδ2    | TCR Vδ2    | IgG1        | Dr. Hercend (26) |
| BB3      | TCR Vδ2    | IgG1        | Dr. L. Moretta (24, 45) |
| A13      | TCR Vδ1    | IgG1        | Dr. L. Moretta (23, 26) |
| δTCS-1   | TCR Vδ1-J61, Vδ1-J62 | IgG1 | T Cell Sciences (45, 46) |
| OKT11    | CD2         | IgG1        | ATCC                |
| 9E8      | CD2         | IgG1        | This laboratory     |
| BW0110   | CD2         | IgG2b       | Dr. Enssle (Behringwerke) |
| M-T910   | CD2         | IgG1        | Dr. Rieber (28)     |
| IOT11    | CD2         | IgG1        | Dionova (Hamburg, FRG) |
| IOT11a   | CD2         | IgG2a (rat) | Dionova             |
| OKT4     | CD2         | IgG2b       | ATCC                |
proliferation and IL-2 production were measured as parameters of cellular activation. To immobilize mAbs in culture plates, wells of 96-well, round-bottomed culture plates (Nunc) were coated for 18 h with 1 μg purified mAb in PBS per well. The plates were washed twice with PBS, and clone cells were added at 3-5 × 10^4 cells per well in a total volume of 200 μl in RPMI 1640/10% FCS without exogenous IL-2. Alternatively, T cell clones were incubated for 30 min on ice with the respective mAb, washed twice, and added at 3-5 × 10^4 cells per well to U-bottomed microculture plates previously coated with 1 μg per well of goat anti-mouse IgG (Tago). All cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 18-24 h, 75 μl of supernatant was removed from each well and transferred to a 96-well flat-bottomed microculture plate for determination of IL-2 content. After an additional 12-24 h, 1 μCi [³H]TdR (specific activity 6.7 Ci/mmol) per well was added to the remaining cells, and incubation was continued for another 6 h. Afterwards, the cultures were harvested and prepared for counting of β emission in a Packard liquid scintillation counter. Results are expressed as mean cpm of triplicate cultures. SD were always <15%.

**Determination of IL-2 Production.** IL-2 produced by mAb-stimulated T cell clones was detected in a colorimetric assay on IL-2-dependent murine CTLL cells as described (29). Briefly, 15,000 CTLL cells per well were added to flat-bottomed culture plates containing supernatants of T cell clones. After incubation for 24 h at 37°C/5% CO₂, the tetrazolium salt MTT (Sigma, Deisenhofen, FRG) was added at 250 μg/ml, and incubation was continued for another 4 h. Formazan crystals were solubilized in 10% SDS/1% HCl, and OD at 570 nm were read on an ELISA reader with the reference wave length set at 690 nm. This assay is highly specific for IL-2; the CTLL clone used does not respond to human rIL1, rIL3, or rIL4 (29).

**Cytotoxicity Assay.** FcR⁺ murine P815 mastocytoma cells were used in a standard 4-h [⁵¹Cr]-release assay to evaluate the cytotoxic effector activity of cloned T cells in the absence or presence of different mAbs. Percentage specific lysis was calculated as follows: Percent specific lysis = 100 × ([cpmExperimental - cpmSpontaneous]/(cpmMaximal - cpmSpontaneous)).

**Results**

**Immobilized Single Anti-CD2 mAbs Stimulates Proliferation of γ/δ⁺ Clones.** We have investigated possible stimulatory effects of anti-CD2 mAbs on cloned γ/δ⁺ T cells. To this end, clone cells were added to mAb-coated culture plates, and [³H]TdR incorporation was determined after 36 h of culture. As shown in Fig. 1, γ/δ⁺ clones strongly proliferated in response to individual immobilized anti-CD2 mAbs. All stimulatory anti-CD2 mAbs depicted in Fig. 1 were directed against the T11.1 epitope. It should be stressed, however, that stimulation of γ/δ⁺ T cell clones could not be achieved with every anti-CD2 mAb. Among the anti-CD2 mAbs tested (see Table 1), OKT11 (ATCC), 9E8, MT910, and BW0110 were stimulatory, whereas IOT11 and IOT11a were not (not shown). There was no obvious correlation between the stimulatory capacity of a given anti-CD2 mAb and its IgG subclass. Thus, stimulatory anti-CD2 mAbs were either IgG1 (OKT11 [ATCC], 9E8, MT910) or IgG2b (BW0110), and nonstimulatory mAbs were either IgG1 (IOT11) or IgG2a (IOT11a). Note that γ/δ⁺ clones expressing different molecular forms of the TCR-γ/δ as deduced by reactivity with anti-γ and anti-δ mAbs (see Table 2 for phenotypic analysis) were all activated by stimulatory anti-CD2 mAbs. In contrast, activation by immobilized anti-γ9 mAb 7A5 was restricted to γ9-expressing clones (D768/6, A92DN3, D768/4, B54; see Fig. 1). As a control, immobilized anti-CD4 mAb OKT4 did not stimulate CD4⁺ γ/δ⁺ clones.

A second protocol was equally efficient in eliciting CD2-dependent activation of γ/δ⁺ clones. Here, clone cells were first incubated with anti-CD2 mAb, then washed, and cul-

![Figure 1](image_url)
tured in microculture plates previously coated or not with goat anti-mouse Ig. Under these conditions, Vγ9-expressing D768/6 clone cells strongly proliferated (Fig. 2). No proliferative response was observed, however, when anti-CD2-labeled γ/δ+ clones were cultured in microculture plates not coated with anti-mouse Ig, indicating that cross-linking of cell surface CD2 molecules was essential to trigger proliferation (Fig. 2).

The stimulatory effect of individual immobilized anti-CD2 mAbs was strikingly specific for γ/δ+ cells. As illustrated in Fig. 3, none of five well-characterized (CD4+ or CD8+) TCRγ/δ+ clones proliferated in response to immobilized single anti-CD2 mAbs OKT11 (ATCC) or 9E8, while all of them were activated by plastic-bound anti-CD3 (OKT3) or anti-TCR-α/β (BMA031) mAbs.

**Immobilized Single Anti-CD2 mAbs Stimulate IL2 Production in γ/δ+ Clones.** The above data showed that triggering by a single immobilized or cross-linked anti-CD2 mAb induced autocrine proliferation of γ/δ+ but not α/β+ T cell clones. To investigate whether this proliferative response was IL2 dependent, we measured IL2 in cell-free culture supernatants of anti-CD2-stimulated γ/δ+ clones. As shown in Fig. 4, IL2 production was induced in all four analyzed γ/δ+ clones by at least two of the tested anti-CD2 mAbs. However, not all anti-CD2 mAbs that stimulated proliferation of a given γ/δ+ clone (Fig. 1) also triggered measurable IL2 production. Of interest, certain anti-CD2 mAbs were effective on some but not other γ/δ+ clones. It is thus evident that the anti-CD2–triggered autocrine proliferation of γ/δ+ clones is not necessarily dependent on the secretion of detectable amounts of IL2 into the culture medium.

**A Single Anti-CD2 mAb Triggers Cytotoxic Ejector Function in γ/δ+ but not α/β+ Clones.** Next we asked whether anti-CD2 mAbs could also trigger cytotoxic effector function in cloned γ/δ+ T cells. Lysis of FeR+ murine P815 target cells is a well-established system to monitor anti-CD3/TCR mAb-triggered activation of CTL. As shown in Fig. 5A, γ/δ+ clone D768/3 expressing a Vγ9/Vδ2 TCR did not kill P815 target cells in the absence of added antibody. Anti-CD3 (OKT3, BMA030), anti-TCR Vγ9 (7A5), and anti-CD3 (OKT11 [ATCC]) mAbs all triggered cytoytic activity, whereas anti-TCR-α/β mAb BMA031 was without effect. A second γ/δ+ clone expressing a different TCR phenotype (Vγ9/Vδ1) displayed some spontaneous cytotoxic activity against P815 target cells (Fig. 5B). Again, the cytotoxic activity was increased by anti-CD2 (OKT11

![Figure 2: Cross-linking is required for anti-CD2-mediated stimulation of γ/δ+ clone cells. Aliquots of γ/δ+ clone D768/6 were incubated for 20 min on ice with the indicated mAbs. After being washed twice, clone cells (5 × 10^6 per well) were cultured in triplicate in microculture plates that had been previously coated (□) or not (■) with goat anti-mouse IgG. [3H]Tdr incorporation was measured after 36 h.](image-url)
Discussion

Our experiments revealed a striking difference between cloned TCR-γ/δ+ and MR-α/β+ T cells with respect to stimulation via the alternative CD2 activation pathway. It is well established that the CD2-dependent activation of α/β+ T cells requires two signals provided by two mAbs directed against different epitopes of CD2 (1, 9, 10). One of the two signals can be replaced by LFA-3 (the natural ligand for CD2), sheep E, or PMA (11-13). We now report that stimulation with a single anti-CD2 mAb in the absence of feeder cells is sufficient to trigger IL-2 production, proliferation, and cytotoxic effector activity in γ/δ+ clones expressing distinct molecular forms of the TCR-γ/δ.

Activation requirements of γ/δ+ T cells have not been thoroughly studied. A proliferative response of freshly isolated resting γ/δ+ cells can be initiated by PHA, anti-CD3 mAb, or alloantigenic stimulation (22, 30). After PHA stimulation, purified γ/δ+ cells produce less IL-2 than α/β+ cells, and proliferation of γ/δ+ cells can be triggered in the absence of detectable amounts of secreted IL-2 (22). In line

Figure 3. Cloned α/β+ T cells do not proliferate in response to immobilized anti-CD2 mAb. Four different CD4+ clones (A, B, D, E) and one CD8+ clone (C) were analyzed. Experimental system as in Fig. 1.
with these results, we found that the anti-CD2 triggered proliferation of cloned γδ+ T cells was not necessarily associated with the secretion of measurable IL-2, although some anti-CD2 mAbs induced strong IL-2 production. It remains to be established whether growth factor(s) other than IL-2 (e.g., IL-4) contribute to the autocrine proliferation of γδ+ clones in response to anti-CD2 signaling.

As shown here, established γδ+ clones are selectively activated by immobilized or cross-linked single anti-CD2 mAbs. The responsiveness of a given γδ+ clone to anti-CD2 signaling does not depend on the experimental protocol used to establish the clone. Although most clones were obtained from purified CD4− CD8− cells after isolation of E-rosetting T cells, several γδ+ clones were established after sorting of 7A5+ (Vγ9+) T cells from unseparated PBMC. These clones were equally susceptible to anti-CD2 signaling (not shown). The present results raise the important issue of whether this selective activation pathway is also operational in resting polyclonal γδ+ T cells. A prerequisite to address this question is the ability to isolate sufficiently pure γδ+ T cells by negative selection, so as not to interfere with CD3/TCR triggering during positive selection (e.g., cell sorting involving anti-TCR mAbs). Experiments to study the CD2-dependent activation pathway of primary γδ+ T cells are now in progress.

Given that single anti-CD2 mAbs directed against the sheep E binding epitope stimulate γδ+ but not α/β+ clone proliferation, one might expect similar effects utilizing sheep E instead of anti-CD2 mAb. However, under various experimental conditions, we did not observe activation (defined as cellular proliferation or IL-2 production) of cloned γδ+ T cells in the presence of sheep E only. It is thus obvious that cross-linking of the T11.1 epitope by a single anti-CD2 mAb provides a stronger activation signal to cloned γδ+ T cells than binding of sheep E to cell surface T11.1. In this context it is of interest that cross-linking of an individual anti-CD2 mAb induces Ca²⁺ mobilization also in α/β+ T cells, without directly stimulating T cell proliferation (31).

TCR-independent "alternative" activation pathways have been previously studied with cloned γδ+ T cells. Interestingly enough, activation via CD28 (32) appears to be deficient in γδ+ cells, due to the lack of CD28 expression on many γδ+ T cells (33). With regard to CD2 activation, Ferrini et al. (33) reported on the activation of γδ+ clones by an appropriate combination of two different anti-CD2 mAbs. However, data reported from other groups support our present results that γδ+, but not α/β+ T cells, can be activated by single anti-CD2 mAbs. Thus, Goedegebuure et al. (34) demonstrated that the lytic machinery can be triggered in γδ+ but not α/β+ T cell clones by bispecific heteroconjugates containing a single anti-CD2 (T11.1) mAb cross-linked to anti-DNP. Recently, Pawelec et al. reported that 2 of 13 anti-CD2 mAbs from the 4th International Leukocyte Typing Workshop (35) singly stimulated autocrine γδ+ T cell clone proliferation (36). In contrast to our results, however, the presence of EBV-transformed LCL feeder cells was absolutely required in their system. Thus, no γδ+ clone activation was observed with immobilized individual anti-CD2 mAbs in the absence of feeder cells (36). The apparent discrepancy to our present results is most likely due to the usage of different anti-CD2 mAbs in the two studies. As a matter of fact, none of the four stimulatory anti-CD2 mAbs used in our experiments (Table 1) was included in the study of Pawelec et al. (36). It is obvious from both studies that not all anti-CD2 (T11.1) mAbs individually activate γδ+ T cell clones, be it in the absence (this study) or presence (36) of feeder cells. It is presently unclear why certain anti-CD2 mAbs are stimulatory while others are not. The Ig isotype does not appear to be critical because IgG1 anti-CD2 included both stimulatory (OKT11 [an IgG1 reagent from AMC], 9E8, M-T910) and nonstimulatory (IOT11, OKT11 [an IgG2A reagent from Ortho Diagnostics, Raritan, NJ]; not shown) mAbs. It will be important to precisely characterize the stimulatory mAbs with respect to the recognized epitope (37). In this context, it is of interest that anti-CD2 (T11.1) mAbs can be classified into two subgroups (T11.1A, T11.1B; reference 38). While both T11.1A and T11.1B mAbs block E rosette formation and inhibit binding of LFA-3, T11.1A antibodies can replace the triggering signal provided by LFA-3, whereas T11.1B antibodies exert suppressive activity (38). In addition, it will also be important to investigate whether the

Figure 5. Soluble anti-CD2 mAb triggers cytotoxic effector function in γδ+ clones. Cloned γδ+ (A, B) or αβ+ (C) cells were tested against P815 target cells in the absence or presence of 1 μg/ml of the indicated mAb. E/T ratio was 5:1.
signal provided by a single cross-linked anti-CD2 mAb can be replaced by LFA-3, the natural ligand for CD2 (12). There is evidence that the CD2 and CD3/TCR activation pathways are functionally linked at least in T cells that express both molecules (27, 39). Activation of α/β+ T cells via CD2 results in tyrosine phosphorylation of the CD3/TCR ε chain (40), and suitable experimental conditions allow the specific co-precipitation of a fraction of CD2 with the CD3/TCR molecular complex (41). The present demonstration that cross-linking by a single anti-CD2 mAb is sufficient to stimulate γ/δ+ but not α/β+ T cells might suggest that CD2 and CD3/TCR molecules are even closer associated in γ/δ+ T cells. Experiments to address these questions are in progress.

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