Helper or Cytolytic Functions Can Be Selectively Induced in Bifunctional T Cell Clones

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Summary

By using bifunctional T cell populations, we have shown in this report that elicitation of helper versus cytolytic function depends on the stimulatory signal at the membrane. Interestingly enough, the transduction of these signals is likely to be achieved via different metabolic pathways. Thus, helper function is associated with intracellular Ca^{2+} mobilization and PLC activation, while cytolysis can occur even in the absence of detectable levels of these second messengers. These results indicate that selective activation through the same membrane-transducing molecule may orientate T cell function through qualitatively or quantitatively different second messengers. This would be an important part of immune regulation.

The two major T cell activities, i.e., helper and cytotoxic functions, are supported by two separate sets of lymphocytes in correlation with CD4 or CD8 expression, respectively. Exceptions to this rule have been reported in different experimental conditions providing evidence for $CD4^+$ T cell clones displaying both helper and cytotoxic activities (1–2). However, it is not clear whether both activities are simultaneously expressed or can be selectively induced by distinct activating signals. Using different combination pairs of anti-CD2 mAbs for T cell activation, we demonstrate that it is possible to induce preferentially one of the two activities. Moreover, helper function is associated with calcium influx and phosphoinositide hydrolysis, whereas cytolysis can be observed in the absence of detectable amounts of these second messengers. This indicates that depending on the mode of activation through a same membrane transducing molecule, a single T cell can express either helper or cytotoxic function. This selective orientation might involve qualitative or quantitative differences in second messenger induction.

Materials and Methods

T Cell Clones. T cell clones were produced as previously described (2–4). They are all helper and cytotoxic, specific for the influenza A/Texas virus, and restricted by HLA-DR molecule (2–4, and unpublished observations). They were maintained in long-term culture by weekly restimulation with PHA and irradiated feeder cells in the presence of rIL-2, as previously described (2–4). In all experiments described herein, T cells were used 7 d after their last restimulation.

Monoclonal Antibodies. The three anti-CD2 mAbs used were anti-TII_1 (OKT11A, IgG2a), anti-GT2 (IgG1), and anti-D66 (IgG2a), which have been previously characterized (5–7). The anti-CD3 mAb used was OKT3 (IgG2a). In dose-response experiments, anti-$T11_1$, anti-GT2, and anti-D66 were purified from ascitic fluids using protein A–Sepharose chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) and titrated by ELISA.

T Cell Proliferation Assays. Cells were cultured in triplicate in 96-well round-bottomed microtiter plates, with various stimulators, as indicated, in a total volume of 200 μl of culture medium for 3 d at 37°C, in 5% CO_2 atmosphere. During the last 6 h of culture [^{3}H]thymidine (0.8 μCi) (Amersham, les Ulis, France) was added.

T Cell Cytotoxicity Assays. Cytotoxicity was assessed by the standard ^{51}Cr-release assay described elsewhere (3). Briefly, effector cells (5×10^4 cells per well) were seeded into round-bottomed microtiter plates, in RPMI 1640 medium supplemented with 10% FCS. mAbs were added 20 min before addition of target cells. ^{51}Cr-labeled K562 target cells (5×10^3 cells per well) were added in a total volume of 200 μl. After centrifugation at 100 *g*, cells were incubated for 4 h at 37°C in 5% CO_2 atmosphere. Plates were then centrifuged again. 100 μl of supernatant was harvested from each well and the radioactivity was measured using a gamma counter.

Lymphokine mRNA Accumulation. Total RNA was isolated by the guanidinium isothiocyanate procedure (8). Equal amounts (10 μg) of RNA (this was confirmed by visualization of ribosomal RNA after staining of the gel with ethidium bromide) were resolved on

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glyoxal-agarose gel, transferred to nylon filter, and sequentially hybridized at high stringency with specific probes as already described (9). These probes (listed on the left of the figure) were obtained after in vitro transcription of T3-T7 Bluescribe vector (Vector Cloning System, San Diego, CA) containing the 1.35-kb PstI–BamHI fragment from huIL-2 R3 cDNA (10), 0.3-kb XbaI–StuI from huIL-2 cDNA (9), 0.72-kb XhoI–HpaI from huIL3 cDNA (11), and 0.28-kb EcoRV–EcoRI from huIL-4 cDNA (12).

Fc Rosettes and Inhibition of Binding by GT2, D66, and T11 Hybridoma Proteins. Rosette assays between IgG-coated SRBC and cells to be investigated have been previously described in detail (13). Briefly, SRBC were coated with two different BALB/c mouse mAbs directed to SRBC (U182.5, IgG1; UN2, IgG2a) kindly provided by Dr. M.D. Scharff (Albert Einstein College of Medicine, New York, NY), and tested for rosette formation with K562 cells. Cells associated with at least five SRBC were scored as $Fc\gamma R^+$. In another set of experiments, hybridoma proteins were purified from ascitic fluids by passing them over protein A–Sepharose columns (LKB-Pharmacia, Uppsala, Sweden). ELISAs were performed as previously described (14) to control the purity of these preparations. Hybridoma proteins were then heat-aggregated by incubation at 60°C for 20 min. Pellets of 2.5×10^5 K562 cells were incubated for 45 min at room temperature with 100 μl of heat-aggregated proteins diluted in PBS. IgG1- or IgG2a-coated SRBC were then added in 0.4 ml of PBS and rosettes were counted after 10 min centrifugation at 900 rpm at 4°C.

Measurement of $[Ca^{2+}]i$. T cells were washed and suspended at $5–7 \times 10^6$/ml in culture medium containing 3 μM acetoxymethyl ester of indo 1, according to the method of Rabinovitch et al. (15). Briefly, after incubation with indo 1 for 30 min at 37°C to effect loading, cells were washed, resuspended in the same medium at 10^6/ml, and analyzed on a cytofluorograph 50 HH cell sorter equipped with a 2150 computer (Ortho Pharmaceutical, Raritan, NJ). Analyses were conducted at 37°C at a flow rate of 500 cells. Cells were then transferred to spectrofluorometer cuvettes and after establishing the baseline $[Ca^{2+}]_i$, anti-D66+$T11_1$ mAbs (*a*) or anti-GT2+$T11_1$ mAbs in the presence of K562 cells (*b*) were added.

PI Turnover. Effector cells were washed twice in a phosphate-free buffer containing 20 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 5 mM KCl, and 0.1% Glc. The final pellet was resuspended in the same buffer and the cell suspension (10^7/ml) was incubated with carrier free ^{32}P orthophosphoric acid (Amersham, 20 μCi/ml) for 1.30 h at 37°C, until isotopic equilibrium. Cloned T cells as well as target cells were then washed twice; 100-μl aliquots of the effector cell suspension were transferred into polypropylene tubes and after adding or not adding the K562 cell suspension (10^5/tube), effector cells were stimulated with anti-CD2 mAbs for different time periods. Then, reaction was stopped as previously described (16). Radiolabeled phospholipids were located by autoradiography and their nature was determined by parallel migration of standard phospholipids visualized by exposure to iodine vapors. Spots concerning PI+PA were subsequently scraped from the plates and evaluated by liquid scintillation counting.

Results and Discussion

Two bifunctional human T cell clones, i.e., TA4 and M3ap20 specific for the influenza virus associated with HLA class II molecule and isolated from two different donors, were selected for the present study. As previously demonstrated, these monoclonal T cell populations exhibit a $CD2^+/3^+/4^+/8^-$ phenotype and have the same specificity and restriction for both

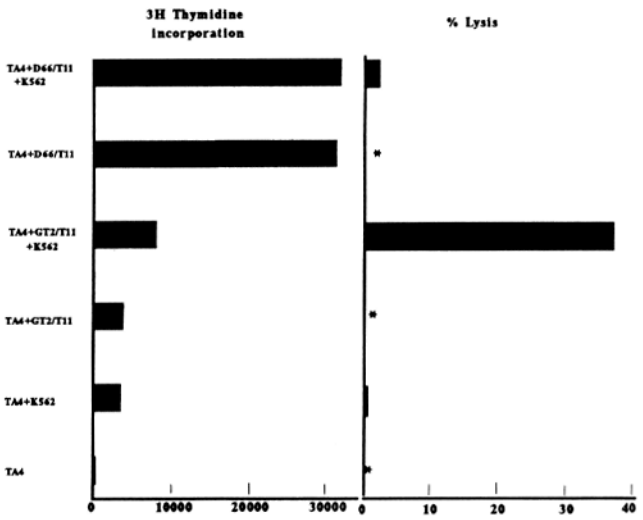

Figure 1. Anti-D66+$T11_1$ or GT2+$T11_1$ mAbs preferentially induce helper or cytolytic functions, respectively. Cells were tested on the same day in proliferation and cytotoxicity assays. They were stimulated with anti-D66+$T11_1$ mAbs (1:200 final dilution for each) or anti-GT2+$T11_1$ mAbs (1:200 final dilution for each). To compare both assays, K562 cells were added in proliferation assay at the same E/T ratio than in cytotoxicity assay (10:1). Results of proliferation assays are expressed as mean counts per minute of triplicate. Results of cytotoxicity assay are expressed as percentage of specific cytotoxicity as calculated by the following formula: Percent cytotoxicity = 100 × [(^{51}Cr release with effector cells) − (spontaneous ^{51}Cr release)/(maximum ^{51}Cr release) − (spontaneous ^{51}Cr release)]. (*Asterisk*) In the absence of K562 cells, lysis could not be tested.

helper and cytolytic activities (2–4). Activation of T cell clones can be induced by mAbs to membrane proteins such as CD3 or CD2 (17–19). In the case of CD2, combination pairs of mAbs are required, such as D66+$T11_1$ and GT2+$T11_1$, which recognize respectively D66 and $T11_1$ or GT2 and $T11_1$ epitopes on CD2 molecules (5–7). Interestingly, stimulation of our T cell clones with the first combination, D66+$T11_1$, was clearly more effective than GT2+$T11_1$ to induce helper function. This observation led us to investigate whether expression of cytolytic function in these bifunctional clones would be reciprocally more efficiently induced by GT2+$T11_1$ combination. Consistent with this hypothesis, Fig. 1 shows that cytotoxic activity of clone TA4, as assessed by short-term cytolysis assay on the nonspecific K562 cell line, is preferentially triggered with anti-GT2+$T11_1$ mAbs, whereas helper function, as assessed by proliferation assay, is preferentially triggered with anti-D66+$T11_1$ mAbs. It must be noticed that identical results were obtained with all T cell clones tested, i.e., TA4, M3ap20, and three more clones (data not shown). This dichotomy was still observed in dose-response experiments since, as illustrated with clone TA4 in Fig. 2, helper function was preferentially triggered with D66+$T11_1$ and K562 lysis preferentially with GT2+$T11_1$, whatever the concentrations of purified mAbs used. Moreover, the opposite effects observed on the cytolytic function of our T cell clones with the two combination pairs of mAbs used cannot be related to a difference in the binding ability of GT2

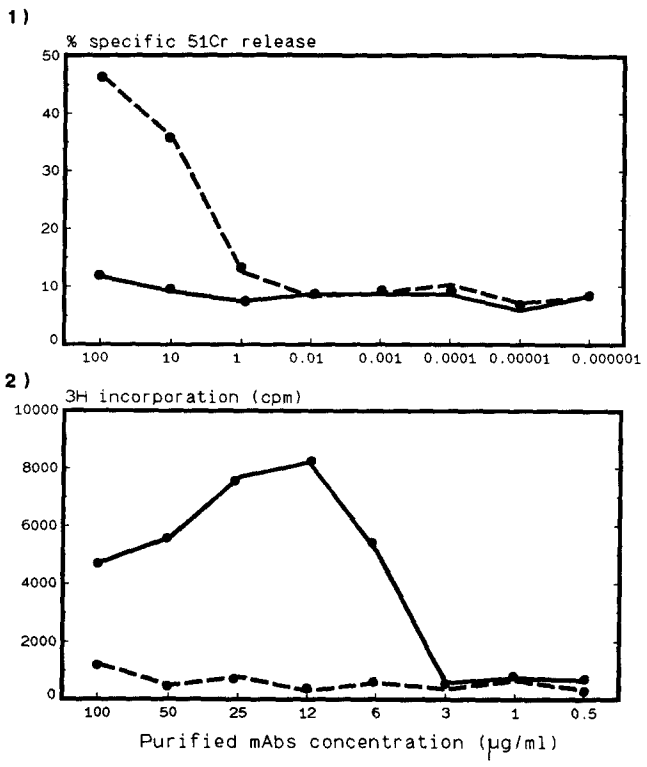

Figure 2. Dose-response experiments. Cells were tested in cytotoxic (*1*) and proliferation assays (*2*). They were stimulated by different concentrations of purified mAbs: D66+T11$_1$ (—) or GT2+T11$_1$ (——). Results of proliferation assay are expressed as mean counts per minute of triplicate. Results of cytotoxicity assay using K562 cells as target, are expressed as percentage of specific cytotoxicity calculated as described in Fig. 1.

(IgG1) and D66 (IgG2a) to the FcγRII present on K562 cells. Indeed, these cells express FcγRII that bind immune-complexed IgG1 and IgG2a, as shown by their ability to form rosettes with both IgG1- and IgG2a-coated SRBC (Table 1). Furthermore, heat-aggregated GT2, D66, and T11$_1$ proteins were able to inhibit the rosette formation between K562 cells and IgG2a- or IgG1-coated SRBC (Table 1). In these functional assays, helper and cytolytic activities were assayed following different experimental conditions. Indeed, proliferation was tested after 3 d of culture in the absence of K562 cells, while cytolysis was analyzed in the presence of the labeled targets, and after a short 4-h period of stimulation. Therefore, these assays did not directly investigate whether both activities could be simultaneously expressed in a single T cell upon a strictly identical mode of stimulation. Thus, helper function was tested in short-term assay (5 vs. 4 h for cytolysis) by analyzing IL-2, IL-3, IL-4, and IL-2R mRNA accumulation. Moreover, K562 cells, which are required for cytolysis, were added at the same E/T cell ratio. As illustrated in Fig. 3 with clone M3ap20, when both functional assays were performed under strictly identical conditions of stimulation, GT2+T11 preferentially induced cytolysis; conversely, D66+T11$_1$ selectively induced helper function. These

Table 1. *Binding of GT2, D66, and T11$_1$ mAbs to FcγRII Expressed on K562 Cells*

	Rosette formation*	
IgG added‡	SRBC-γ1	SRBC-γ2a
	%	%
None	47.1	42.0
T11 (IgG2a)	15.5	12.6
D66 (IgG2a)	15.1	20.5
GT2 (IgG1)	20.7	19.8

* Rosette assays between K562 cells and IgG1- or IgG2a-coated SRBC and inhibition of binding by hybridoma proteins were performed as described in Materials and Methods.
‡ Heat-aggregated hybridoma proteins were added at a final concentration of 20 µg/ml.

results strongly suggest that induction of either function depends upon different signals delivered through the same transducing CD2 molecule.

Concomitantly, we explore the second intracellular messengers induced by the two mAbs pairs. T cell helper function induction via CD2 molecule (20) or CD3/Ti antigen receptor complex (TCR) (21) has already been described to be associated with membrane phosphoinositide-derived second messengers: inositol 1, 4, 5 triphosphate (IP3) and diacylglycerol (DG). These two second messengers are the products of a rapid, transient hydrolysis of membrane phosphatidylinositol 4, 5 biphosphate (PIP2) by phospholipase C (PLC) and act synergistically to activate protein kinase C (PKC), leading to protein phosphorylation and modulation of gene expression. IP3 is responsible for Ca^{2+} mobilization from intracellular stores, whereas DG increases enzyme affinity for Ca^{2+} (22–24). Phosphatidylinositol (PI) turnover induced by PIP2 hydrolysis, resulting in phosphatidic acid (PA), and PI accumulation has been described to be associated with extracellular Ca^{2+} influx (25–27). In contrast, PKC activation, in the absence of free cytosolic $[Ca^{2+}]_i$ and PIP2 hydrolysis, has been reported to be absolutely required for cytotoxic triggering. Indeed, PMA can trigger T cell-mediated cytolysis with no increase in free cytosolic $[Ca^{2+}]_i$ (28).

In our model of bifunctional T cells stimulated via CD2 molecule by anti-D66+T11$_1$ or anti-GT2+T11$_1$ mAbs, cytosolic free Ca^{2+} increment was investigated by measuring fluorescence with the indo-1 indicator, and phospholipid hydrolysis was determined by analyzing PI+PA accumulation. As shown in Fig. 4 *a*, the helper function of M3ap20 clone triggered with anti-D66+T11 mAbs was accompanied by $[Ca^{2+}]_i$ increase. Interestingly, and as also reported by Alcover et al. (29) in other human T cell clones, a several minute latency in $[Ca^{2+}]_i$ rise after mAb addition was observed. This contrasts with the rapid increase observed in a Jurkat T cell line stimulated through CD2 molecule, as reported by Pan-

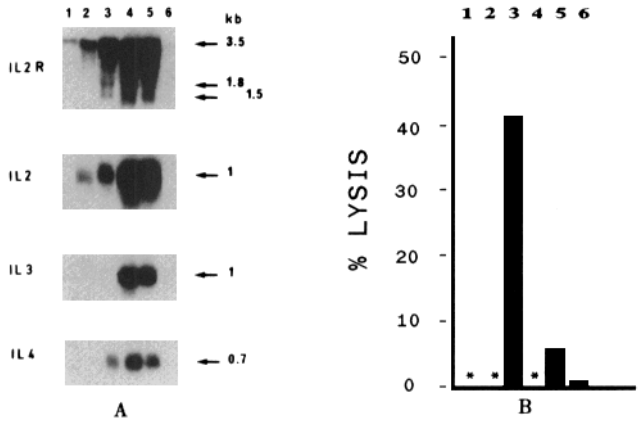

Figure 3. Expression of IL2R and lymphokine mRNA compared with cytotoxic activity in bifunctional T cell clones. (*A*) Cloned T cells were unstimulated (lane *1*); stimulated for 5 h with anti-GT2+T11$_1$ mAbs (lane *2*), anti-GT2+T11$_1$ and K562 (lane *3*), anti-D66+T11$_1$ (lane *4*), anti-D66+T11$_1$ and K562 (lane *5*). K562 was cultured alone as control (lane *6*). Culture conditions are identical as those described in Fig. 1. Then, total cellular RNA was extracted as detailed in Materials and Methods and analyzed by Northern Blotting. 10 μg of RNA were transferred on nylon membranes and hybridized with IL-2, IL-3, IL-4, IL-2R riboprobes. (*B*) In the same experiment, the cloned T cells were tested for cytolytic activity under the same conditions of stimulation. Results are expressed as percentage of specific cytotoxicity calculated as described in Fig. 1. (*Asterisk*). In the absence of K562 cells, lysis could not be tested.

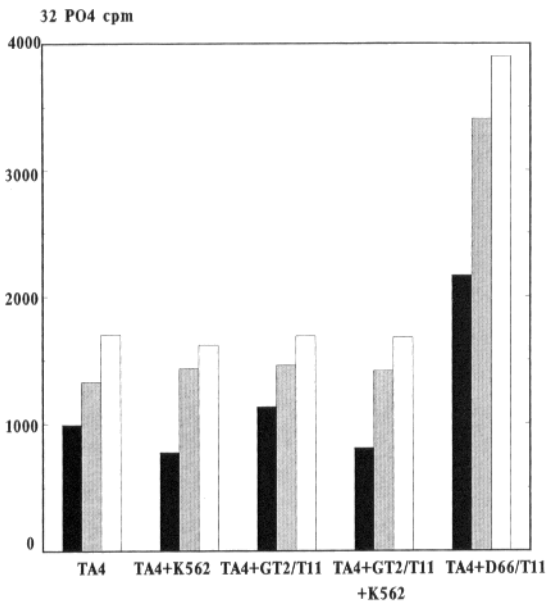

Figure 5. PA and PI accumulation. Cells were labeled with $^{32}PO_4$ as described in Materials and Methods. After stimulation for 15 min (*filled bars*), 1 h (*dotted bars*), or 3 h (*open bars*) with anti-GT2+T11$_1$ or anti-D66+T11$_1$ in the presence or absence of K562 cells, radiolabeled phospholipids were extracted and located by autoradiography. Results are presented as the cpm of (PA+PI) $^{32}PO_4$ incorporation.

taleo et al. (20). In both clones, helper function was associated with PIP2 hydrolysis as determined by PI+PA accumulation and as illustrated with clone TA4 in Fig. 5. This is in good agreement with other reports, indicating that $[Ca^{2+}]_i$ rise and phospholipid hydrolysis are associated with acquisition of T cell helper function through CD2 stimulation (20, 29). In contrast, cytotoxic activity triggered with anti-GT2+T11 mAb, even in the presence of K562 cells, was not associated with detectable changes in $[Ca^{2+}]_i$ (Fig. 4 *b*) nor with PI+PA accumulation (Fig. 5). It must be noticed that chronic treatment of our bifunctional T cell clones with phorbol ester abolished their ability to express helper and cytotoxic functions when subsequently stimulated by anti-CD2 mAbs (data not shown). Since this treatment is known to inactivate PKC (30, 31), one may assume that PKC activation is involved in the induction of both functions.

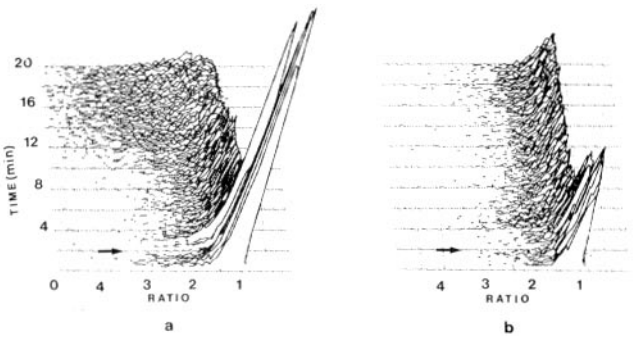

Figure 4. Measurement of $[Ca^{2+}]_i$. Cells were loaded with Indo 1 for 30 min at 37°C, washed, resuspended, and transferred to spectrofluorometers cuvettes. Anti-D66+T11$_1$ (*a*) or anti-GT2+T11$_1$ mAbs in the presence of K562 cells (*b*) were added. Data are presented as isometric plots of the ratio of indo 1 violet/blue fluorescence (proportional to $[Ca^{2+}]_i$ vs. time, vs. number of cells, according to Grynkiewicz et al. (33).

Altogether, these data suggest that different second messenger metabolic pathways are triggered, depending on the combination pairs of anti-CD2 used for stimulation. This clearly correlates with subsequent expression of selective functions. Nevertheless, one cannot rule out that the GT2+T11$_1$ combination might be less effective at increasing steady-state levels of phosphoinositide turnover and Ca^{2+} influx, both detected by relatively low sensitive methods. In that case, the same metabolic pathways would be involved but the threshold required for cytolytic function induction would be lower than that for helper function. Consistent with this hypothesis, we recently demonstrated that a secondary signal provided by IL-2 in addition to GT2+T11$_1$ can lead to helper function (16, 32) and PA+PI accumulation (16). In addition, it must be noticed that in the present study, K562 cells in association with anti-GT2+T11$_1$ provide a secondary signal leading to some proliferation (Fig. 1) associated with low IL-2, IL-4, IL-2R mRNA accumulation (Fig. 3, lane *3*).

Finally, the induction of helper but not cytotoxic function after D66+T11$_1$ activation strengthens the assumption that GT2+T11$_1$ or D66+T11$_1$ mAbs trigger different functions through two distinct metabolic pathways. Indeed, if the low levels of GT2+T11$_1$-activated second messengers are

Table 2. *D66 + T11 Combination Does Not Abolish the CD3/TCR-mediated Cytolysis*

Exp.	Reagents added at time 0	Reagents added 30 min after	Percent lysis at different E/T ratio 20/1	6/1	3/1
1	Anti-D66 + T11	K562	1.1	0.8	1
	Anti-CD3	K562	71	53	41.5
	Anti-CD3	Anti-D66 + T11 + K562	52.5	47	35.3
	Anti-D66 + T11	Anti-CD3 + K562	50.5	47	33
	Anti-CD3 + K562	Anti-D66 + T11	ND*	ND	ND
2	Anti-D66 + T11	K562	ND	0	0
	Anti-CD3	K562	38	40	27
	Anti-CD3	Anti-D66 + T11 + K562	27	27	15
	Anti-D66 + T11	Anti-CD3 + K562	26	26	11
	Anti-CD3 + K562	Anti-D66 + T11	44	38	15

Cloned T cells were tested for lysis of K562 cells in different conditions of stimulation. 30 min after triggering with anti-D66 + $T11_1$ or CD3 mAbs, target cells alone (lanes *1* and *2*, respectively) or in association with anti-D66 + $T11_1$ (lane *3*) or anti-CD3 (lane *4*) were added. Then cytolysis was performed during 4 h. In Exp. 2, the same clone was tested in other conditions of stimulation: anti-D66 + $T11_1$ was added 30 min after triggering of cloned T cells with anti-CD3 plus target cells (lane *5*). Results are expressed as percentage of specific cytotoxicity, as calculated in Fig. 1.

sufficient to trigger cytotoxic activity but not helper function, the strong $[Ca^{2+}]_i$ and PA+PI increment observed after D66+$T11_1$ activation could not account for the absence of cytolytic activity. Thus, one hypothesis would be that the D66+$T11_1$ combination would inhibit cytolytic activity. This is quite unlikely, since, as shown in Table 2 with the M3ap20 clone, D66+$T11_1$ combination, which does not by itself induce any cytolysis, is unable to abolish the CD3/TCR-mediated cytolysis, even though a slight inhibition is occasionally observed.

Even if the exact biochemical mechanisms by which CD2 can differently orientate T cell functions are not fully understood, one may speculate that this phenomenon could be an important part in the human in vivo immune regulation, by directing the response towards cellular and/or humoral immunity. Although experiments are more difficult to carry out, further studies are now in progress to determine whether a similar dichotomy exist in TCR/CD3-mediated T cell activation.

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