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Non-Fc Receptor-Binding Humanized Anti-CD3 Antibodies Induce Apoptosis of Activated Human T Cells¹ ✓

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Non-Fc Receptor-Binding Humanized Anti-CD3 Antibodies Induce Apoptosis of Activated Human T Cells¹

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Human trials in organ allografts have demonstrated that murine anti-CD3 mAbs are immunosuppressive. By mimicking Ag, anti-CD3 can produce T cell activation, anergy, or death. Activation of resting T cells in vivo results in dose-limiting cytokine release and is caused by Ab-mediated cross-linking of T cells and Fc γ receptor (FcR)-bearing cells. With the goal of minimizing cytokine-induced toxicity, anti-CD3 have been engineered to lower Fc binding avidity. Preclinical murine studies have indicated that non-FcR-binding anti-CD3 can induce apoptosis of Ag-activated T cells. Since induction of T cell apoptosis may be an important mechanism of immunosuppression by anti-CD3, we tested whether Fc mutations affect the ability of anti-human CD3 to induce apoptosis of activated T cells. We compared wild-type murine anti-CD3, M291, and OKT3 and their humanized, FcR- and non-FcR-binding structural variants in quantitative assays of T cell apoptosis. Non-FcR-binding variants produced more sustainable phosphorylation of extracellular signal-regulated kinase-2, greater release of IFN- γ , and more effectively caused activation-dependent T cell apoptosis. Non-FcR-binding variants dissociated more quickly from the T cell surface and caused less internalization of the TCR, which then remained available in greater abundance on the cell surface for signaling. Cross-linking of non-FcR-binding variants by antiglobulin enhanced TCR internalization and minimized induction of T cell apoptosis. We conclude that non-FcR-binding, humanized anti-CD3 have improved ability to induce apoptosis of activated T cells, presumably by allowing durable expression of the TCR and sustained signaling. *The Journal of Immunology*, 2000, 165: 6205–6213.

Targeting the TCR-CD3 complex on human T cells with the murine mAb OKT3 has been used for almost 20 years in the treatment of organ allograft rejection (1). A second generation of genetically engineered anti-CD3 mAbs has been developed not only by grafting complementarity-determining regions (CDRs)³ of murine anti-CD3 mAb into human IgG sequences (2–5), but also by introducing non-FcR-binding mutations into the Fc (6, 7). Humanization of the mAb results in decreased immunogenicity and improved mAb half-life. Furthermore, non-FcR-binding mAbs do not activate resting T cells (5, 7–9) and have limited potential for inducing cytokine release and acute toxicity in vivo (10–12). Based on preclinical data in mice, we have been interested in the possibility that such mAbs might provide improved immunosuppression by inducing apoptosis of Ag-activated T cells (13).

The mechanism of immunosuppression mediated by anti-CD3 mAb is complex, but it is believed to depend largely on TCR internalization (14–16) or unresponsiveness to Ag restimulation (17–20). T cell responses are influenced by the status of T cell activation and the Ag load. Proliferating T cells undergo proapoptotic regulation after stimulation with high-dose Ag (21). Solid phase-bound anti-CD3 mAb also induces apoptosis in murine and human T cells (22, 23) beyond early G₁ in the cell cycle (24–27). Induction of apoptosis requires the up-regulation of extracellular signal-regulated kinase 2 (ERK2) (28) and expression of death effector molecules Fas or TNF receptor and their ligands (29–31). Soluble anti-CD3 mAbs have been reported to cause T cell apoptosis in one study (32) in addition to our own observations in murine T cells (13, 28, 33).

T cell responses, including apoptosis, are influenced by the dynamic interaction between TCR and peptide-MHC complexes (34, 35). Cognate and altered peptide ligands signal through the TCR with a potentially different outcome on T cell activation by virtue of subtle differences in affinity and/or dissociation rates (36–41). Agonist peptide-MHC ligands induce both apoptosis and lymphokine production (42), whereas partial agonist altered peptide ligands may induce apoptosis without lymphokine secretion (43). Engagement of the TCR-CD3 complex leads to phosphorylation of its component subchains and internalization of the TCR (44), ultimately limiting T cell hyperstimulation (45). Anti-CD3 variants may, like altered peptide ligands, interact differently with the TCR with variable effect on T cell activation and fate (46). Anti-CD3 mAbs that bind with high avidity and dissociate from TCR at a slow rate may engage CD3 in a way to phosphorylate and internalize all available TCR molecules. Since signaling depends on sustained triggering through TCR above a minimum threshold (47–50), in the absence of TCR these high-avidity anti-CD3 mAbs are expected to be poor stimulants. On the contrary, anti-CD3 mAbs with lower avidity, that is, those that dissociate from the

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³ Abbreviations used in this paper: CDR, complementarity-determining region; ERK2, extracellular signal-regulated kinase 2; CM, complete medium; PI, propidium iodide; A_o, apoptotic DNA.

TCR at a faster rate and induce less internalization of TCR, may induce more sustained T cell triggering. These lower avidity anti-CD3 may be better agents to trigger T cell death.

In this report, we demonstrate that soluble anti-human CD3 mAbs can induce apoptosis of preactivated T cells. Second, anti-CD3 mAbs differ in their ability to induce apoptosis. Third, stimulation with low-avidity, non-FcR-binding, anti-CD3 mAbs does not induce the rapid modulation of surface CD3/TCR that is characteristic of high-avidity FcR-binding anti-CD3 mAbs. The persistence of CD3/TCR on the cell surface is required for sustained signaling. Finally, non-FcR-binding anti-CD3 mAbs induce sustained phosphorylation of ERK, enhanced IFN- γ production, and death by apoptosis. We surmise that non-FcR-binding anti-CD3 mAbs might be candidates for producing T cell clonal deletion *in vivo*.

Materials and Methods

Anti-CD3 mAbs

Three murine anti-human CD3 mAbs were used in these experiments: IgG2a OKT3 (Ortho, Raritan, NJ), IgG2a M291 (5) (Protein Design Laboratories, Fremont, CA), and IgG2b BC3 (14) (Fred Hutchinson Cancer Research Center, Seattle, WA). All three mAbs bind to human CD3 ϵ , but they have different variable region sequences (5, 18). Using the murine DNA sequences derived from OKT3 and M291 hybridomas, a panel of genetically engineered anti-CD3 mAbs was produced (5, 7). Structural differences among the anti-CD3 mAbs used in this study are compared in Table I. Briefly, the OKT3 variants are chimeric, whereas the HuM291 variants are more fully humanized. The anti-CD3 IgG1 mAb contains human IgG1 Fc, which binds FcRs. Anti-CD3 IgG2M3 contains human IgG2 Fc with mutations at amino acid residues 234 and 237 (Val \rightarrow Ala), rendering the mAb incapable of binding FcRs (5, 7). Finally, anti-CD3 Fos are F(ab')₂ made by fusing the dimer-forming, leucine zipper Fos to the hinge of anti-CD3 mAbs (51). The two anti-CD3 Fos fragments used in this study contain human IgG1 CH1, hinge, and CK sequences (52).

Preparation of activated T cells

Human PBMC from adult normal donors were separated by Ficoll-Hypaque (Lymphoprep; Nycomed, Oslo, Norway) density gradient centrifugation of heparinized whole blood. Cells recovered from the interface were washed twice with RPMI 1640 and suspended in complete medium (CM) which comprised: RPMI-HEPES medium supplemented with 4 mM L-glutamine, 1 mM pyruvate, 50 mM 2-ME, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% heat-inactivated pooled human serum. Primary cultures were established in 24-well plates (Costar, Cambridge, MA), precoated with anti-CD3 mAb BC3 at 16 μ g/ml in bicarbonate (35 mM)-carbonate (15 mM) buffer (pH 9.6), for 2 h at room temperature. Freshly isolated PBMC were plated at 5–7.5 \times 10⁵ cells/well in 2 ml of CM and incubated for 72–96 h at 37°C and 5% CO₂.

Quantitation of T cell death induced by stimulation with soluble anti-CD3 mAbs

Activated cells were harvested, dead cells were removed by density gradient centrifugation, and viable cells were resuspended in fresh CM with 50 IU/ml recombinant human IL-2 (Chiron, Emeryville, CA). Preactivated T cells were not coated with anti-CD3 mAb BC3, indicating that the Ab had not been shed from the plastic. Furthermore, CD3 expression was maintained (data not shown). Secondary stimulation with the panel of anti-CD3 mAbs was conducted for up to 48 h in 96-well flat-bottom plates with 10⁵ cells/well in a final volume of 200 μ l. In separate experiments, secondary stimulation was with HuM291-IgG2M3 alone, anti-human IgG2 mAb (clone HP-6002; Sigma, St. Louis, MO), or both agents together. Following restimulation, cells were harvested and stained at 4°C in PBS containing 0.1% BSA, 0.1% sodium azide, and 1 μ g/ml propidium iodide (PI; Sigma) immediately before analysis. A FACScan analyzer and CellQuest software (Becton Dickinson, San Jose, CA) were used in all experiments. Fluorescence data were collected using logarithmic amplification from 2 \times 10⁴ cells that were determined to be lymphocytes by forward and right angle light scatter plots. PI uptake indicated dead cells. For experiments of timed acquisition, cells were incubated with FITC-conjugated CD4 or CD8, PE-conjugated CD56 plus CD16, or control mAbs (Becton Dickinson) for 30 min at 4°C. Cells were then washed once and resuspended in 300 μ l of PI (1.6 μ g/ml). Ungated cells were acquired for 45 s at a constant flow rate using a FACScan, and the number of viable T cells and their phenotypes

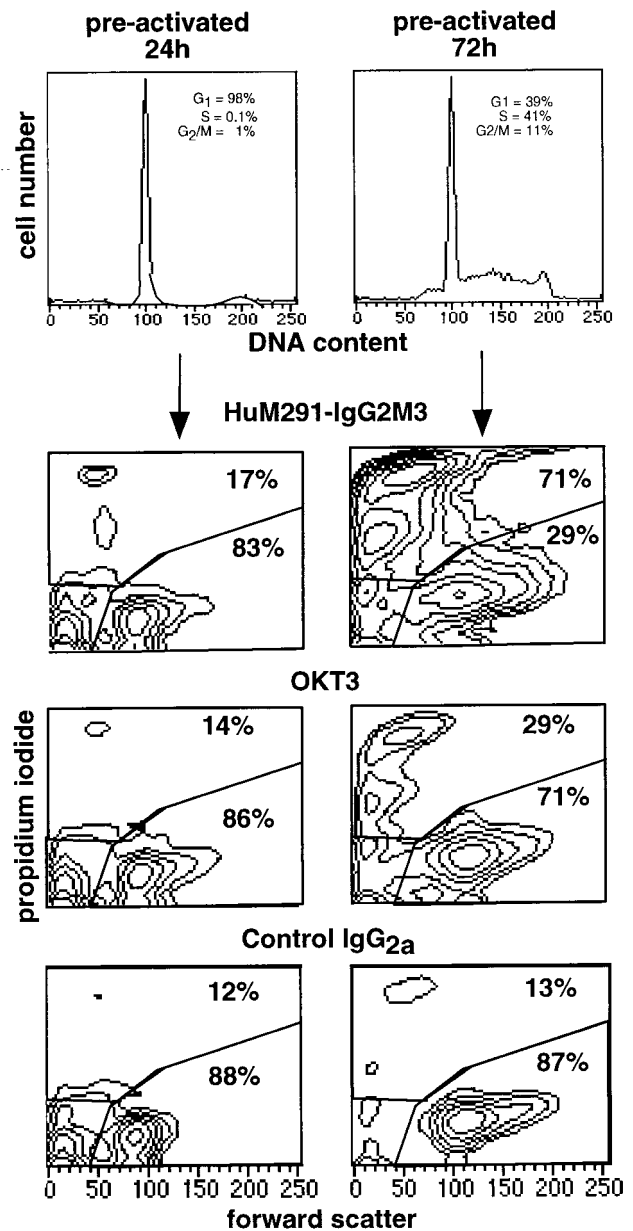


FIGURE 1. Soluble anti-CD3 mAbs induce death of preactivated and cycling human peripheral T cells. T cells were preactivated for 24 h (*left panel*) or 72 h (*right panel*) by solid phase-bound anti-CD3 mAb, BC3. T cells were harvested, one aliquot was used for cell cycle analysis, and other aliquots were exposed for 24 h to 0.2 μ g/ml of the anti-CD3 mAbs, HuM291-IgG2M3 or OKT3, or to a murine IgG2a mAb of irrelevant specificity in the presence of 50 IU/ml IL-2 in CM containing 10% normal human serum. Cells were then harvested and tested by microfluorometry as described in *Materials and Methods*.

were determined (53). In other experiments, 1.5 \times 10⁵ cells were prepared for cell cycle analysis (13). Cells were lysed with 1% Nonidet P-40 and incubated with 10 μ g/ml RNase for 30 min at 37°C. Nuclear DNA was stained with 100 μ g/ml PI, and samples were allowed to equilibrate in the dark for 1 h before analysis. Hypodiploid DNA was scored as apoptotic DNA (A₀) after gating out doublets. T cell apoptosis was also detected by dual staining of activated T cells with FITC-conjugated annexin V and PI (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cells were analyzed immediately by FACScan.

Quantitation of CD3/TCR modulation by flow cytometry

Freshly isolated PBMC (1.5 \times 10⁵ cells/ml) were exposed to medium alone or to different anti-CD3 mAbs at varying concentrations with or

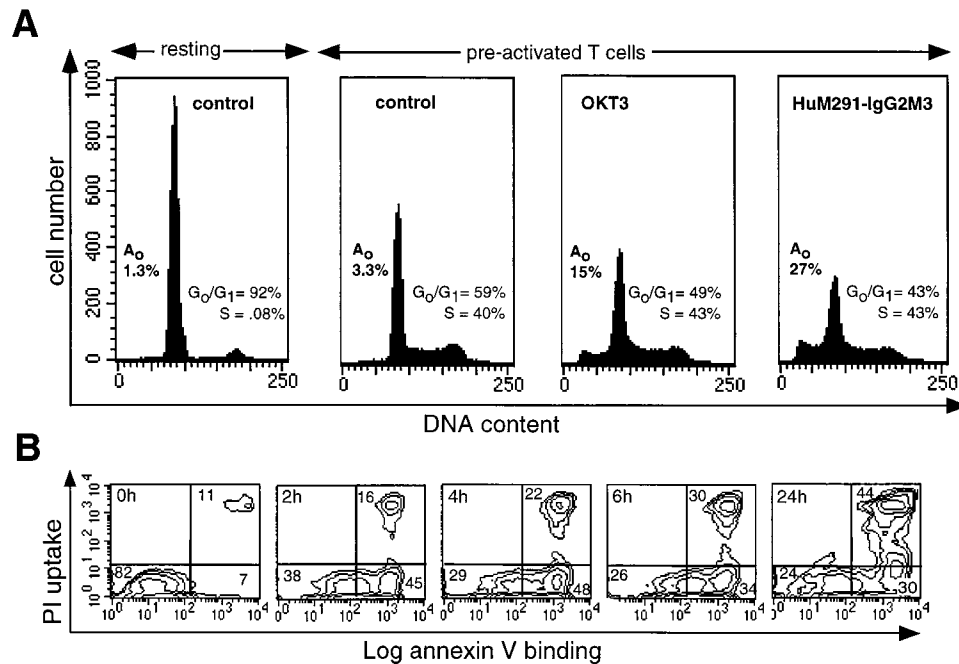


FIGURE 2. Soluble anti-CD3 mAbs induce apoptosis in activated human peripheral human T cells. *A*, Freshly isolated T cells or T cells preactivated in culture with solid phase-bound anti-CD3 mAb, BC3, for 72 h were exposed to HuM291-IgG2M3, murine OKT3, medium alone containing 10% normal human serum, or a murine IgG2a mAb of irrelevant specificity for up to 24 h. Cells were then prepared for cell cycle analysis by staining with PI (*top*). *B*, Preactivated T cells were exposed to HuM291-IgG2M3 and then stained with FITC-conjugated annexin V and PI at the time points indicated (*bottom*). Flow cytometry was used to determine the percentage of cells in early apoptosis (annexin V positive and PI negative), late apoptosis (annexin V and PI positive), and viable cells (annexin V and PI negative).

without anti-human IgG2 mAb for up to 24 h at 37°C in 5% CO₂. Modulation was stopped by cooling cells at 4°C. Saturating amounts (10 μg/ml) of mAb were added to ensure that any remaining unbound CD3 molecule was coated with anti-CD3 mAb. After 30 min at 4°C, cells were washed twice and stained with an appropriate Fcγ chain-specific antglobulin (PE-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) and FITC-conjugated F(ab')₂ goat anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA)). Cells were washed, fixed in 1% paraformaldehyde, and analyzed by FACScan. Lymphocytes were gated by forward and side scatter characteristics, surface staining of CD3 was analyzed in histogram mode, and the logarithmic median fluorescence intensity was recorded. The percentage of CD3/TCR modulation was calculated as: (control cells MFI_{anti-Ig} - anti-CD3 mAb-treated MFI_{anti-Ig}) / (control cells MFI_{anti-Ig}) × 100, where anti-Ig represents the PE- or FITC-conjugated goat anti-mouse or goat anti-human antoglobulins as appropriate. Control cells were PBMCs not incubated with anti-CD3.

Dissociation of anti-CD3 mAbs from T cells

Anti-CD3 mAbs were labeled with ¹²⁵I by chloramine-T-catalyzed iodination and separated from unreacted iodide using PD-10 columns (Pharmacia, Piscataway, NJ; 17-0851-01) (54). Jurkat T cells were coated with ¹²⁵I-labeled anti-CD3, washed three times, and recultured at 37°C. After different incubation times, cells were centrifuged, and the supernatants were harvested and mixed with 25% TCA to precipitate protein-bound ¹²⁵I shed from the cell surface. The TCA-soluble portion of the supernatant represents free ¹²⁵I that derives from ¹²⁵I-labeled mAbs that have been internalized and degraded in the lysosomal compartment. Surface-bound mAb was measured after stripping the pelleted cells with RPMI/BSA that was titrated with 5 N HCl to pH 1.0. The remaining cell pellet contained the intracellular mAb. Relative contributions of radioactivity from these four components of the T cell culture were determined (55, 56).

IFN-γ, IL-4, and IL-10 ELISA

Supernatants from activated T cell cultures were harvested at 24 h and assayed for cytokines by an ELISA. Capture and biotinylated secondary detector Abs to human IFN-γ, IL-4, and IL-10 were all obtained as matched pairs (Endogen, Boston, MA). The colorimetric detecting agent for the captured cytokine sandwich for IL-4 and IL-10 was PolyHRP:SA20 conjugate (Research Diagnostics, Flanders, NJ) and that for IFN-γ was

HRP-conjugated avidin D (Vector Laboratories, Burlingame, CA). The substrate was 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD). Reactions were stopped with 1 M H₃PO₄. OD was determined at 450 nm using a microplate reader (Vmax; Molecular Devices, Sunnyvale, CA). IL-4, IL-10, and IFN-γ values were calculated, respectively, from standardized curves using recombinant human IL-4, IL-10 (Endogen), or IFN-γ (BioSource International, Camarillo, CA) in assay buffer by log-log or four-parameter analysis, using SoftMax Pro machinery (Molecular Devices). Inter- and intraassay coefficients of variation were <10% with an assay sensitivity of <0.5 pg/ml for IL-4, <0.3 pg/ml for IL-10, and <0.3 pg/ml for IFN-γ. All samples, standards, and controls were run in duplicate.

Cell stimulation and preparation of cell lysates

Preactivated human T cells were resuspended in serum-free RPMI-HEPES medium, prewarmed at 37°C for 10 min before stimulation, and used for stimulation experiments under the conditions described in *Results*. After stimulation, cells were washed in cold PBS containing 400 μM EDTA and 400 μM Na₃VO₄. Activated cells were solubilized for 30 min at 4°C in 150 μl of lysis buffer (20 mM Tris, 2 mM EDTA, 137 mM NaCl, 1% Triton X-100, and 10% glycerol supplemented with 10 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, and 1 μg/ml aprotinin/leupeptin). Insoluble material was removed by centrifugation at 13,000 rpm (10 min), and protein content was quantified with Bradford assay reagents (Bio-Rad, Hercules, CA).

Immunoblotting reagents

Anti-phosphoERK (sc-7383) and anti-ERK2 (sc-154) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PMA, ionomycin, and anti-human IgG2 mAb (I4139) were purchased from Sigma. Anti-mouse IgG2a Ab (103-01) was obtained from Southern Biotechnology Associates. Peroxidase-conjugated AffinPure donkey anti-mouse IgG and anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories.

Immunoblot analysis

Detergent-solubilized whole-cell lysates (20 μg) were resolved through a 10% polyacrylamide-SDS gel and transferred to nitrocellulose membrane. The membrane was blocked overnight (4°C) in PBST (1× PBS plus 0.1% Tween 20) containing 5% nonfat dry milk. The membrane was briefly washed with PBST and then incubated for 2 h with specific primary Abs

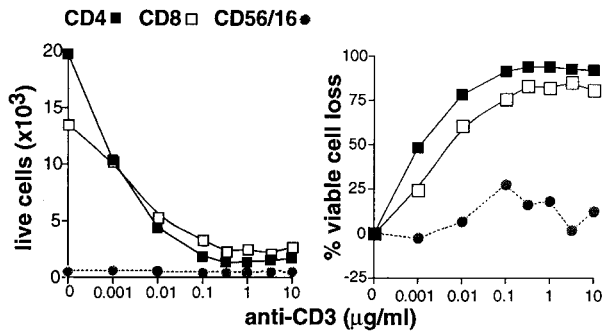


FIGURE 3. CD4 and CD8 T cells are both susceptible to in vitro apoptosis induced by soluble anti-CD3 mAb. Cells were activated as described in *Materials and Methods*. Preactivated T cells were exposed to various concentrations of HuM291-IgG2M3 for 24 h. Cells were stained with fluoro-chrome-conjugated anti-CD4 (■), CD8 (□), or CD16/56 (●) mAb and PI. These samples were acquired on a FACScan at a constant flow rate for 45 s. The phenotype of cells remaining viable (PI negative) was determined.

diluted in PBST plus 1% BSA. Afterward, it was washed several times with PBST and incubated (50 min) with peroxidase-conjugated donkey anti-mouse or anti-rabbit IgG Ab (1/30,000) in PBST plus 1% BSA. The membrane was extensively washed with PBST and developed by enhanced chemiluminescence.

Statistical analysis

Generalized estimating equations were used to regress cell death on the type of Ab and the dose of Ab. Data were analyzed by clustering on experiment. A linear link function was used assuming a normal error distribution and an independent working correlation matrix. Cell death at concentrations ≥ 333 ng/ml was also analyzed using these methods, where the slope of cell death was estimated and tested with respect to the concentration.

Results

Activated and cycling peripheral human T cells undergo apoptosis following exposure to soluble anti-CD3

T cells contained in freshly isolated human PBMC were activated in tissue culture by the plastic-bound anti-CD3 mAb, BC3. At 24 h $< 2\%$ of the cells were in S-G₂; however, by 72–96 h, at least 50% of cells were in S-G₂. Preactivated T cells were harvested and recultured in the presence of soluble anti-CD3 mAbs plus exogenous IL-2 for 24–48 h. T cell death was then quantified by PI staining and flow microfluorometry. Anti-CD3 mAbs induced death in T cells preactivated for 72 h, most of which were in cycle, but not in T cells preactivated for 24 h, which were not in cycle (Fig. 1). The murine FcR-binding anti-CD3 mAb, OKT3, induced T cell death, and the fully humanized, non-FcR-binding anti-CD3 mAb, HuM291-IgG2M3, appeared to be even more effective.

Table I. Panel of anti-CD3 mAbs used

Name	Type	Murine Sequence	Human Sequence	Isotype	FcR Type Bound	Ref.
OKT3	Mouse mAb	All	None	IgG2a	I–II	1
BC3	Mouse mAb	All	None	IgG2b	II	12
M291	Mouse mAb	All	None	IgG2a	I–II	8
OKT3-IgG1	Chimeric mAb	V	CH1,2,3 C κ	IgG1	I–II	7
OKT3-IgG2M3	Chimeric mAb	V	CH1,2,3 C κ	IgG2M3 ^a	None	7
OKT3-Fos ^b	Chimeric mAb	V	CH1 C κ	N/A	None	7
HuM291-IgG1	Humanized mAb	CDR	Non-CDR	IgG1	I–II	8
HuM291-IgG2M3	Humanized mAb	CDR	Non-CDR	IgG2M3*	None	8
HuM291-Fos	Humanized F(ab') ₂	CDR	Non-CDR	N/A	None	8

^a Anti-CD3 IgG2M3 contains human IgG2 Fc with mutations at amino acid residues 234 and 237 (Val→Ala) (7).

^b Fos represents a F(ab')₂ version of the anti-CD3 molecules containing the leucine zipper Fos (51). V, Variable region; N/A, not applicable.

To assess whether T cells were dying by apoptosis, we measured the proportion of cells with hypodiploid DNA (A₀). Preactivated T cells underwent apoptotic death after a 24-h exposure to soluble anti-CD3 mAb OKT3 or HuM291-IgG2M3 (Fig. 2A). An earlier stage of apoptosis can be measured by the detection of plasma membrane phosphatidylserine with fluorescein-labeled annexin V and microfluorometry (57, 58). Using this method apoptosis was detectable as early as 2 h following exposure of preactivated T cells to soluble anti-CD3 mAb HuM291-IgG2M3, when most cells were still able to exclude PI (Fig. 2B). These results demonstrate that soluble anti-CD3 mAbs can induce apoptosis in activated human peripheral T cells.

CD4 and CD8 T cells are susceptible to anti-CD3 mAb-mediated apoptosis

We examined the phenotype of cells that were susceptible to death after exposure to variable concentrations of soluble anti-CD3 mAb. Preactivated cells were $> 98\%$ CD4 or CD8 $\alpha\beta$ T cells, with the remaining cells including very small numbers of NK cells, B cells, and $\gamma\delta$ T cells (data not shown). After treatment with HuM291-IgG2M3, we found that both CD4 and CD8 T cells were susceptible to anti-CD3 mAb-induced cell death (Fig. 3, left panel), with up to 85% of CD8 and 93% of CD4 T cells dead in the presence of HuM291-IgG2M3 at 0.33 $\mu\text{g/ml}$ (Fig. 3, right panel).

Humanized anti-CD3 mAbs induce more T cell apoptosis than their murine anti-CD3 counterparts

Our goal was to determine whether humanized, non-FcR-binding anti-CD3 retained the ability to induce T cell apoptosis in the face of their potential to have improved clinical safety and pharmacokinetics. We exposed preactivated human T cells to the murine anti-CD3 mAbs, OKT3 and M291, and a panel of their respective FcR-binding and non-FcR-binding variants (Table I). Cell death was induced in T cell cultures by all anti-CD3 mAbs tested. However, two questions arose from the comparison of murine anti-CD3 mAbs with their humanized or chimeric variants. First, after 24 h chimeric and humanized anti-CD3 induced significantly more T cell death than murine anti-CD3 mAbs OKT3 or M291 ($p < 0.0001$; Fig. 4, top). A rank order existed among mAbs in terms of their ability to induce apoptosis, with HuM291-IgG2M3 being the most effective and OKT3 being the least effective (Table II). Murine M291 also induced T cell death more effectively than murine OKT3 ($p = 0.0004$). Second, both murine anti-CD3 mAbs exhibited a marked pro-zone, with less or no T cell death occurring at concentrations of mAb ≥ 0.33 $\mu\text{g/ml}$. At concentrations of mAb ≥ 1 $\mu\text{g/ml}$, a very slight, but significant, pro-zone was exhibited by humanized M291 ($p = 0.009$) and chimeric OKT3 ($p = 0.01$) variants.

Table II. Functional characteristics of anti-CD3 mAbs used

Name	Mean Cell Death ^a (%)	IFN- γ (pg/ml)	Modulation ^b ([anti-CD3] ₅₀ μ g/ml)	Avidity ^c ($K_a \times 10^9 M^{-1}$)
HuM291-IgG2M3	60.6	160	>10	0.5
OKT3-IgG2M3	56.7	150	0.1–0.5	0.5
OKT3-Fos	56.5	150	ND	1.0
HuM291-Fos	55.8	135	ND	1.2
HuM291-IgG1	53.8	100	1.0–10	1.6
OKT3-IgG1	51.7	125	0.05–0.1	1.1
M291	44.1	87	0.01–0.05	1.6
OKT3	27.8	72	0.01–0.02	1.2

^a Mean cell death is an average of the maximal amounts of cell death observed in each experiment ($n = 6-13$) at optimal Ab concentration.

^b [anti-CD3]₅₀ defined as the concentration of anti-CD3 which induces 50% modulation of surface T cell CD3 after 12 h. ND, Not done.

^c Avidity constants were derived by Cole et al. (5, 7).

To address the first question of whether the potency of anti-CD3 mAb in inducing cell death was a function of differential signaling through the CD3-TCR complex, we measured the levels of secreted cytokines. IL-2, IFN- γ , IL-4, and IL-10 were tested in supernatants from samples cultured in parallel to those used for cell death assays but without exogenous IL-2. IL-2, IL-4, and IL-10 were measurable at barely detectable levels (data not shown), while anti-CD3 mAbs, which induced the most apoptosis *in vitro*, also induced the most IFN- γ production (Fig. 4, lower panel). We interpreted these results to indicate that anti-CD3 mAbs most effective at inducing T cell apoptosis do so because of a stronger activation signal via their interaction with the CD3-TCR complex.

Humanized anti-CD3 mAb HuM291-IgG2M3 induces sustained phosphorylation of ERK2

Because we and others had previously demonstrated that induction of apoptosis by TCR signaling requires activation of ERK2 (28, 59, 60), we examined the effect of anti-CD3 mAb on phosphorylation of ERK. ERK became phosphorylated in greater amounts after stimulation with soluble HuM291-IgG2M3 compared with OKT3. Moreover, phosphorylation of ERK persisted for at least 3 h after stimulation with HuM291-IgG2M3, while there was a progressive decline in ERK phosphorylation that was observed beyond 30 min following stimulation with OKT3 (Fig. 5). These data support the concept that humanized anti-CD3 mAb HuM291-IgG2M3 transduces a stronger signal through the Ras-mitogen-activated protein kinase/ERK pathway.

Rapid TCR modulation induced by anti-CD3 mAbs is associated with reduced T cell apoptosis

To determine why apoptosis is subjected to a marked prozone after stimulation with either murine OKT3 or M291 we tested the following hypothesis. The biphasic apoptosis dose-response curves might be due to a net loss of TCR molecules from the T cell surface, occurring preferentially after protracted exposure to high mAb concentrations. This was based on previous observations that internalization and recycling of TCR-CD3 complexes to the T cell surface is a constitutive T cell function (44, 61); however, net internalization during the course of T cell stimulation through the TCR ultimately limits ongoing T cell signaling.

We were interested in the possibility that anti-CD3 mAbs that bind sufficient ligands for signaling, without exceeding the rate of re-expression to the surface, may induce more T cell death by allowing protracted TCR signaling. Modulation experiments could not be conducted in preactivated T cells because T cell apoptosis *per se* induces a decline of TCR expression, making results uninterpretable (62). Therefore, we conducted modulation experiments in resting T cells. HuM291-IgG2M3 does not induce appreciable

internalization of surface CD3 after 12 h (Fig. 6A, lower panel, and B, left panel). In contrast, the murine mAbs OKT3 and M291 induce 50% internalization of surface CD3 at mAb concentrations as low as 10–50 ng/ml after 12 h and at 10 μ g/ml show 85–90% internalization of CD3. Similarly, the concentration of anti-CD3 mAb required to induce 50% internalization of CD3 is ~5-fold higher for OKT3-IgG1 compared with OKT3, and 5-fold higher again for OKT3-IgG2M3 compared with the OKT3-IgG1 (Fig. 6B, right panel). Therefore, the rank order of the abilities of the OKT3, OKT3 IgG1, and OKT3-IgG2M3 variants to induce T cell death is the inverse of the rank order of their abilities to induce CD3/TCR internalization at 12 h (Table II). In other experiments in which the kinetics of CD3 modulation by a fixed mAb concentration (10 μ g/ml) was followed over 3 h, murine M291 modulated CD3-TCR

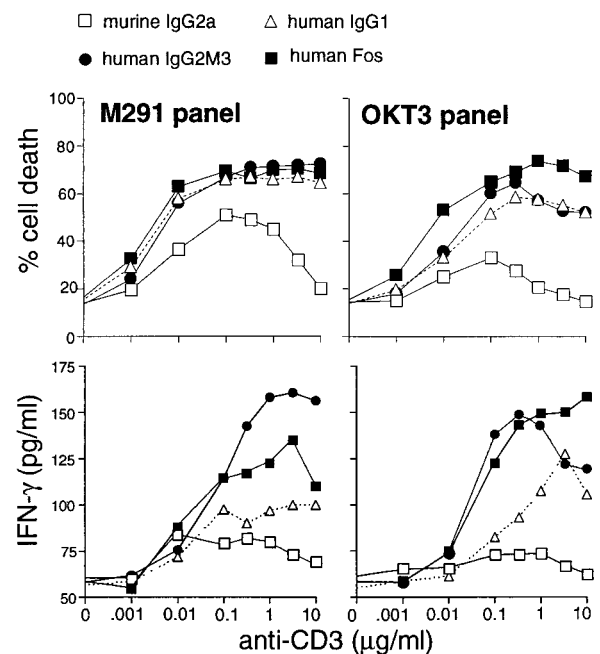


FIGURE 4. Anti-CD3 mAb variants induce more T cell death and release of IFN- γ than do murine anti-CD3 mAbs OKT3 or M291. After preactivation for 96 h by solid phase-bound anti-CD3 mAb, BC3, T cells were harvested and re-exposed for 24 h to a soluble anti-CD3 mAb in the presence of 50 U/ml IL-2. The Abs are described in Table I. Cell death was detected by uptake of PI, and cells were analyzed by microfluorometry (top). Supernatants from samples cultured in parallel to those for the cell death assays, but without exogenous IL-2, and with CM containing FCS instead of pooled human serum were assayed for the cytokines indicated (bottom).

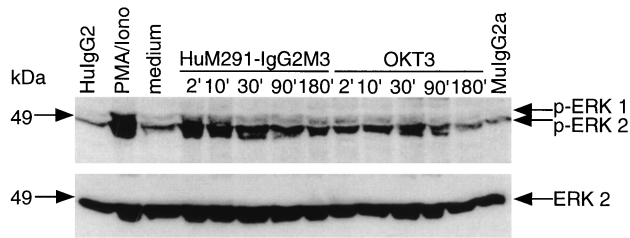


FIGURE 5. HuM291 induces greater and more sustained ERK phosphorylation in activated human T cells than OKT3. Cells were cultured in medium alone or were stimulated at 37°C with 50 $\mu\text{g}/\mu\text{l}$ HuM291 or 50 $\mu\text{g}/\mu\text{l}$ OKT3 for the indicated time in minutes. Activated cells stimulated with PMA (15 ng/ml) plus ionomycin (700 ng/ml) for 10 min were analyzed as a positive control for ERK phosphorylation. Cells receiving 50 $\mu\text{g}/\mu\text{l}$ anti-human IgG2 or 50 $\mu\text{g}/\mu\text{l}$ anti-mouse IgG2a for 10 min were analyzed as additional negative controls for ERK phosphorylation. Whole-cell lysates were prepared, and 20 μg of protein was separated by SDS-PAGE as described in *Materials and Methods*. ERK phosphorylation (p-ERK1 and p-ERK2) was determined by Western blotting with an anti-phospho-ERK mAb (*top panel*). Subsequently, the same nitrocellulose membrane was stripped and reprobed with an anti-ERK2 mAb to determine ERK2 expression for each time interval and condition (*bottom panel*). A representative experiment of three performed is shown. The M_r of standard proteins (kilodaltons) is indicated on the left.

complexes to a greater extent than HuM291-IgG2M3 or HuM291-IgG1 (Fig. 6C). We conclude that non-FcR-binding mAbs cause slower modulation of the TCR over time. This probably contributes to more sustained T cell signaling above the threshold, which results in a greater ability of humanized anti-CD3 to cause activation-induced apoptosis.

Enhanced anti-CD3 mAb shedding from the T cell surface is associated with decreased TCR modulation

Although modulation experiments followed the fate of surface CD3 molecules after continuous exposure to anti-CD3, we used an

RIA to follow the fate of ^{125}I -labeled anti-CD3 mAb after pulse labeling Jurkat T cells. HuM291-IgG2M3 was shed at a faster rate and in greater amounts and was less likely to be internalized than HuM291-IgG1 or M291 (Fig. 7). These data indicate that HuM291-IgG2M3 has a faster off rate than HuM291-IgG1 or M291. The rank order for the OKT3 panel confirms that the OKT3-IgG2M3 variant is shed at a faster rate than OKT3. As expected, anti-CD3 mAbs with a faster off rate are also less likely to induce rapid and extensive TCR modulation.

Multivalent cross-linking of non-FcR-binding, humanized anti-CD3 inhibits T cell apoptosis

To test directly the hypothesis that non-FcR-binding anti-CD3 are more potent inducers of apoptosis because they modulate the TCR poorly, we measured CD3 modulation and T cell apoptosis in cells exposed to HuM291-IgG2M3 with or without antiglobulin. We found that Ab cross-linking enhanced CD3 modulation (Fig. 8A) and inhibited apoptosis of preactivated T cells (Fig. 8B). These data are consistent with the model that Fc-FcR interactions produce multivalent cross-linking of anti-CD3, stabilize binding, and enhance CD3 internalization (63). By avoiding Fc cross-linking, non-FcR-binding anti-CD3 are less potent at inducing CD3 modulation and are more likely to produce sustained signaling.

Discussion

In this study, we have demonstrated that soluble anti-CD3 ϵ mAbs can induce the death of activated human T cells, and mAb variants containing human IgG constant regions are more effective at inducing T cell death than the native murine mAbs OKT3 and M291. Among the anti-CD3 mAbs tested, the non-FcR binding, IgG2M3 variants are the least effective at inducing cytokine release and proliferation of resting peripheral T cells due to their lack of interaction with APCs (5, 7). Furthermore, we show here that apoptosis induced by soluble anti-CD3 mAbs is improved by avoiding FcR binding on APCs, since IgG2M3 and F(ab')₂ variants are the

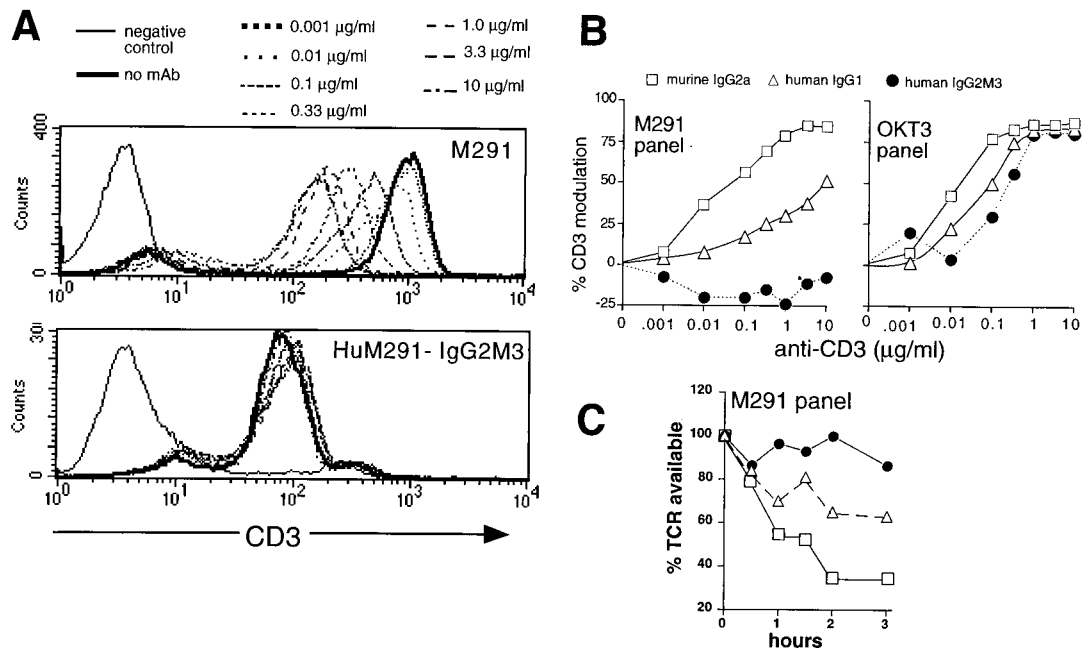


FIGURE 6. Anti-CD3 mAbs variants induce less TCR modulation than do murine anti-CD3 mAbs OKT3 or M291. PBMC were incubated at 37°C with a soluble anti-CD3 mAb in graded concentrations (A and B) or were fixed at 10 $\mu\text{g}/\mu\text{l}$ (C). After 12 h (A and B) or at different time points (C), TCR modulation was stopped by cooling the cells at 4°C. Any remaining unbound surface CD3 molecules were saturated by additional anti-CD3 mAb. All bound anti-CD3 mAb was labeled with fluorochrome-conjugated antiglobulin. Representative FACSscan histogram data (A) were used to quantitate the Ab concentration dependence of CD3 modulation as described in *Materials and Methods* (B and C).

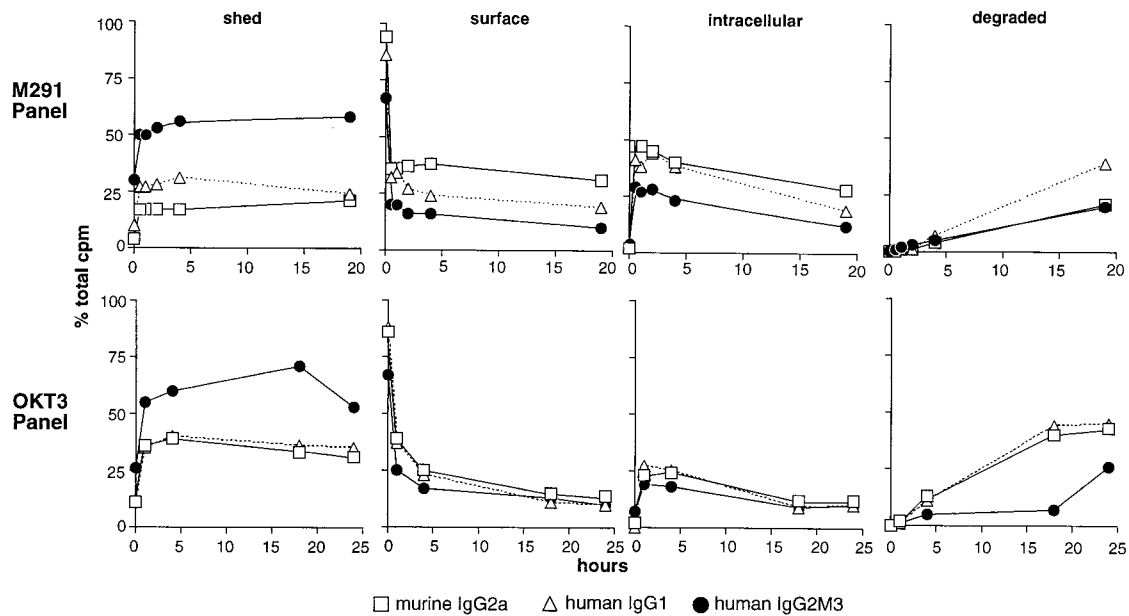


FIGURE 7. Anti-CD3 IgG2M3 variant mAbs are more likely to be shed from the T cell surface and less likely to be internalized than murine M291 or OKT3. Anti-CD3 mAbs were labeled with ¹²⁵I as described in *Materials and Methods*. Jurkat T cells were coated with ¹²⁵I-labeled mAb and then washed. After different incubation times cells were pelleted by centrifugation, and the supernatants were harvested and mixed with 25% TCA to precipitate protein-bound ¹²⁵I shed from the cell surface (*left*). The TCA-soluble portion of the supernatant represents free ¹²⁵I that derives from ¹²⁵I-labeled mAbs that have been internalized and degraded in the lysosomal compartment (*right*). Surface-bound mAb was measured after stripping the pelleted cells with hydrochloric acid wash (*left middle*). The remaining cell pellet contained the intracellular mAb (*right middle*).

most effective at inducing T cell apoptosis. Thus, soluble IgG2M3 non-FcR-binding anti-CD3 mAbs can induce apoptosis of previously activated, cycling T cells, a characteristic previously described for solid phase-bound anti-CD3 mAbs or high-dose peptide presented by competent APCs (21–23).

TCR activation-induced cell death requires the initial expression of molecules mediating apoptosis, including Fas and Fas ligand on CD4 cells, or TNF- α and TNF- α receptor on CD8 cells (29, 31, 64). On CD4 cells, simultaneous signals by TCR and Fas induce activation of caspase 8 and immediate T cell death (33, 65–68). Ag or solid phase-bound, but not soluble, anti-CD3 mAbs are required for the induction of Fas ligand expression (33, 69). Soluble anti-CD3 mAbs, however, can trigger apoptosis of CD4 cells, if the TCR has been engaged by Ag, Fas ligand is expressed, and T cells are cycling (13, 32, 33). The data presented here show that human T cells prestimulated with solid phase-bound anti-CD3 for 24 h are not in cycle and are not susceptible to death induced by restimulation with soluble anti-CD3. In contrast, T cells prestimulated for 72 h are cycling and are readily susceptible to apoptosis induced by soluble anti-CD3.

The ability to induce T cell death varied among the anti-CD3 mAbs tested and correlated with the mAb ability to induce IFN- γ release, suggesting that both functions depend on the strength of the activation signals transduced by the TCR. That the strength of T cell signaling is important for the induction of apoptosis is also supported by our observation that non-FcR-binding HuM291-IgG2M3, which induced the most T cell apoptosis, also induced brisk and durable phosphorylation of ERK2. By contrast, FcR-binding murine OKT3, which induced the least apoptosis, did not sustain phosphorylation of ERK2.

Productive engagement of the TCR by Ag or anti-CD3 mAbs induces tyrosine phosphorylation of CD3 $\gamma\delta\epsilon$ and ζ -chains, and internalization of the TCR. When the rate of TCR internalization exceeds the rate of TCR re-expression to the surface, the total number of TCR molecules on the cell surface decreases, a process designated modulation. In this study radioimmunolabeling experiments showed that HuM291-IgG2M3 had a lower ability to be internalized and a higher off rate than HuM291-IgG1 or M291. As expected, the low rate of mAb internalization correlated with the

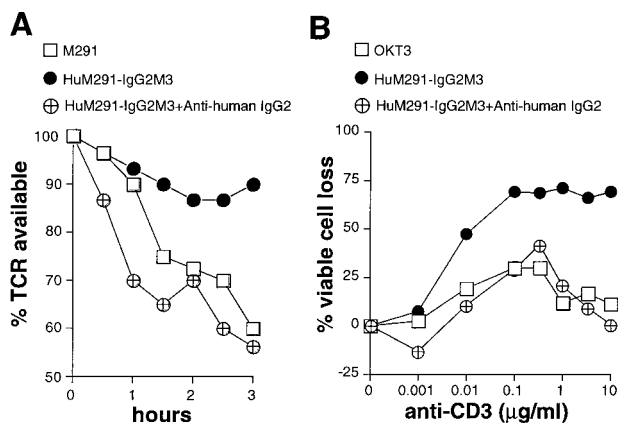


FIGURE 8. Antigliobulin cross-linking of HuM291-IgG2M3 enhances modulation of TCR and inhibits apoptosis. *A*, PBMC were incubated at 37°C with 10 $\mu\text{g/ml}$ of soluble anti-CD3 mAb with or without 15 mg/ml anti-human IgG2. TCR modulation was stopped at different time points by cooling the cells at 4°C. Any remaining unbound surface CD3 molecules were saturated by additional anti-CD3 mAb. All bound anti-CD3 mAb was labeled with fluorochrome-conjugated antiglobulin and quantitated by flow cytometry as described in *Materials and Methods*. *B*, After preactivation for 96 h by a solid phase-bound anti-CD3 mAb, BC3, T cells were harvested, and re-exposed for 20 h to soluble OKT3 or HuM291-IgG2M3 with or without antihuman IgG2 (15 $\mu\text{g/ml}$) in the presence of 50 U/ml IL-2. Cell samples were stained with PI and were acquired on a FACScan at a constant flow rate for 45 s. Cell death was determined by averaging the number of viable (PI-negative) cells remaining in triplicate samples.

poor ability to induce TCR modulation. Thus, the mAb most effective at inducing phosphorylation of ERK2, secretion of IFN- γ , and T cell death was also the least effective at modulating the TCR, whereas the mAb that was unable to sustain phosphorylation of ERK and induced low IFN- γ secretion and T cell death also induced extensive modulation of the TCR. This apparent paradox can be explained by the model that T cell activation requires signaling by a number of TCR molecules above a threshold and for a sufficient time (36, 40, 50). Therefore, anti-CD3 mAbs that induce only partial modulation of the TCR are more likely to produce sustained signaling and lead to T cell death than other anti-CD3 mAbs, which, because they induce extensive modulation of the TCR, are capable of only transient signaling.

We sought to understand how alterations in the anti-CD3 mAb structure might relate to the ability of the Ab to induce T cell death. Our observation that M291 induces significantly more T cell death than OKT3 demonstrates that the variable mAb region is important in determining the potential for induction of apoptosis, since both Abs bind to human CD3 ϵ and are murine IgG2a. It is not excluded that M291 and OKT3 mAbs might bind preferentially to either the $\gamma\epsilon$ or the $\delta\epsilon$ heterodimers associated with the TCR complex. Since phosphorylation of the γ -chain regulates TCR internalization and recycling (46, 70), preferential binding to $\gamma\epsilon$, rather than $\delta\epsilon$, may be associated with an increased rate of TCR internalization. Alternatively, decreased avidity or fine structural differences among the anti-CD3 ϵ mAbs might be responsible for altering the dynamics of the interaction with the TCR and the resulting signal.

Ab engineering by replacement of the murine Fc domain with one of human origin, or substitution of the murine amino acid sequence conserved within the variable region leads to a variable loss in CD3 binding avidity (Table II). Even if the substitution was restricted to the Fc tail, it is likely that the engineering process altered the conformation or the flexibility of the CDR (71–73), resulting in changes in mAb avidity. Anti-CD3 mAbs HuM291-IgG2M3 and OKT3-IgG2M3, which induced the most T cell apoptosis, also had the lowest K_a . However, among the entire mAb panel tested, there was no precise correlation between K_a and mAb potency in activating T cell apoptosis.

The four anti-CD3 variants with no FcR binding were more potent at inducing T cell apoptosis and release of IFN- γ than the two FcR-binding variants and the two FcR-binding murine mAbs. The results of radioimmunolabeling and flow cytometry experiments showed that non-FcR-binding mAbs were less likely to be internalized or cause TCR modulation. This led to the hypothesis that Fc-FcR interactions produce multivalent cross-linking of anti-CD3 that enhance mAb-CD3 interactions, thereby facilitating internalization of the immune complex. To test this hypothesis, we provided multivalent cross-linking of non-FcR-binding HuM291-IgG2M3 with a specific antiglobulin. Coincubation of resting cells with HuM291-IgG2M3 and anti-human IgG2 mAb forced the TCR to modulate extensively and diminished the induction of apoptosis in preactivated T cells, thus mimicking the respective responses seen after exposure to FcR-binding murine anti-CD3 alone. These observations are consistent with the concept that multivalent cross-linking of anti-CD3 via Fc-FcR interactions facilitates productive engagement and receptor internalization, thereby limiting the duration of TCR signaling and the resulting apoptosis.

Non-FcR-binding anti-CD3 mAbs are unable to activate resting T cells (5, 7). In this study, we show that non-FcR-binding anti-CD3 mAbs are especially potent at inducing apoptosis selectively in activated T cells. It is attractive to speculate that IgG2M3 variants of OKT3 or M291, or other anti-CD3 mAbs with similar properties, might be able to induce peripheral T cell tolerance in humans by selective depletion of activated pathogenic T cells.

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