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IL-1α IS PRODUCED BY T LYMPHOCYTES ACTIVATED VIA THE CD2 PLUS CD28 PATHWAYS[1]

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In addition to activation via the TCR complex, resting purified T cells can be activated to proliferate by mAb directed against the two surface molecules, CD2 and CD28. We demonstrate here that only the CD2 plus CD28 combined activation induces the expression and secretion of IL-1α, a cytokine classically considered as a monokine. In contrast, neither IL-1β nor IL-6 were produced. A second monokine, TNF-α was transiently expressed by T cells activated with either CD2 or CD28 mAb, but was expressed to higher levels and with a prolonged kinetics in cells activated by the CD2 plus CD28 combination. The prolonged expression of the IL-1α gene could account, at least in part, for the monocyte-independent and long lasting T cell proliferation induced by the CD2 plus CD28 co-stimulation. Secretion of monokines, such as IL-1α, by activated T cells, could play a regulatory role in immune responses, as well as contribute to autoimmune processes.

T cell activation requires the participation of the TCR, and of other T cell accessory molecules, as well as the presence of several soluble or membrane bound accessory cell-derived growth factors. Human T cells can be driven to proliferate in vitro by mAb directed against either the CD3-TCR complex, or other surface molecules such as CD2 (1). Both pathways are dependent on the presence of either accessory cells or exogenous cytokines, such as IL-1α and β, IL-6, and TNF-α (2–4). In a murine system, IL-1α was shown to be predominantly involved in IL-2 secretion, whereas IL-6, and, to a lesser extent, TNF-α, appeared involved in induction of IL-2 receptivity (5). Accessory cells and/or their secreted products seemed no longer required when in vitro T cell stimulation with either mAb or PHA was performed in the presence of protein kinase C activators such as the phorbol ester PMA.

A PMA-like effect was recently demonstrated when triggering purified T cell populations via the CD28 molecule (6). Under conditions where neither cross-linked CD3 mAb nor soluble CD2 mAb, used alone, activated purified resting T cells, the simultaneous triggering via CD28 led to activation, i.e., T cell proliferation and IL-2 secretion. The CD2 plus CD28 proliferation was independent from accessory (monocytic) cells, long-lasting (more than 3 wk), and autocrine and/or paracrine for IL-2 (6–8). Triggering by CD28 in the presence of PMA was also shown to induce the secretion of several lymphokines, IL-2, IFN-γ, TNF-α, and lymphotoxin (9), and to regulate the mRNA stability of four lymphokines, IL-2, granulocyte-macrophage CSF, IFN-γ, and TNF-α, induced by CD3 activation (10). Altogether, these results showed that CD28 played an important regulatory function in T cell activation, possibly through an increase in the secretion of T cell-derived cytokines (11).

The accessory cell-derived cytokines, or monokines, IL-1α, IL-1β, IL-6, and TNF-α therefore appear as major cofactors in T cell activation. We have looked for their expression and secretion by purified resting T cells activated via the CD2 pathway with or without CD28 costimulation. We report that, in contrast to triggering by either molecule alone, activation by the CD2 plus CD28 combination is associated with the expression and secretion of a monokine, IL-1α, but not of two other monokines, IL-1β and IL-6. Although IL-1α is produced by various cell types, its production, in the immune system, was classically thought to be restricted to APC (2–4). In addition, we show that another monokine, TNF-α, is expressed to high levels and with a prolonged kinetics in CD2 plus CD28-stimulated T cells. IL-1α production, coupled to the prolonged expression of the IL-2 gene in the CD2 + CD28 activation, could account, at least in part, for the monocyte-independent T cell proliferation induced by activation via the CD2 + CD28 pathways, and explain the IL-1-like effect of CD28 triggering in combination with CD2 mAb.

MATERIALS AND METHODS

Cells. PBL were isolated from healthy blood donors, and T cells purified as described previously (12). Controls for the absence of monocytic, B and NK cell contamination were performed by flow cytofluorimetry analysis using specific FITC-labeled mAb, 10G3.3 (CD14), IOB4 (CD19, Immunotech SA, Marseille, France) and NKH1 (Coulter Electronics, Hialeah, FL), respectively. Contamination by B or NK cells was less than 1%. Preparations were considered as depleted of monocytes when less than 1% of the cells were labeled with the 10G3.3 mAb, and when no proliferation was observed upon optimal stimulation with either PHA or CD3 mAb. In addition, we previously reported the absence of expression of the c-*fms* proto-

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oncogene in purified stimulated T cell preparations (12).

Long term cultures of mAb-activated T cells were obtained as follows. After an initial stimulation with the mAb described below, cells were fed twice a week with RPMI medium containing 10% FCS. They were kept at a density of 1×10^6 cells/ml and harvested once a week for RNA preparations. Proliferation was assessed by [^{3}H]TdR incorporation as well as by cell counting.

CD4$^+$ and CD8$^+$ T cell subsets were purified by negative selection using biotinylated CD4 or CD8 mAb and avidine-coated immunomagnetic beads (Immunotech SA) and stimulated for 4 days with the mAb described below.

Several cell lines (GCT, 5637, MLA 144, HSB2, Jurkat) were used as positive and negative controls for cytokine gene expression. They were grown in RPMI medium containing 10% FCS, and when indicated, treated with TPA[3] (10^{-8} M) before harvesting.

mAb and T cell activation. The following mAb were used, alone or in combination, to activate T cells: mAb 39C1.5 (rat IgG2a) and 6F10.3 (mouse IgG1) (anti-CD2) (13), mAb 248 (anti-CD28, mouse IgM), and 289 (anti-CD3, mouse IgG2a) (both obtained from Dr A. Moretta, Cancer Institute, Genoa, Italy). CD2 and CD3 mAb were used as purified mAb at 10 μg/ml each; CD28 mAb was ascites fluid used at a 1/400 dilution.

Assays for secreted and cell bound IL-1α and IL-1β. Determinations were performed with EIA (Immunotech SA), specific for IL-1α and IL-1β; their limit of detection is less than 5 pg/ml. Results were expressed in pg IL-1α/10^6 cells initially seeded. T cells submitted to the various stimuli were harvested by centrifugation at the indicated times. The 100-μl aliquots of the supernatants were tested directly for their IL-1α and β contents. Cell pellets were resuspended in 500 μl lysis buffer (0.1 M sodium phosphate, pH 7.2, containing 10 mM sodium azide, 10 mM PMSF, and 10 mg/ml of Nonidet P-40), incubated at room temperature for 5 min and centrifuged at 10,000 rpm for 15 min. The 50- or 100-μl aliquots of the supernatants were tested for their IL-1α and β contents by EIA. The detergent in the lysis buffer did not affect either the linearity or the sensitivity of the EIA. Samples of international reference standards NIBSC 86/632 (IL-1α) and NIBSC 86/552 (IL-1β) were included in all the assays.

RNA preparation and Northern blot analysis. Total RNA was prepared from cell pellets by lysis in guanidium isothiocyanate (14). The 10-μg aliquots of total RNA were submitted to electrophoresis in agarose/formaldehyde gels (14), and transferred to nylon sheets (Nytran, Schleicher & Schull, Keene, NH). Blots were prehybridized and hybridized (14), with 1×10^6 cpm/ml of [^{32}P] labeled RNA probes synthesized in vitro as described below. They were washed in 2 × SSC, 0.1% SDS, RNAse-treated (final concentrations: RNAse A, 5 μg/ml and RNAse T1, 10 U/ml) in 2 × SSC for 30 min at 37°C and washed successively in 1 × SSC, 0.1% SDS at 42°C down to 0.1 × SSC, 0.1% SDS at 42°C. They were autoradiographed on Kodak XAR-5 films (Eastman Kodak, Rochester, NY) at −80°C using intensifying screens.

RNA probes and specificity controls. All the probes were human cDNA: a 1.7-kb IL-1α insert in pGEM-3 (a gift of R. de Waal Malefyt, DNAX, Palo Alto, CA), a 0.8-kb IL-1β insert in pGEM-1, a 0.3-kb IL-2 insert in pSK (both gifts of C. Vaquero, Paris, France), and a 0.7-kb IL-6 insert in pSPT18 (a gift of H. Merz, Würzburg, Germany). The probe for TNF-α was pGTNF14 (15). Plasmid DNA were linearized with the appropriate restriction enzyme, purified by agarose gel electrophoresis, and complementary RNA was synthesized in vitro with either SP6, T3, or T7 polymerases using a transcription kit (Stratagene, La Jolla, CA) and [α^{32}P]UTP (Amersham Inte plc, Amersham, England). The specificities of the probes had been previously checked by hybridization to RNA blots prepared with RNA from control cell lines, GCT for IL-6, 5637 for IL-1α and β, HSB2 cells activated with TPA for TNF-α, and MLA 144 cells activated with TPA for IL-2. Jurkat cells were used as a negative control.

RESULTS AND DISCUSSION

Late but prolonged expression of IL-1α compared to IL-2 gene in T cells activated by CD2 plus CD28 mAb. We looked for expression of the IL-1α and IL-1β genes by purified resting T cells activated with either CD2 (anti-T11.1 + anti-T11.2) or CD28 mAb separately, or with the CD2 + CD28 combination of mAb. Triggering T cells via either CD2 or CD28 pathway alone did not induce detectable levels of expression of the IL-1α gene, for 6 to 96 h post-stimulation (Fig. 1 *A* and *B*). In contrast, CD2 + CD28 co-stimulation induced the expression of the IL-1α gene, from 48 to 96 h post-stimulation (Figure 1 *A* and *B*), but not of the IL-1β gene (see below). Densitometer scanning of the autoradiograms showed a progressive increase of the IL-1α mRNA steady-state levels, from 48 to 96 h (Fig. 1*C*).

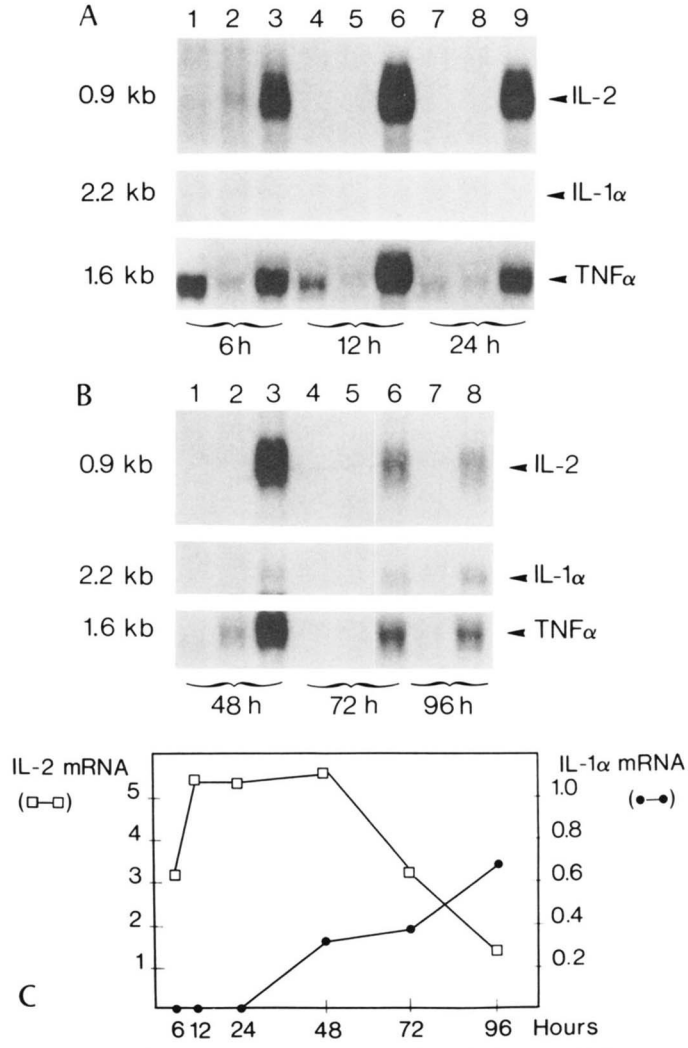

Figure 1. Kinetics of expression of the IL-2, IL-1α, and TNF-α genes by purified T cells. *A* and *B*, Purified T cells were stimulated with either CD2, CD28, or CD2 + CD28 mAb (see *Materials and Methods*), and harvested at 6, 12, and 24 h (*A*), and 48, 72, and 96 h (*B*). Ten-μg aliquots of total RNA were used to prepare RNA blots, which were hybridized as described under *Materials and Methods*. The various lanes contained RNA extracted from T cells stimulated with CD2 (*lanes 1, 4,* and *7, A, lanes 1* and *4, B*), CD28 (*lanes 2, 5,* and *8, A, lanes 2, 5,* and *7, B*), and CD2 + CD28 mAb (*lanes 3, 6,* and *9, A, lanes 3, 6,* and *8, B*). *C*, Levels of expression of the IL-2 and IL-1α genes by CD2 + CD28 stimulated T cells. Densitometer scans of the above autoradiograms were integrated, and the levels of the two transcripts (in densitometric units) were plotted relative to the levels of 28 S rRNA.

As mentioned above, APC were thought to be the main source of IL-1α (2–4). However, other cells of the immune system were shown to produce IL-1: peripheral B and NK cells stimulated with LPS (16, 17), murine (18), and human T cell clones stimulated either with Con A or CD3 mAb in the presence of TPA (19) (R. de Waal Malefyt and H. Spits, personal communication). It was to rule out the possibility that IL-1α was expressed by contaminating cells, that all our T cells preparations had been checked for absence of monocytic, B, and NK cells.

To determine the duration of the expression of the IL-1α gene, we derived and analyzed long term cultures of

[3] Abbreviations used in this paper: TPA, 12-0-tetradecanoyPphorbol-13-acetate; EIA, enzyme immune assays.

CD2 + CD28-activated T cells. Figure 2 shows that the IL-1α transcript was still present at 7 days after stimulation, but no longer detected at 14 days, although CD2 + CD28-activated T cells proliferated for up to 18 days (Table I). Expression of the IL-1α gene appeared specifically associated with the CD2 + CD28 combination, as it was not detected in T cells optimally activated with plastic-coated CD3 mAb, which proliferated and expressed the TNF-α transcript (Fig. 2). This delayed but sustained expression of the IL-1α gene suggests either a secondary effect resulting from delayed synthesis of either proteins or second messengers, or alternatively, the expansion of a T cell subpopulation.

We compared the kinetics of expression of the IL-1α gene to that of the IL-2 gene in the same preparations of activated T cells. As shown in Figure 1 *A* and *B*, activation via either pathway alone did not lead to significant expression of the IL-2 gene, for 6 to 96 h post-stimulation, a result in agreement with the absence of T cell proliferation when activation is performed in the absence of either PMA or IL-2 (8). A weak, early, and transient expression of the IL-2 gene was occasionally observed upon activation with CD28 mAb alone (Fig. 1*A*). In contrast, CD2 + CD28 activation induced an early (6 h) and prolonged expression of the IL-2 gene (Fig. 1 *A* and *B*). Densitometer scanning of the autoradiograms showed a plateau value from 12 to 48 h, with a subsequent decrease (Fig. 1*C*). The high levels of expression of the IL-2 gene, together with the duration of this expression, could explain the strong and prolonged T cell proliferation induced by this co-stimulation (8). These results additionally suggest a synergy in the induction of the transcription of the IL-2 gene by the CD2 + CD28 combination. CD28 was shown to stabilize the IL-2 transcript in T cells activated with a cross-linked CD3 mAb (10); whether it plays a similar role in the expression of the IL-2 gene upon triggering with the CD2 + CD28 combination remains to be determined.

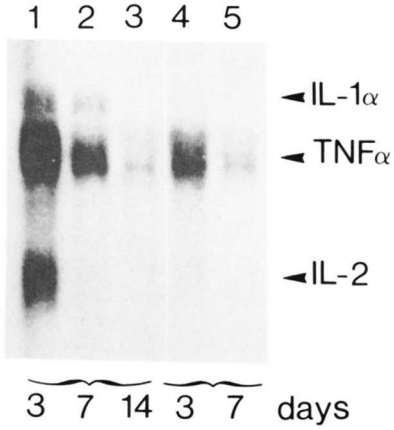

Figure 2. Long term expression of the IL-2, IL-1α, and TNF-α genes by activated T cells. Purified T cells, activated with either CD2 + CD28 (*lanes 1* to *3*) or plastic-coated CD3 (*lanes 4* and *5*) mAb, were maintained in culture as described in *Materials and Methods*. Blots were prepared with total RNA from cells harvested at 3, 7, and 14 days after initial stimulation, and hybridized as described in the legend to Figure 1.

TABLE I

Long term proliferation of activated T cells[a]

	Cell proliferation (cells × 10^{-6})			
Expt. 1[b]				
mAb	D0	D7	D14	D21
CD2 + CD28	75	89	148	10
Coated CD3	75	70	0	ND

Expt. 2	Cell proliferation (cpm × 10^{-3})		
mAb	D4	D7	D14
CD2 + CD28	50	49	0.16
Coated CD3	33.5	7.2	0
Coated CD3 + CD28	133	12.3	0.1
	Cell proliferation (cpm × 10^{-3})		
mAb	D0	D7	D14
CD2 + CD28	80	150	270
Coated CD3	80	74	0
Coated CD3 + CD28	80	855	154

[a] The proliferation of purified T cells stimulated with different mAb combinations was determined either by cell count determination or by [^{3}H]thymidine incorporation at different time intervals (day 7 to 21). D0 refers to the day of seeding of the cells. Two separate experiments with different donors are shown.

[b] The RNA preparations analyzed in Figure 2 were obtained in this experiment.

CD2 plus CD28-activated T cells synthesize and excrete IL-1α protein. The presence of the IL-1α transcript was associated with synthesis of the protein, first detectable by EIA in cell lysates at 48 h and in cell supernatants at 72 h (Table II). No IL-1α protein was detected in either cell lysates or supernatants of T cells activated with either CD2 or CD28 mAb alone. The kinetics of synthesis of the IL-1α protein thus paralleled that of its transcript. IL-1α is a potent co-inducer of several genes expressed upon T cell triggering, including IL-2, which plays a key role in T cell proliferation (5). However, this kinetics of expression favors an involvement of IL-1α in late rather than early stages of T cell activation via the CD2 + CD28 pathways. The late expression of the IL-1α gene contrasts with the early expression of other cytokine genes, such as IL-2 (Fig. 1*A*) and macrophage CSF (12), and of cytokine receptor genes, such as the IL-2R α-chain (CD25) (our unpublished observations) in CD2 + CD28 co-stimulated T cells. This kinetics of expression also contrasts with the early transcription of the IL-1α and β genes in LPS or TNF-α-stimulated mononuclear cells (20, 21).

IL-1α is produced by CD4$^+$ T cell subset. T cells can be separated into two exclusive subsets, based on the expression of the CD4 and CD8 molecules. The CD4$^+$ population produces IL-2 and most T cell-derived cytokines. Purified T cells were separated into CD4$^+$ and CD8$^+$ subsets as described in *Materials and Methods*, activated with mAb and analyzed for IL-1α production. Synthesis of IL-1α appeared almost exclusively restricted to the CD4$^+$ population (Table III), which is also the population that predominantly expresses CD28.

Expression of TNF-α but not of IL-1β and IL-6 genes by activated T cells. The expression of IL-1β, IL-6, and

TABLE II

Levels of secreted and cell-bound IL-1α in purified mAb-activated T cells[a]

mAb		12 h	24 h	48 h	72 h	96 h
None	SN	<5	<5	<5	<5	<5
	Cell	<5	<5	<5	<5	<5
CD2	SN	<5	<5	<5	<5	<5
	Cell	<5	<5	<5	<5	<5
CD28	SN	<5	<5	<5	<5	<5
	Cell	<5	<5	<5	<5	6
CD2 + CD28	SN	<5	<5	<5	20	160
	Cell	<5	<5	50	250	700

[a] The levels of secreted (SN) and cell-bound (Cell) IL-1α were determined with an EIA as described in *Materials and Methods*, and expressed in pg IL-1α/10^6 cells initially seeded.

TABLE III

Levels of secreted and cell-bound IL-1α in mAb-activated CD4⁺ and CD8⁺ T cell subsets[a]

mAb		Total T Cells	CD4⁺	CD8⁺
CD2	SN	6	<5	<5
	Cell	<5	<5	<5
CD28	SN	<5	<5	<5
	Cell	<5	<5	<5
CD2 + CD28	SN	120	9	<5
	Cell	1170	510	15
CD3	SN	ND	<5	<5
	Cell	ND	<5	<5

[a] The levels of secreted (SN) and cell-bound (Cell) IL-1α were determined by EIA (see legend to Table II) in T cell subsets purified as described in *Materials and Methods*, and expressed in pg IL-1α/10^6 cells.

TNF-α, all known as accessory cell-derived cytokines, was also analyzed. Activation with either CD2 or CD28 mAb alone, or with the CD2 + CD28 combination, did not lead to detectable expression of the IL-1β and IL-6 genes. These results were obtained with different donors, using RNA prepared at 6, 12, 24, 48, and 72 h post-stimulation, as well as at 7 and 14 days (data not shown). In addition, no IL-1β was detected in either cell supernatants or cell lysates by EIA (data not shown). Thus expression of the IL-1α gene, on the one hand, and of the IL-1β and IL-6 genes, on the other, was dissociated in T cells triggered with CD2 + CD28 mAb. This result additionally confirms that the expression of the IL-1α gene that we observed in activated T cells was not the result of monocytic contamination, as activated monocytes synthesize both the IL-1α and β transcripts, in a different (1:9) ratio (22). The expression of the two IL-1 genes, which encode two proteins that bind equally well to the different IL-1R characterized so far (23–26), is thus differentially regulated in activated T cells, as already shown in the case of monocytes (20, 27).

TNF-α is expressed by various cell types, including lymphocytes (28). CD28 triggering in the presence of PMA was reported to induce TNF-α expression (9). As shown in Figure 1, stimulation with CD28, and to a lesser extent, with CD2 mAb, induced an early (6 h) expression of the TNF-α gene. The transcript was still detected at 72 h under these conditions of activation. This expression of the TNF-α gene was not associated with proliferation of the activated cells (see above). CD2 + CD28 co-stimulation induced higher levels of TNF-α expression than either stimulus alone (Fig. 1). The transcript was still detectable 14 days after the initial stimulation with CD2 + CD28 mAb; it was also expressed late by T cells activated with coated CD3 mAb (Fig. 2). TNF-α is a potent inducer of the expression of CD25 (29, 30), and could thus contribute, in combination with IL-1α, to the sustained proliferation induced by CD2 + CD28 activation, an hypothesis suggested by the partial inhibition of this T cell proliferation by anti-TNF-α mAbs (our unpublished observations).

Altogether, our results demonstrate that T cells co-stimulated with CD2 + CD28 mAb, but not via either pathway alone, express the IL-1α gene, and synthesize and excrete the IL-1α protein. The $CD4^+$ T cell subset appears as the major producer of IL-1α. Both the IL-1α transcript and protein were only detected at late times after activation, which does not exclude the possibility that this monokine is produced earlier, at subdetectable levels. The combination of CD28 plus coated CD3 mAb also induced purified T cells to produce IL-1α (data not shown), thus showing that the production of this monokine is not restricted to the CD2 plus CD28 pathways. Similar results have recently been obtained by others (L. Aarden, personal communication).

These data, which refer to purified T cells activated by the CD2 plus CD28 co-stimulation, could be relevant to both physiologic and pathophysiologic immune responses. In particular, taking into account the recently described relationship between the CD28 T cell molecule and a B cell ligand (31), our observations, together with the already known properties of the CD28 molecule, could explain some of the parameters involved in the collaboration between Th and B cells in T-dependent Ag responses, such as the local release of cytokines such as IL-1α or IL-2, both involved in B cell proliferation and/or differentiation (31–34) in normal and/or disease states.

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