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Eur. J. Histochem.
44: 89-99, 2000
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Molecules that inhibit T-cell functions: cytochemical localization and shuttling*

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Key words: immune system T-cell functions, down-regulation, inhibitory molecules, CTLA-4, HP-F1

SUMMARY

Adaptive immune responses to antigens are mediated by specific receptors expressed on B cells (BCR's) and T cells (TCR's). Effector cells and memory cells are produced following a proliferative wave that accounts for clonal expansion. If not down-regulated, clonal expansion might lead to uncontrolled lymphoproliferation that would be harmful for the organism. Several mechanisms that account for the down-sizing of activated lymphocyte clones are briefly reviewed here. We next consider in detail one such mechanism that deals with the functional characterization and the immunocytochemical localization of two T-cell inhibitory molecules, namely the Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) and the HP-F1 antigen, both present in all T lymphocytes . CTLA-4 and HP-F1 inhibit $CD4^+$ T-helper cell proliferation and the lytic ability of $CD8^+$ T-cytotoxic cells in non-specific and in antigen-specific cytolytic assays. Interestingly, a clonal distribution exists as for the ability of CTLA-4 and HP-F1 to inhibit T-cell functions. In resting and activated T cells, both molecules are largely confined in the endosomal compartment, as shown by immunofluorescence analyses. However, upon interaction of T cells with Antigen-Presenting Cells (APC's) or with target cells that must be killed, CTLA-4 molecules are transported to the plasma membrane, at the site of cell-to-cell contact where, following interaction with ligands, they trigger inhibitory signals.

INTRODUCTION

Adaptive immune responses mediated by B and T cells via their antigen receptors (BCR's and TCR's) are the result of a sequence of events that can be summarized as follows: (i) cell activation via antigen receptor and its associated activatory and co-activatory molecules (the antigen receptor complex and the so-called second signal), (ii) entry into the cell cycle and cell proliferation leading to expansion of the antigen-specific clone, (iii) generation of effector cells and of memory cells.

A successful immune response that has led to clearance of antigen/pathogen must be terminated and, in the case of chronic antigenic stimulation (such as in autoimmune disorders or in chronic viral infections, cytomegalovirus for instance), the immune response must be anyhow contained. In the case of cell-mediated responses, it has been estimated that the doubling time of a T cell can be as short as 4.5 hours. Thus, in a week's time, one cell could multiply to almost $1x10^{12}$ cells, thereby doubling the total number of T cells in a human organism. A half of these cells would belong to a single clonotype, therefore hampering the clonal diversity and the possibility to recognize a large panel of antigens. This hypotetical example emphasizes the need for a

Presented at the 28th national Congress of the Italian Society of Histochemistry June 2-4, 1999 Camerino (Italy)

containment of lymphoproliferation in the course of immune responses.

Several mechanisms subserve this function and a major role is played by apoptosis, the programmed cell death process that occurs at several levels in the course and at the end of immune responses. Apoptosis in the immune system has been reviewed recently (Lenardo *et al.*, 1999) and is beyond the scope of our work. However, we will briefly summarize the main check points where apoptosis is triggered in order to contain the expansion of a responding lymphocyte clone. Effector cells are short-lived elements that undergo apoptosis in days or months. Plasma cells or effector T cells (either helper or cytotoxic), once the immune response has succeeded, die by starvation (i.e. lack of growth factors) and down-regulation of anti-apoptotic genes; this process is termed passive apoptosis and implies cytochrome c release from mitochondria and triggering of the caspase cascade. In the case of chronic antigenic stimulation, the same T cell expresses surface molecules of the TNF family and their ligands (e.g. TNF and TNFR-1, Fas and Fas L) the interaction of which triggers a suicidal program termed active apoptosis that again occurs via activation of caspases. Another example that underlines the role of apoptosis for the containment of cell-mediated responses is the event that occurs in the T-cell areas of peripheral lymphoid organs. Here, T lymphocytes trigger apoptosis of APC's, thus reducing or abolishing T cell exposure to antigens. This process is also mediated by TNF family members (TRAIL and its receptors DR4 and DR5).

The relevance of apoptosis for the containment of lymphoproliferation that follows antigen stimulation is shown by animal models and by a human disease. Two mice strains, *lpr* and *gld*, carry autosomal recessive mutations in the death receptors Fas or FasL, respectively. These mice develop autoimmune disorders and a lymphoproliferative syndrome. The human Autoimmune Lymphoproliferative Syndrome (ALPS) is a rare disorder characterized by autoimmunity and lymphoproliferation and is due to mutations of Fas, FasL or caspase 10 genes, all leading to an impairment of apoptosis.

INHIBITION OF T-CELL MEDIATED RESPONSES

Besides apoptosis, briefly considered in the introduction, other molecular mechanisms exist that terminate or prevent immune responses, thus limiting T-cell clonal expansion.

As mentioned already, in order to activate specifically a T cell, two signals are necessary; signal 1 is provided by the TCR complex and signal 2 (also called co-activatory signal) is mediated by the interaction between CD28 constitutively expressed by T cells and CD80/CD86 present on APC's and on some target cells that must be killed (Lenschow *et al.*, 1996; Bluestone *et al.*, 1997). Other molecules can substitute for the CD28-CD80/CD86 system (e.g. CD40). In the absence of signal 2, the T cell does not respond.

The best characterized mechanism that terminates T-cell responses and down-regulates T-cell functions (see below) is mediated by the CTLA-4 (CD152) molecule. Interestingly, CTLA-4 recognizes the same ligand of the co-activatory molecule CD28, i.e. CD80/CD86, but with much higher affinity. In both resting and activated T cells, CTLA-4 is stored largely in the endosomal compartment of the cytoplasm. However, when T cells interact with APC's or target cells, CTLA-4 is quickly shuttled to the plasma membrane, at the site of cell-to-cell contact. Given its higher affinity for CD80/CD86, it is feasible that CTLA-4 competes with CD28, thus quenching signal 2. In addition, in activated T cells, a tyrosine phosphorylated motif in the cytoplasmic tail of CTLA-4 interacts with an SHP-2 protein phosphatase which dephosphorylates critical tyrosine residues of the activatory TCR complex (ζ chains).

It is of note that mice knocked-out for CTLA-4 develop a lymphoproliferative disorder. This is similar to that observed in mice and humans with mutated genes that encode for molecules of the apoptosis machinery (Waterhouse *et al.*, 1996).

A second inhibitory receptor, that we are currently investigating in our laboratories, is a molecule recognized by the mAb HP-F1 (Saverino *et al.*, submitted). This antibody has been originally described as specific for LIR1/ILT2, an inhibitory receptor that belongs to the LIR family and that is widely expressed by several types of leukocytes, including a small subset of T cells (Colonna *et al.*, 1997). We have shown that the HP-F1 moAb also recognizes a molecule which, at variance from that originally described for LIR1/ILT2 is expressed by all T cells, both resting and activated. The molecule has been termed the HP-F1 antigen. In addi-

tion to the widespread distribution in T cells, T-cell clones that do not express LIR1/ILT2 mRNA are positive for the HP-F1 antigen. As detailed in the two following sections, both the functional role and the cellular localization of the HP-F1 antigen are very similar to those of the CTLA-4 molecule.

At variance from CTLA-4 and HP-F1, other inhibitory receptors are expressed by small subsets of T cells. These receptors, typically found on NK cells, recognize molecules of the major histocompatibility complex (MHC). A summary of inhibitory receptors expressed by some or all T cells is provided in Table I.

FUNCTIONAL STUDIES ON MECHANISMS THAT INHIBIT T-CELL MEDIATED IMMUNITY

KIR inhibitory molecules down-regulate effector functions of cytotoxic T lymphocytes

Killer cell immunoglobulin (Ig)-like receptors (KIR) comprise a family of inhibitory molecules that recognize HLA-Class I alleles belonging to the A, B and C loci. Interactions between KIR and their ligands expressed on target cells lead to delivery of inhibitory signals that prevent NK cell lysis. In normal subjects, CTL's that express KIR account for less than 5% of the T cells, but they are expanded considerably (>40%) in the reconstitution phase that follows bone marrow transplantation from three loci incompatible donors (Albi *et al.*, 1996; Ciccone *et al.*, 1996). The presence on these cells of both Class I restricted TCR's and of KIR molecules is an apparently contradictory event. In fact, HLA-Class I molecules could mediate two opposite effects on this lymphocyte subpopulation: activation, when appropriate peptides are presented to the TCR, and inhibition as a consequence of interaction between Class I molecules and KIR. Thus, the functional outcome in these cells may depend on a balance between two opposite effects. Two distinct possibilities exist (Albi *et al.*, 1996; Ciccone *et al.*, 1996). In Fig. 1 panel A, a KIR^+CTL characterized by a prevalence of the inhibitory effect mediated by KIR is depicted. Accordingly, this cell type is able to lyse HLA-Class I^- target cells and is unable to recognize antigen via TCR. In contrast, in panel B, a Class I-restricted T lymphocyte does not exhibit cytolytic NK-like function in spite of the expression of KIR. We suggest that, in this case, the TCR is able to mediate a prevailing activation signal.

The CTLA-4 (CD152) molecule down-regulates T-cell mediated cytotoxicity

At variance from KIR molecules, CTLA-4 (CD152) is expressed by all T lymphocytes (Bluestone *et al.*, 1997). Therefore, this inhibitory receptor could play a more general role in regulating function of all T cells. To investigate the ability of CTLA-4 to regulate T cell function, we analyzed a number of CTL clones. In a non-antigen-specific assay, such as the redirected killing test, anti-CTLA-4 mAb was able to inhibit activation induced by anti-CD3 or anti-CD28 or anti-CD2 mAb. It is of note that, in all clones, the same level of inhibition was not observed. In 8 clones inhibition was >40%, in 10 it ranged between 40 and 20%, and in 4 it was <20% (see Table 2). This observation suggests that a clonal heterogeneity exists as for the ability of CTLA-4 to inhibit

Table I
Inhibitory receptors and their expression on T lymphocytes

Inhibitory receptors	Ligands	Expression on T-cells
KIR Family	HLA-Class I loci and alleles	2-4%
LIR/ILT Family	HLA-Class I	10% (some family members only)
CD94/NKG2A	HLA-E	10-15%
CTLA-4 (CD152)	CD80/CD86	All T cells
HP-F1 antigen	?	All T cells

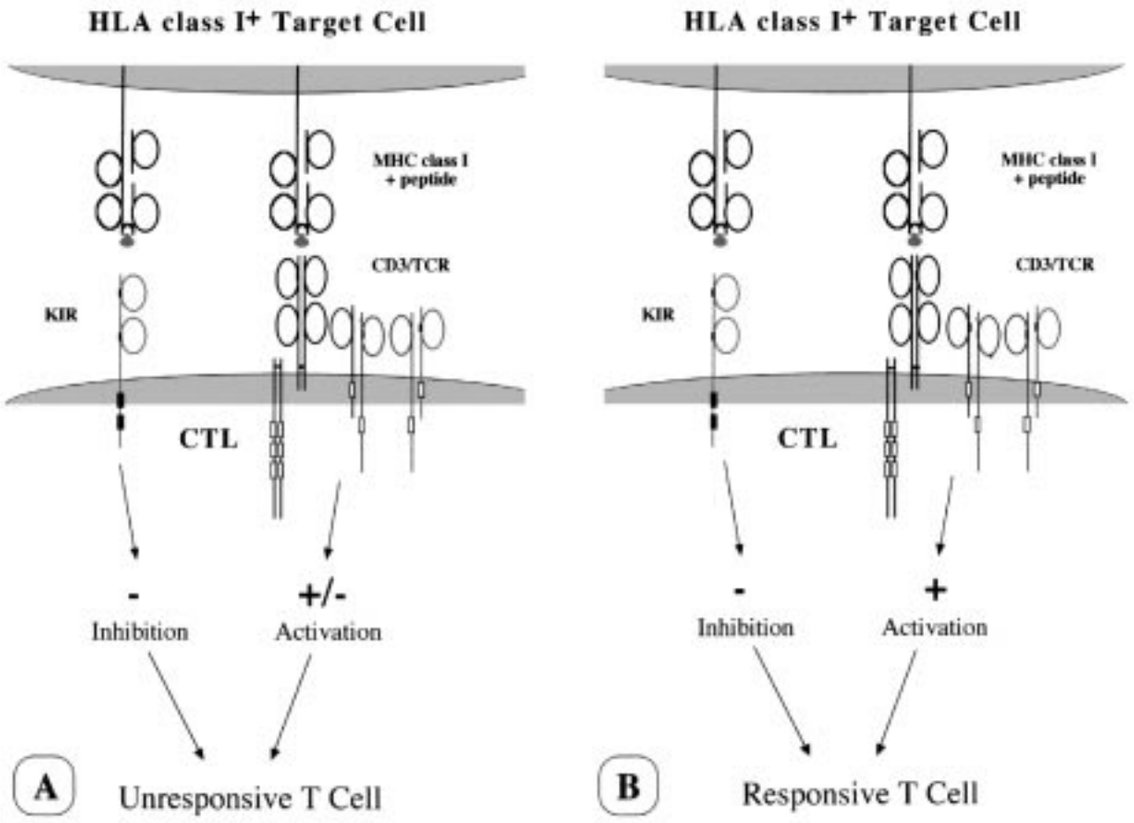

Fig. 1 - HLA Class I mediates two opposite effects on KIR$^+$ T lymphocytes.

The functional outcome in CD3$^+$CD8$^+$KIR$^+$ T lymphocytes depends on a balance between two opposite effects mediated by KIR and T-cell receptors (TCRs). In Panel A, a cytotoxic T cell (CLT) is characterized by a prevalence of the inhibitory effect mediated by the interaction of HLA-Class I molecules and KIR. In contrast, panel B shows a case where TCR mediates a prevaling activatory signal, in spite of KIR expression on T cell.

CD3/TCR- or CD28- or CD2-mediated CTL activation. To lend support to this hypothesis, we performed experiments using antigen-specific CTL's since this model closely mimics a physiologic condition. We selected eight antigen-specific T cell clones which kill autologous EBV-infected B-lymphocytes, but that are unable to lyse allogeneic B-EBV cell lines. These CTL clones were used in a cytolytic assay in which anti-CTLA-4 mAb, soluble recombinant receptor (*i.e.* CTLA-4 Ig) or anti-CD80/86 mAb were included. In this system, at variance from the redirected killing assay, cross-linking of surface molecules by mAb does not occur. Addition of anti-CTLA-4 mAb or of CTLA-4 Ig or of anti-CD80/CD86 mAb in the assay is expected to result in a blockade of receptor/ligand interactions. As a consequence, blockade of an inhibitory signal, such as that delivered by CTLA-4, should enhance lysis. A >40% increment of lysis was achieved in 3 out of 8 clones studied. Since all CTL clones do not equally display it, this feature also appears to be clonally distributed.

In conclusion, this study shows that, in some clones, inhibition is prevalent, in others activation predominates, suggesting the existence of a clonal heterogeneity. Questions arise from this observation: is only a proportion of T lymphocytes rendered anergic by this mechanism? Or else, do all

Table II
Different degree of inhibition of CD3-induced lysis by cross-linking of CTLA-4 molecules

Clones	P815[#]	P815 + αCD3	P815 + αCD3+ αCTLA4 ([##])	P815 + αCD3+αDR[###]	P815 + αCTLA-4
GE-03	22	62	29 **(82)**	60	22
BA-25	10	88	39 **(63)**	89	21
BA-99	7	95	51 **(50)**	94	9
CO-04	12	95	56 **(47)**	96	20
GA-02	3	50	29 **(45)**	55	0
CO-01	4	100	59 **(44)**	98	8
GE-02	2	99	59 **(41)**	98	1
RP-02	11	90	58 **(41)**	94	17
CO-03	10	90	59 **(39)**	88	22
BA-10	29	82	60 **(39)**	80	20
BC-28	0	85	54 **(36)**	80	n.d.
RP-01	1	55	36 **(35)**	59	4
GA-04	0	100	70 **(30)**	97	n.d.
CO-02	18	91	70 **(29)**	95	22
AK-01	8	96	74 **(25)**	94	10
GE-01	8	94	72 **(25)**	92	14
EA-08	5	100	77 **(24)**	95	27
EA-07	8	91	71 **(24)**	89	25
BA-28	2	70	58 **(18)**	74	9
GB-01	0	58	48 **(17)**	60	8
CG-08	2	46	40 **(14)**	44	1
BA-31	9	66	60 **(11)**	66	22

[#]The E:T ratio was 2:1.
[##]Percent of lysis inhibition in parenthesis was calculated using the following formula:

$$\frac{(\%\ \text{lysis with}\ \alpha CD3) - (\%\ \text{lysis with}\ \alpha CD3 + \alpha CTLA\text{-}4)}{(\%\ \text{lysis with}\ \alpha CD3) - (\%\ \text{spontaneous lysis})} \times 100$$

[###]Positive control using a mAb specific for HLA-DR (isotype IgG2a): lysis induced by anti-CD3 mAb is unaffected.

T cells, sooner or later, become anergic following chronic antigenic challenge? Although we do not have an answer to these questions, this type of regulation could play a crucial role in autoimmunity and in the prevention of an uncontrolled proliferation of T lymphocytes, as it occurs in CTLA-4 knockout mice (Waterhouse *et al.*, 1996).

The HP-F1 antigen down-regulates T cell functions

The HP-F1 antigen inhibits CD3/TCR-mediated activation in both $CD4^+$ and $CD8^+$ clones (Saverino *et al.*, submitted). This observation has been achieved by using non antigen-specific tests such as the redirected killing assay for CTL or a prolif-

Table III

Different degree of inhibition of CD3-induced proliferation by the HP-F1 mAb in its cross-linked form

T cell clones	No Ag[#]	αCD3	HP-F1 mAb	αCD4	GAM	αCD3 HP-F1 +mAb	αCD3+ HP-F1 mAb+GAM[##]	αCD3+ αCD4[###]
CT-30	6	44	4	6	7	58	6 **(99)**	49
DZ-36	6	63	3	5	12	63	8 **(98)**	65
DZ-28	5	70	4	4	4	112	6 **(98)**	80
DZ-26	23	85	27	22	20	90	24 **(97)**	82
DZ-05	46	112	50	45	45	132	50 **(94)**	123
BC-06	33	79	34	35	34	89	36 **(93)**	81
DZ-04	2	72	2	3	3	89	11 **(88)**	79
DZ-18	5	24	6	6	6	24	8 **(86)**	24
CT-36	29	82	32	30	28	121	40 **(79)**	74
DZ-08	17	38	18	19	17	45	24 **(69)**	40
DZ-19	3	30	5	4	4	37	12 **(66)**	32
SB-08	3	44	5	4	3	37	28 **(40)**	45
DB-05	41	136	39	41	42	125	99 **(39)**	141
DZ-37	17	43	15	15	14	51	33 **(39)**	45
SB-24	6	37	4	8	7	37	27 **(34)**	35
FM-04	43	122	38	40	39	123	103 **(24)**	122
SB-01	0	13	1	2	1	12	11 **(21)**	11
SB-05	1	12	2	1	2	14	10 **(20)**	12
DZ-20	0	6	3	2	2	7	5 **(18)**	7
SB-33	1	11	6	2	2	15	9 **(16)**	14
SB-03	6	50	35	8	9	53	45 **(12)**	54
SB-30	1	11	1	2	1	17	10 **(3)**	12

[#]Proliferative responses are express as Kcpm (cpmx1000).

[##]The percentage of proliferation inhibition in parenthesis was calculated using the following formula:

$$\frac{\text{(Kcpm proliferation with αCD3) - (Kcpm proliferation with αCD3 + HP-F1 mAb)}}{\text{(Kcpm proliferation with αCD3) - (Kcpm proliferation with no stimulus)}} \times 100$$

[###]Positive control using a mAb specific for CD4 (isotype IgG1): proliferation induced by anti-CD3 mAb is unaffected.

eration assay for helper T lymphocytes. Similarly to that observed for CTLA-4, and according to the level of inhibition induced via HP-F1 antigen, three groups of $CD4^+$ T cell clones could be distinguished. In the first group inhibition of proliferation was >40%, in the second group it ranged

between 40% and 20% and in a third group it was negligible (<20%) (Table III). In conclusion, cross-linking of the HP-F1 mAb inhibits proliferation triggered via the CD3/TCR pathway; however this feature is variable among different clones. This suggests that a clonal heterogeneity also exists as for the ability of the receptor recognized by the HP-F1 mAb to inhibit CD3/TCR-mediated proliferation. In spite of different levels of inhibition observed for the various clones, again there was no relationship between the amount of surface HP-F1 expression and the level of inhibition. Similarly, the HP-F1 antigen down-regulates antigen recognition by $CD8^+$ cells in a clonally distributed fashion. The HP-F1 mAb increases target cell lysis mediated by CTL's, whereas its cross-linking reduces cytotoxicity (see Table IV). The HP-F1 antigen also controls responses to recall antigens such as Tetanus Toxoid (TT), *Candida albicans* (Ca) and purified protein derivative (PPD). Three groups of responders could be identified. In a first group of 4 donors the HP-F1 mAb did not affect significantly proliferative responses induced by recall antigens. In a second group of 7 donors, the HP-F1 mAb increased proliferation by blocking receptor-ligand interactions, and a decreased proliferation was observed when the HP-F1 mAb was cross-linked by GAM (goat anti-mouse immunoglobulin antiserum). In a third group of 4 donors we detected a low response to recall antigens, and the addition of the HP-F1 mAb increased sharply T-cell proliferation.

CYTOCHEMICAL LOCALIZATION AND SHUTTLING OF CTLA-4 AND HP-F1 INHIBITORY MOLECULES

As described above, both CTLA-4 and HP-F1 are mainly intracytoplasmic proteins (Fig. 2). However, the intracellular localization contrasts with their presumed function on the cell surface as plasma membrane receptors. These apparently contradictory findings may have at least two possible explanations: (i) the intracellular localization may serve as a reservoir to directionally express the inhibitory molecules on discrete surface areas (i.e. at the site of effector/target cell interaction); (ii) the intracellular localization may be the result of regulatory mechanisms of inhibitory molecule activity, based on the equilibrium between receptor storage vs degradation, and export to the cell surface vs retrieval by the endocytic pathway; the possibility that both explanations may be correct cannot be ruled out.

Plasma membrane receptor recycling and intracellular traffic are regulated by specific signals defining their fate. It is conceivable that both CTLA-4 and HP-F1 traffic may use these common pathways.

Clathrin-mediated vesicle budding is one of the possible mechanisms of protein export from the TGN (Trans Golgi Network), and of receptor endocytosis from the plasma membrane. In the clathrin vesicles, the adaptor proteins AP1, AP2 and AP3 link receptors to cargo. Specific tyrosine-based motifs (YXXØ or NPXY) in the cytoplasmic tail of receptors, mediate their interactions with the μ1 and μ2 chains of AP1 and AP2. While AP1 appears to play a role in the sorting from the TGN to endosome/lysosome, AP2 plays a role in the plasma membrane endocytic pathway (Le Borgne and Hoflack, 1998).

The demonstration that CTLA-4 is exported from intracellular stores to discrete plasma mem-

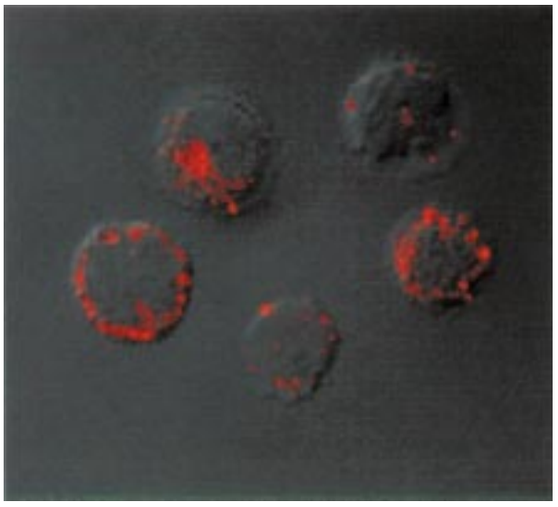

Fig. 2 - The HP-F1 antigen is localized in the cytoplasm of all T cells.

The HP-F1 antigen has been detected in all clones. As shown in this figure the HP-F1 antigen is localized in the cytoplasm of T cells. T lymphocytes were fixed with paraformaldehyde, permeabilized with saponin and incubated with the HP-F1 mAb. TRIC-conjugated goat anti-mouse antiserum and Cy3-conjugated donkey anti-goat antiserum were the secondary reagents. T cells adherent to polilysine-coated slides were analyzed by confocal microscopy.

Table IV

Different degree of inhibition of CD3-induced lysis by HP-F1 mAb in a redirected killing assay

$CD3^+CD8^+$ clones	P815[#]	P815 + αCD3	P815 + αCD3+ HP-F1 mAb[##]	P815 + αCD3+ αCD4[###]	P815 + HP-F1 mAb
CO-16	19	85	20 **(98)**	88	17
BA-28	2	53	3 **(98)**	55	0
CO-10	1	60	2 **(98)**	59	0
BC-04	20	70	22 **(96)**	47	6
BC-03	17	60	22 **(88)**	51	13
SB-05	15	88	32 **(77)**	90	0
CO-12	26	90	42 **(75)**	88	30
CO-14	0	53	14 **(74)**	55	0
EA-02	15	95	46 **(61)**	96	16
DZ-11	10	73	35 **(60)**	75	5
GA-03	8	79	38 **(58)**	n.t.	18
FG-09	10	98	48 **(57)**	98	8
CO-01	7	94	47 **(54)**	n.t.	1
CO-03	11	95	55 **(48)**	97	22
CO-04	3	85	47 **(46)**	n.t.	1
DZ-34	7	68	40 **(46)**	61	9
BC-02	0	98	55 **(44)**	99	5
AK-03	6	66	42 **(40)**	64	8
RP-02	14	90	60 **(39)**	n.t.	12
CO-01	0	67	41 **(39)**	40	0
CO-02	2	75	49 **(36)**	n.t.	1
BC-01	0	100	62 **(38)**	97	0
AK-01	6	87	58 **(36)**	n.t.	0
BA-10	0	73	47 **(36)**	60	0
GC-01	0	75	51 **(32)**	n.t.	10
RP-01	12	88	66 **(29)**	n.t.	15
CO-10	6	88	65 **(28)**	91	10
AK-02	8	96	79 **(19)**	n.t.	16
GA-01	3	77	65 **(16)**	n.t.	0
GC-10	9	88	81 **(9)**	n.t.	0

[#]The E:T ratio was 2:1.

[##]The percentage of lysis inhibition in parenthesis was calculated using the following formula:

$$\frac{(\%\ \text{lysis with}\ \alpha CD3) - (\%\ \text{lysis with}\ \alpha CD3 + \text{HP-F1 mAb})}{(\%\ \text{lysis with}\ \alpha CD3) - (\%\ \text{spontaneous lysis})} \times 100$$

[###]Positive control using a mAb specific for CD4 (isotype IgG1): lysis induced by anti-CD3 mAb is unaffected.

brane domains at the site of T lymphocyte/target cell interaction, came from immunofluorescence studies performed by Linsley *et al.* (1996). These authors, even though unable to identify the precise route followed by the exported receptor, described the nature of the intracellular stores as Golgi compartments and endosomes, due to their immunofluorescence staining with LcL or Texas red conjugated transferrin, respectively (Leung *et al.*, 1995; Linsley *et al.*, 1996).

The evidence that 11 cytoplasmic residues containing a YVKM sequence were found to be responsible for the CTLA-4 intracellular localization signal (Leung *et al.*, 1995), and the more recent demonstration (Scheinder *et al.*,1999) of the binding of the YVKM motif to the AP1 adaptor, suggest a role for AP1 in regulating the shuttling of receptor from the Golgi to degradation compartments. This mechanism may function by controlling the ratio of intracellularly stored vs exported CTLA-4.

At this point, it appears feasible that T lymphocytes may be able to specifically control the rate and the site of CTLA-4 surface expression, upon target cell recognition.

However, a down regulatory mechanism to abort the inhibitory effect of both CTLA-4 and HP-F1 has to be envisaged, in order to explain the ability of T lymphocytes to undergo several cycles of activation by target cells. To this respect, the demonstration that fluorescent antibody bound to CTLA-4 is internalized in activated T lymphocytes (Linsley *et al.*, 1996), and that clustering of HP-F1 by antibody bridging, activates both T lymphocytes inhibitory events (Saverino *et al.*, submitted) and HP-F1 internalization (Tacchetti et al, unpublished data), as demonstrated by immunogold studies at the ultrastructural level, suggest that both molecules may be retrieved upon T cell activation.

Furthermore, by coupling HP-F1 bridging antibodies with protein A-gold, we could demonstrate that, upon activation, HP-F1 is internalized and accumulates in endosome compartments. Furthermore, immunogold staining of ultrathin cryosec-

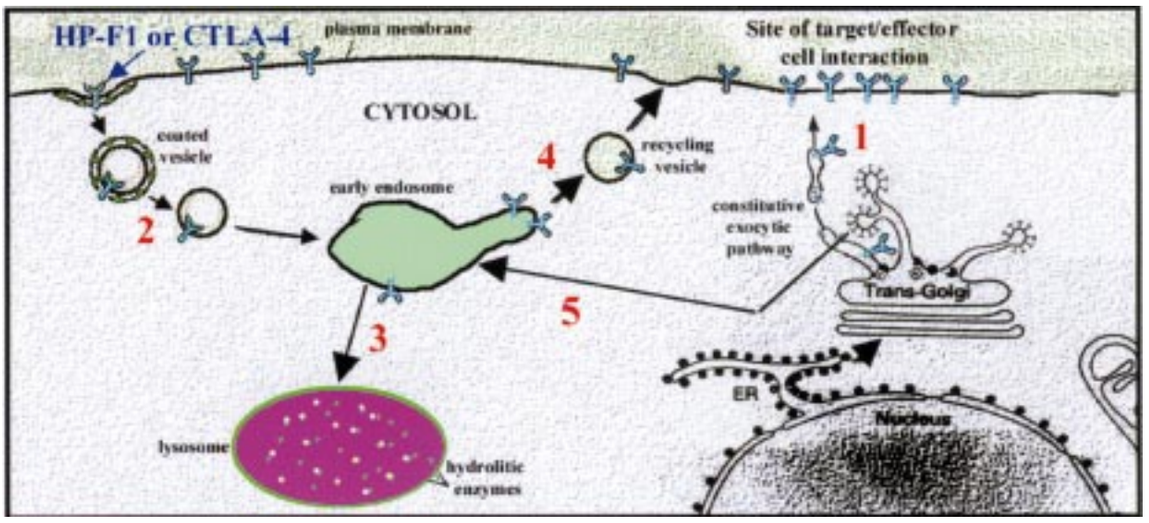

Fig. 3 - Modulation of inhibitory molecule activity can be mediated by controlling the level of surface expression.

This cartoon depicts the main possible pathways involved in receptor export to, and retrieval from the plasma membrane, as well as recycling and degradation. As described in the text, there are evidences in the literature that CTLA-4 is trafficked through some of these pathways. It is conceivable, but not proven yet, that HP-FI may follow the same pathways.

1. De novo synthesis and export to plasma membrane.
2. Receptor internalization.
3. Receptor degradation.
4. Receptor recycling.
5. TGN to endosome traffic.

tions demonstrates that at least a fraction of the internalized molecules is subsequently targeted to lysosomes (Tacchetti et al, unpublished data).

Altogether, these data suggest that the intracellular localization of inhibitory molecules may serve both to directionally express the inhibitory molecules on discrete surface areas, and to regulate inhibitory molecule activity, by controlling the rate of degradation and the ratio of exported vs internalized molecules.

The question at this point deals with the mechanisms involved in inhibitory receptor internalization. CTLA-4 has been shown, by several authors, to bind the adaptor complex AP2 (Shiratori *et al.*, 1997; Chuang *et al.*, 1997; Zhang and Allison, 1997), suggesting that the tyrosine-based internalization sequence at the cytoplasmic domain may be involved in the clathrin-mediated endocytic retrieval as well.

Furthermore, since the phosphorylation state of the tyrosine in the motif has been suggested to modulate the interaction with the μ2 chains of AP2 (Shiratori *et al.*, 1997; Bradshaw *et al.*, 1997), it is conceivable that, at least the CTLA-4 endocytic process, may be subjected to regulatory restriction.

These data suggest that the clathrin-mediated endocytosis, is the likely way of CTLA-4 retrieval from the plasma membrane. However, since no sequence is yet available for HP-F1, it is not possible at this time establish whether this process can be considered as a common pathway for all inhibitory molecule internalization.

CONCLUSIONS

Besides programmed cell death, down-regulation or termination of lymphoproliferation and activation linked to cell-mediated immune responses, largely depend on inhibitory molecules. Among them, only CTLA-4 (CD152) and the HP-F1 antigen are present in all T cells. In both resting and activated T cells these molecules are largely confined to the endosomal cytoplasmic compartment. However, following T cell/ APC or target cell adhesion that is mandatory for the effector functions of cell-mediated immunity, these molecules reach the plasma membrane at the site of cell-to-cell contact. Subsequently they are internalized and, at least in part, they reach a degradation compartment. This molecular shuttling ensures that T cell activation is successfully terminated and that, on the other hand, the cell is anergized only temporarily and ready for another encounter with antigen.

ACKNOWLEDGMENTS

This study was supported by grants from Associazione Italiana Ricerca sul Cancro (AIRC) to C.E.G. and to E.C.

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