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REVIEW

Recent advances in adult T-cell leukemia therapy: focus on a new anti-transferrin receptor monoclonal antibody

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HTLV-I is an endemic retrovirus responsible for the adult T-cell leukemia/lymphoma (ATLL). This aggressive lymphoid proliferation is associated with a bad prognosis due to the resistance of HTLV-I-infected cells to most classical chemotherapeutic agents. Here we review recent advances in ATLL immunotherapy. We particularly focus on promising data from our group, characterizing a new mouse monoclonal antibody (mAb A24) against the human transferrin receptor (TfR-1). Monoclonal antibodies to target cell differentiation markers on ATLL cells have already been proposed as therapeutic agents. However, in clinical trials acute forms of ATLL were resistant to these immunotherapies. A24 binds TfR-1 (K_d 2.7 nM) and competes with transferrin for receptor binding. It blocks the proliferation of malignant cells (TfR-1^{high}), such as HTLV-I-infected T cells but not of resting cells. A24 induces TfR-1 endocytosis in lysosomal compartments where the receptor is degraded leading to intracellular iron deprivation. In HTLV-I-infected cells, A24 targets and induces apoptosis of both chronic and acute ATLL forms, independent of antibody aggregation, antibody-dependent cellular cytotoxicity and/or complement addition. The antibody efficacy was confirmed in animal models. We are currently developing strategies to use A24 in clinical trials.
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Introduction

Adult T-cell leukemia/lymphoma (ATLL) was an aggressive lymphoid proliferation associated with the human T-cell lymphotropic virus type I (HTLV-I).[1] ATLL is the first human disease to be linked with a retroviral infection. The clinical spectrum of HTLV-I infections include tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM), uveitis and infective dermatitis in children.[2] HTLV-I is endemic in many regions of the world (for example, Southern Japan, the Caribbean, sub-Saharan west Africa and northern Iran) affecting an estimated 20–30 million individuals, but only a subpopulation of carriers (6% male and 2% female subjects) develops ATLL after a long latent period.[3] HTLV-I is transmitted intravenously, by sexual contact, or through breast-feeding from mother to child, and epidemiological evidence predicts that ATLL development occurs following childhood infection.[4]

ATLL exhibits diverse clinical features: the acute, the subacute or smoldering, the chronic forms and the ATL lymphoma.[5] In the two most aggressive forms (acute leukemia and lymphoma), the tumor syndrome comprises massive lymphadenopathy, hepatosplenomegaly, lytic bone lesions and multiple visceral lesions with skin and lung infiltration.[6] The difference between these two aggressive forms is mainly based on the peripheral blood picture: acute ATL is characterized by a massive infiltration of the peripheral blood by malignant CD4$^+$ T cells (flower cells with convoluted nuclei and basophilic cytoplasm) (Figure 1), while ATL lymphoma is characterized by the presence of less than 1% of leukemic cells in the blood smear. ATLL is preceded by oligoclonal expansions of activated T-cells that are infected with HTLV-I, probably following cell-to-cell transmission.[7] These clonal expansions probably result, at least in early stages of the disease, from expression of the viral oncogenic transactivator protein Tax, since Tax activates the viral promoter and several cellular genes.[8,9]

ATLL diagnosis is generally made on morphological analysis (histological or cytological infiltration by flower cells) and on immunophenotype.[6] No specific chromosomal abnormalities have been associated with ATLL, but the cytogenetic analysis of leukemic cells usually shows multiple nonspecific abnormalities.[10] HTLV-I serology is always positive and Southern blot analysis shows clonal integration of the provirus within tumor cells (peripheral leukemic cells or involved organ biopsies).[11]

Smoldering and chronic forms exhibit a relatively good prognosis with an indefinite or 36-months median survival time, respectively. In contrast, acute leukemic and lymphomatous forms of ATL have a very bad prognosis with a median survival of only 6 and 10 months, respectively and a 4-year survival of about 5%. Indeed, HTLV-I cells are resistant to most apoptosis-inducing agents, and treatment of ATLL patients using conventional chemotherapy has limited benefit. Poor survival prognosis factors have been described such as a high LDH value, high leukemic counts (both reflecting an important tumor burden), hypercalcemia and a poor clinical performance status.[6]

Conventional chemotherapy are efficient in promoting complete remission in a number of cases but did not improve significantly the survival rate since eventually all patients relapse. Recent studies using reduced intensity conditioning allogeneic bone marrow transplantation showed encouraging results. However, ATLL patients are often aged, have severe immunodeficiency, do not achieve complete remission or have no sibling or unrelated donors and therefore are not eligible for allogenic transplantation. Anti-retroviral therapy increases significantly the response rate particularly in chronic and acute

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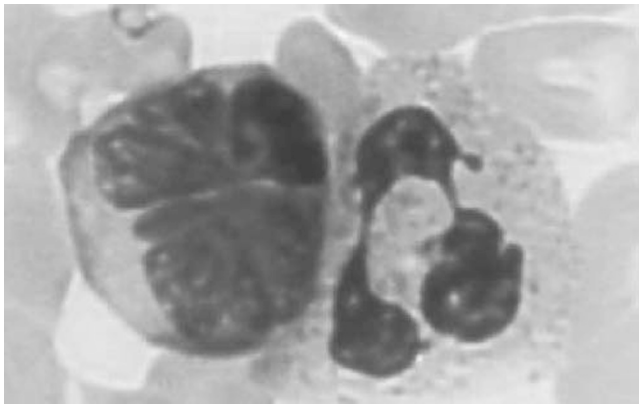

Figure 1 May Grünwald Giemsa staining of a flower cell near a polymorphonuclear neutrophil found in the peripheral blood of an ATLL patient.

forms of ATLL. These patients may experience long survival, particularly those presenting the wild type form of p53 within tumor cells. New alternative targeted therapy against NF-κB pathway (constitutively activated in ATLL cells) such as arsenic and proteasome inhibitors showed their efficacy *in vitro* alone or in combination with interferon-α and chemotherapy, respectively. They are currently tested in clinical trials. All these aspects have been extensively reviewed recently.[12] In this paper we will discuss recent advances in ATLL immunotherapy particularly presenting encouraging results that we have obtained using a novel anti-transferrin receptor antibody.

Advances in ATLL immunotherapy

Monoclonal antibodies (mAbs) are a very attractive approach against ATLL cells. Several authors have proposed to target cell differentiation markers on ATLL cells. The most important advantage of this strategy is the decreased adverse effects in comparison with chemotherapy. The first molecule used as a target for ATLL immunotherapy was the IL-2α receptor (CD25, also called the Tac antigen). However, in clinical trials anti-CD25 antibody was not effective against acute forms of ATLL.[13] Furthermore, radio-immunotherapy using yttrium labeling of the anti-CD25 resulted only in a small improvement of response rates.[14]

The newly identified autocrine loop between IL-15 and its receptor, in addition to the established loop between IL-2 and its receptor, might contribute to the proliferation of HTLV-I infected cells.[15] mAbs against CD2 have shown efficient activities in ATLL mice,[16] and antibodies to IL-15 or to its receptor inhibited the proliferation of peripheral blood mononuclear cells from patients with HTLV-I-associated myelopathy,[15] but their clinical efficacy in ATLL is still unknown.

Another tested immunotherapy is the humanized anti-CD52 mAb, alemtuzumab, currently used against chronic lymphocytic leukemia and non-Hodgkin's lymphoma. Results using this antibody were promising in an ATLL mouse model.[17] However, it mainly acts by antibody-dependent cellular cytotoxicity (ADCC), which could explain the disappointing results in immunocompromised patients (O Hermine, unpublished observation). In addition, ATLL patients treated with alemtuzumab reactivate CMV replication in virtually all cases (O Hermine, unpublished observation). A recent report shows that alemtuzumab induces cell apoptosis by a p53-independent mechanism in human clinical trial.[18] Therefore, this mAb could be used as an adjuvant therapy, in addition to AZT, to prevent selection of p53 mutated clones.

We have recently reported that malignant ATLL cells highly express surface transferrin receptor (TfR-1 or CD71) and that its expression positively correlates with the disease aggressiveness.[19] TfR-1 has already been described in other malignancies and several experiments have validated the efficacy of TfR-1 targeting to block cancer cell proliferation. We have characterized a novel anti-TfR-1 mAb (named A24) that targets and induces apoptosis of malignant cells. Thus, A24 could act both by its intrinsic capacity and by ADCC. This antibody could represent an alternative immunotherapy particularly for immunocompromised patients in which ADCC may be impaired.

The transferrin receptor in normal and malignant cells

Iron is involved in essential cellular functions such as energy transport and DNA synthesis.[20] Transferrin is the main protein involved in serum iron transport and iron uptake is essentially dependent on receptor-mediated endocytosis, involving the transferrin receptor.[21]

TfR-1 is a type II transmembrane glycoprotein largely expressed on proliferating cells.[22] This homodimeric receptor (180 kDa) allows internalization of iron-bound transferrin in clathrin-coated pits.[23,24] In endosomal vesicles, iron is then released by compartment acidification (pH 5–5.5), while apotransferrin and TfR-1 are recycled into the blood or to the cell surface, respectively (Figure 2). TfR-1 expression is strictly regulated by intracellular iron level: TfR-1 mRNA is stabilized and abundant in iron-deficient cells to increase extracellular iron uptake. This post-transcriptional regulation is allowed by the presence of iron responsive elements (IRE) in the 3′-untranslated region of the TfR-1 transcript that are recognized by two iron regulatory proteins (IRP).[25]

TfR-1 expression is ubiquitous but expression levels can be strongly different. Indeed, the majority of cells express this receptor at low level whereas highly proliferating cells, like cells of intestinal epithelium, erythroid precursors and malignant cells, express it at a greater level.[26] TfR-1 is particularly abundant on tumor cells but, most of the time, is not found on their normal counterparts, and is correlated with cancer progression and prognosis.[27,28] During hematopoiesis, TfR-1 is not detected on pluripotent stem cells but has been observed on erythroid progenitors and is again undetectable on mature erythroid cells.[29,30] In leukemia, lymphoma and myeloma, TfR-1 is expressed at higher level on circulating cancer cells.[31]

Recently, a new TfR, TfR-2, was cloned.[32,33] The extracellular domain of TfR-2 shares some homology with TfR-1 but there is no similarity between their intracytoplasmic regions. TfR-2 mRNA does not contain IRE sequences and the expression of this receptor does not appear to be regulated by intracellular iron level.[32,34] Nevertheless, TfR2 protein levels seem to be correlated positively with Tf saturation.[35] By contrast with TfR-1, TfR-2 expression is very restricted and was first described on hepatocytes and enterocytes of the small intestine only.[36] TfR-2 mRNA was detected in erythroid precursors, but expression of the protein in these cells remains controversial and TfR-2 gene inactivation does not affect erythroid cells production.[37] Interestingly, this receptor has been described on several cell lines, derived from solid tumors or myeloid hemopathies even if it is less frequent than TfR-1 on malignant cells.[38] Thus, as TfR-1 is particularly expressed on malignant cells, several groups have tried to target this receptor with mAbs in order to induce apoptosis or to reduce proliferation of cancer cells.

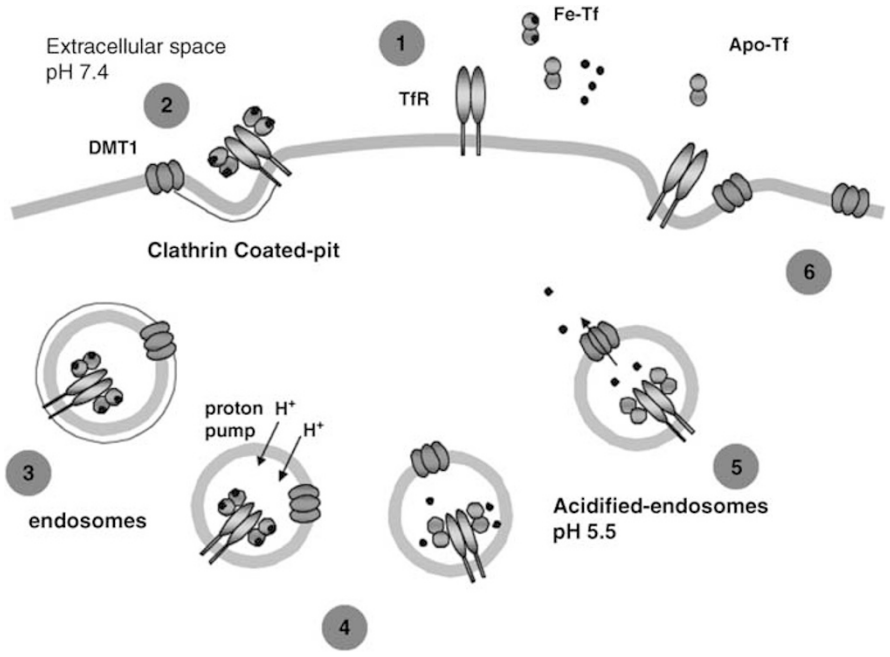

Figure 2 Endocytosis of TfR-1 induced by Fe-Tf. At physiological pH, membrane TfR-1 binds to Fe-loaded (holo)-transferrin (1). Endocytosis starts through clathrin-coated pits (2) that invaginate and give rise to vesicles containing ligand-receptor complexes (3). Following endosomal acidification (pH 5–5.5) iron is released from transferrin (4) and pumped to cytosol through the DMT1 transporter (5). TfR-1-apo-transferrin complexes are recycled to the cell membrane and dissociate at physiological pH (6).

Tumors and anti-TfR-1 antibodies

Targeting the TfR-1 with mAbs has been considered an attractive therapeutic alternative against cancer cells since 1980s. However, it still remains a field of intense investigations. In this section we will summarize different antibodies that have been used to target cancer cells trying to compare their efficiency and disadvantages.

In the initial studies targeting TfR in cancer cells, rat anti-mouse TfR-1 antibodies were used to block malignant cell proliferation. The advantage of this system is its ease of use in murine cell lines and in well-established *in vivo* murine tumor models. Several cell lines were tested, predominantly of hematopoietic origin, and anti-TfR-1 antibodies impaired proliferation of these cells by blocking iron uptake.[39,40] All antibodies did not induce the same effect and their cytotoxic efficiency differed probably due to their different isotypes. Antibody activity was apparently dependent on the cross-linking of the cell surface receptors. In this regard, IgM proved particularly efficient to stop cellular proliferation as compared to IgG.[41] Animal model studies confirmed the ability of anti-TfR-1 antibodies to prevent the development of leukemia and lymphoma and allowed the demonstration of synergistic effects with iron chelators, such as deferoxamine.[42] However, anti-TfR-1 antibodies failed to block the proliferation of the same pre-established tumors.

The majority of studies were realized with murine anti-human TfR-1 antibodies, particularly in normal and pathologic hematopoietic cells.[43] These studies confirmed the major role of receptor dimerization in the induction of apoptosis and demonstrated the higher sensitivity of leukemic progenitors compared to their normal counterparts.[44,45] Work with hematopoietic cell lines demonstrated some heterogenous sensitivity to TfR antibodies that is not well elucidated.

One murine antibody demonstrated a notable cytotoxicity against human malignancies: the murine monoclonal anti-human TfR-1 antibody 42/6. This mAb (curiously an IgA isotype) deprives cells of iron by a noncompetitive blocking of transferrin binding to its receptor.[46] 42/6 was tested on normal T cells, lymphoid and myeloid leukemia cell lines. It blocks cell proliferation in a manner reversible 48 h after its removal from the culture medium.[43] CFU-GM isolated from chronic myelogenous leukemia patients appeared more sensitive to the cytotoxic effects of 42/6 than CFU-GM isolated from healthy volunteers. First *in vitro* promising results encouraged a clinical trial in 27 patients with diverse refractory cancers. Even if there were minimal side effects, intravenous infusions of 42/6 demonstrated partial anti-tumoral effects only in three patients because of the rapid clearance of IgA in circulation and human anti-mouse IgA immune response.[47] Indeed, murine monoclonal anti-TfR-1 antibodies were found to have significant limitations such as short half-life, immunogenicity and lower effector functions because their constant regions are not recognized by the human effector system for ADCC and complement-dependent cytotoxicity (CDC). This underlined the need to develop some chimeric or humanized anti-TfR-1 antibodies.[48] Several approaches were started to develop chimeric antibodies from murine anti-TfR-1 antibodies using chicken avidin that can interact with numerous biotinylated therapeutic agents, especially against hematopoietic malignancies.[49] An anti-human transferrin receptor IgG3-avidin fusion protein was reported to inhibit the proliferation of malignant cells dependent of antibody cross-linking.[50] This anti-hTfR IgG3-Av was also shown to induce TfR internalization into lysosomal LAMP-1 positive compartments and induction of apoptosis through caspases 3, 8 and 9 activation.[51]

A24, a new anti-TfR-1 antibody and perspectives in ATLL treatment

Our group has previously reported the characterization of a new mouse mAb against the human TfR-1 (A24, an IgG2b kappa)[52]

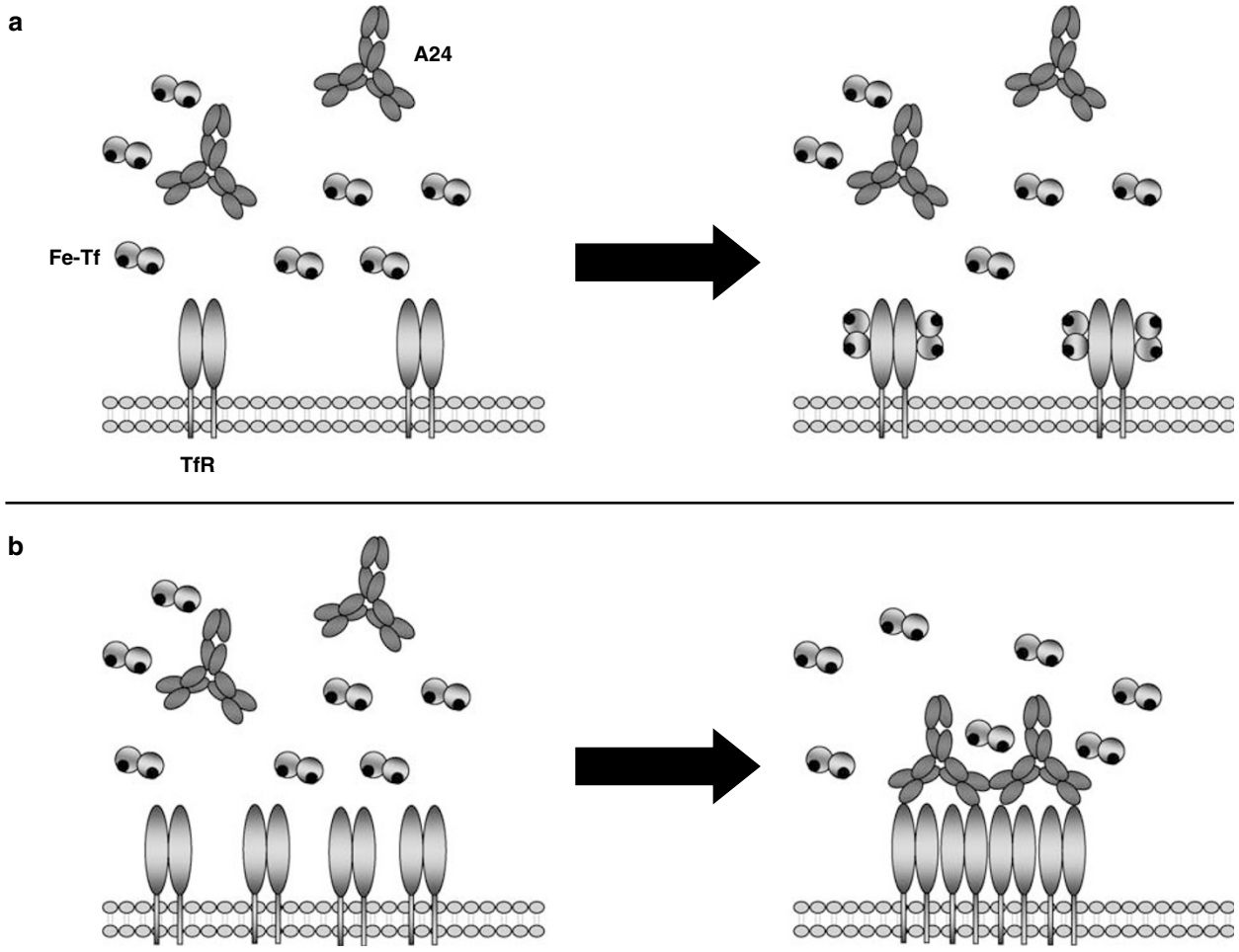

Figure 3 A24 competes with Fe-Tf for receptor binding. Surface plasmon resonance experiments were performed to determine the binding properties of A24 and Fe-Tf to the TfR-1 ectodomain. The affinity of A24 for a low-density surface of TfR-1 was three times lower than that of Fe-Tf. Thus, in cells expressing low density of TfR-1 Fe-Tf binding to its receptor is favored (**a**). However, unlike the monovalent ligand Fe-Tf, A24 possesses two binding sites, and therefore the avidity of this bivalent IgG is dependent on the available density of the receptor on the cell surface. We showed that the dissociation rate (k_{off}) of the A24/TfR-1 complex was six times lower when TfR-1 was available at higher densities. In these conditions, like in high proliferative cells (TfR-1 high positive) A24 binding to TfR-1 is favored (**b**).

that blocks the proliferation of malignant cells.[19] Using surface plasmon resonance analysis, we showed that A24 associates with TfR-1 and competes with Fe-Tf for receptor binding. We also determined that A24 has a lower affinity to TfR-1 than Fe-Tf (2.69 versus 0.98 nM, respectively). However, under high receptor density A24 binding to TfR-1 was higher than that of Fe-Tf due to avidity interactions of bivalent antibody. Thus, A24 can specifically target high proliferating (TfR-1 high) cells (Figure 3). Our first experiments, made with normal activated T cells since TfR-1 expression is required for T-cell growth, demonstrated that A24 inhibits the proliferation of activated T cells. In addition, A24 inhibited ^{55}Fe-Tf uptake inducing cell apoptosis. TfR-1 expression is tightly regulated and essential for malignant T-cell proliferation. Furthermore, since *in vitro* infection of T cells with HTLV-I induced upregulation of TfR-1 on the cell surface, we decided to evaluate the potential effect of A24 against ATL cells. We observed that HTLV-I-infected T cells from acute forms of ATL expressed TfR-1 at levels higher than those observed in chronic ATL forms. *In vitro,* A24 blocked both acute and chronic forms of ATL in contrast to IL-2-Rα-mAb (daclizumab) and inhibited the emergence of HTLV-I-infected T-cell clones. The association of IFN-α, AZT or etoposide with A24 improved the inhibition of tumor cells proliferation. Observations in normal activated T-cells were confirmed in HTLV-I infected T-cells in which A24 abrogates the TfR-1 recycling inducing iron deprivation and apoptosis. Altogether, these results demonstrated that efficient therapeutic tools to treat acute forms of ATL might be derived from A24.[19]

We have recently extended experiments to other lymphomas, particularly in Mantle cell lymphoma (MCL) that is one of the most frequent non-Hodgkin's lymphomas. TfR-1 was highly expressed on both MCL biopsies and freshly isolated tumor cells from MCL leukemic patients, whereas TfR-1 expression was low in resting B cells. A24 blocked cell proliferation without the need for antibody aggregation or cell-mediated cytotoxicity. A24 reduced and impaired TfR-1 cell surface expression and recycling by receptor sequestration in lysosomal compartments (Figure 4). In addition, one single antibody injection totally prevented xenografted MCL tumor establishment in nude mice and delayed tumor progression of established tumors, prolonging mice survival. A24 also synergized with both chemotherapeutic agents (etoposide and cytarabine) and rituximab. The effect of A24 on cell proliferation was mediated through the induction of programmed cell death determined by the caspase-3 and caspase-9 activation. Finally, A24 induced TfR-1

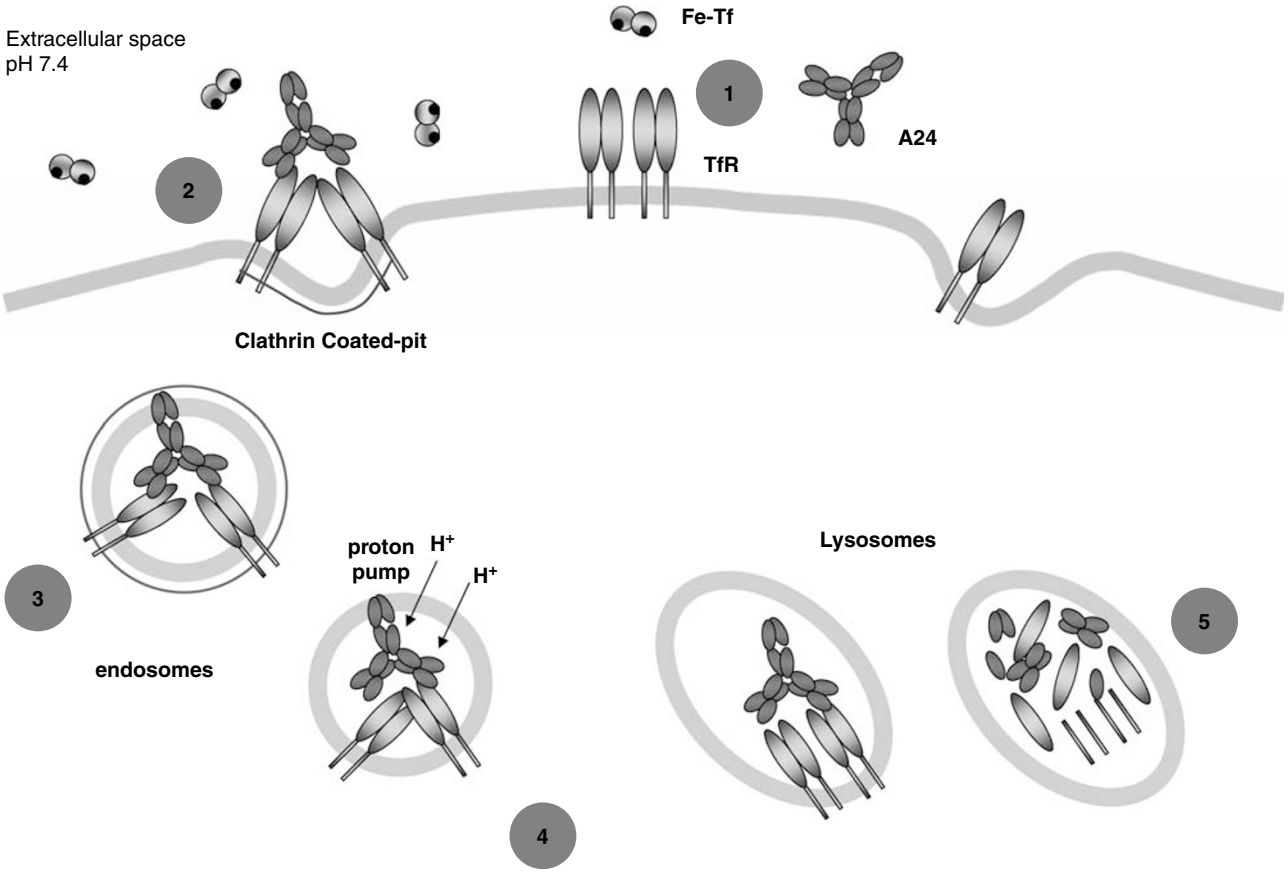

Figure 4 Endocytosis of TfR-1 induced by A24. In cell membranes containing high density of TfR-1, like in tumor cells, A24 binds to TfR-1 (1). A24/TfR-1 complexes are endocytosed through the classical clathrin-coated AP-2-dependant pathway (2–3). Endosomes are acidified (4) and TfR-1/A24 complexes instead of recycling back to cell membrane finally end up in lysosomes where they are degraded (5).

endocytosis via the clathrin adaptor protein complex AP-2 pathway followed by transport to lysosomal compartments.[53]

Altogether, these results identify A24 as a potential new therapeutic agent, alone or in combination with other mAbs and/or chemotherapeutic agents. Furthermore, our *in vivo* data suggest that A24-based treatments could be promising to prevent relapse in ATLL.

Concluding remarks

ATLL represents an important health problem since the population infected by HTLV-I is estimated to be of 20–30 millions individuals. However, since only a subpopulation of carriers will develop ATLL after a long latent period, approaches in HTLV-1 infection, prevention are still few, especially in developing countries. Despite recent advances in the use of conventional chemotherapy, allogeneic bone marrow transplantation and new-targeted therapies, ATLL therapeutic approaches are still a major challenge for clinicians because patients outcome have been disappointing. Alternatively, immunotherapeutic approaches were also suggested in ATLL but acute forms are resistant to antibodies against the α chain of the IL-2 receptor and the anti-CD52 antibody, alemtuzumab, exacerbates immunosuppression.

Efficient therapeutic tools could also be derived from the targeting of the TfR. We have recently reported a new anti-TfR-1 antibody (A24) that targets and induces apoptosis in both chronic and acute ATL forms. In addition, A24 has a synergistic effect with chemotherapy suggesting that it could be used alone or in combination with these drugs to eradicate ATLL cells. Importantly, A24 acts on its own independent of antibody aggregation, ADCC and/or complement addition. Our results, *in vivo* and *in vitro*, demonstrate that efficient ATLL therapies might be derived from this antibody. Therefore, A24 seems to be a new attractive therapy to be used in ATLL. We are at the present developing strategies to use it in clinical trials.

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