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Immune dysfunction in chronic lymphocytic leukemia T cells and lenalidomide as an immunomodulatory drug

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Developing more effective therapeutic options and treatment regimes for patients with chronic lymphocytic leukemia (CLL) is the subject of on going international clinical studies involving chemo-immunotherapy approaches that incorporate monoclonal antibodies such as rituximab (anti-CD20). Results have demonstrated remarkably improved clinical response rates for CLL patients receiving such treatment regimes.[1] To aid the pursuit of curative treatment strategies, particularly for relapsed patients who are unlikely to respond to standard approaches, novel agents for CLL are required. Immune therapy represents a promising treatment approach,[2] as demonstrated by the improved results in CLL with chemo-immunotherapy and successful demonstration of a graft-versus-leukemia effect after allogeneic stem cell transplantation leading to long-term clinical remissions.[3] However, CLL is associated with immune dysfunction and it is becoming increasingly clear that CLL tumor cells co-opt immunosuppressive mechanisms to evade immune recognition. For example, although CLL cells express tumor antigens that can be presented by major histocompatibility complex (MHC) class I and class II molecules, an effective immune response is not elicited against the tumor cells.[4,5] This likely contributes to the clinical pattern of a progressively growing tumor population over time. The failure to mount an effective immune response can be explained, in part, by a lack of effective antigen presentation, as manifested by low levels of expression of adhesion and co-stimulatory molecules essential for the induction of effective immune responses. In addition, CLL cells are known to secrete immunosuppressive cytokines such as interleukin (IL)-6 and IL-10. Thus, repairing the immune dysfunction in CLL is an essential step in order to harness and promote immune cell-mediated anti-cancer responses.

A new agent that is being used in CLL and is receiving considerable interest is the second- generation immunomodulatory drug, lenalidomide (Revlimid; Celgene). Lenalidomide is designed to enhance the immunological and anti-cancer properties of its parent drug thalidomide, while attenuating neurotoxic adverse reactions. Lenalidomide has been shown to be clinically effective as a single agent in relapsed and refractory CLL patients,[6,7] and ongoing clinical trials are also assessing its efficacy in previously untreated patients. The precise anti-CLL mechanism of action of lenalidomide is not yet completely defined. Potential mechanisms of action include blockade of angiogenesis and pro-tumor cytokines, inhibition of stromal cell-CLL cell interactions, and enhancement of immune cell function including that of T cells, monocytes and NK cells. Of note, in contrast to lenalidomide's anti-tumor activity in multiple myeloma, no direct *in vitro* pro-apoptotic effect of lenalidomide has been observed using primary CLL cells.[8]

Uniquely in CLL, the use of lenalidomide is associated with a tumor flare reaction that has been postulated to be associated with a drug-induced, immune-mediated anti-tumor response. This tumor flare reaction is manifested as an acute onset of swelling of involved

lymph nodes with inflammation of the overlying skin, rash and fever. Aue *et al.*, in a study published in this issue of the Journal,[9] show that when relapsed patients are treated with lenalidomide this flare reaction is associated with induction of CLL cell co-stimulatory molecules and T-cell activation. Their findings highlight the need to combine the current understanding of CLL biology, including immune dysfunction, with the results of correlative functional studies in order to identify the critical mechanisms of action of lenalidomide as a new agent in CLL.

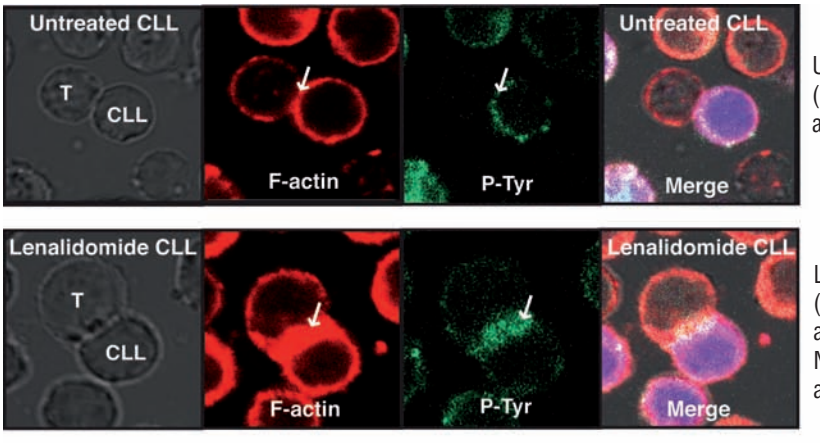
Dysregulated T-cell cytoskeletal gene expression pathways in chronic lymphocytic leukemia

We characterized T-cell defects in CLL by analyzing global gene expression of highly purified CD4 and CD8 T cells from peripheral blood from patients with CLL compared with age-matched healthy donor T cells.[10] This analysis revealed differentially expressed genes, mainly involved in cell differentiation and cytoskeletal formation pathways in CD4 T cells, and in cytoskeletal formation, vesicle trafficking, and cytotoxicity pathways in CD8 T cells. In addition, T-cell gene expression profiling using a well-characterized transgenic mouse model of CLL mice (Eμ-TCL1 transgenic mice) identified commonly dysregulated genes in both T-cell populations from CLL mice and CLL patients.[11] These studies highlighted that among the differentially expressed genes, many were involved in the actin cytoskeletal pathways.

Reversible induced T-cell dysfunction of chronic lymphocytic leukemia cells following lenalidomide treatment

The T-cell actin cytoskeleton is essential for controlling immune activation and effector activity.[12] As dysregulated actin cytoskeletal genes were identified in CLL T cells, we speculated that T cells from CLL patients would have defective immunological recognition and function. Using cell-cell conjugate functional assays with primary patients' T cells and confocal microscopy, CLL T cells were shown to exhibit defective F-actin immunological synapse formation and recognition of autologous tumor cells.[13] None of the recognized prognostic factors in CLL, such as Rai stage and IgV_H mutation status, was shown to be associated with the extent or presence of this immune synapse defect. Of note, this T-cell defect could be induced in healthy allogeneic T cells following short-term *in vitro* or *in vivo* contact with CLL cells. Following co-culture with CLL cells, previously healthy T cells showed suppressed F-actin synapse formation and defective recruitment of critical synapse signaling proteins including early T-cell signaling tyrosine-phosphorylated proteins. The finding that direct contact of CLL cells with allogeneic healthy T cells induces subsequent suppression of T-cell synapse formation may have important clinical relevance for the use of lymphocyte infusions to treat bulky CLL disease. Of importance, defects in the antigen-presenting function of CLL cells were equally as significant as the identified CLL T-cell defects in contributing to autologous immune dysfunction in CLL and treatment of both CLL cells and T cells with lenalidomide was required to repair the T cell defect. These findings are in keeping with those reported by Aue *et al.*, demonstrating upregulation in co-stimulatory molecules by CLL cells. This mechanism of immune suppression also likely contributes to defective anti-tumor T-cell responses and CLL cells were shown to induce defective CD4 T-cell proliferation and both global and antigen-specific T-cell killing of CLL target T cells. The clinical activity of lenalidomide in clinical trials involving poor prognosis CLL patients together with pre-clinical findings showing activation of immune effector cells by lenalidomide lent strong support for testing this agent in CLL functional immune synapse assays. Treatment of both CLL cells and autologous T cells with lenalidomide (0.5 μM) repaired formation of the mature F-actin immune synapse and T-cell signaling (Figure 1).[13]

Moreover, *ex vivo* lenalidomide treatment of autolo-



Untreated primary CLL patient cells (CLL cell interacting with an autologous CD8 T-cell)

Lenalidomide-treated CLL patient cells (Treated CLL cell interacting with a treated autologous CD8 T-cell)
Note: enhanced F-actin immune synapse and tyrosine phosphorylated signaling.

Figure 1. Lenalidomide repairs chronic lymphocytic leukemia T-cell immune synapse dysfunction. Treatment of both chronic lymphocytic leukemia cells and autologous T cells is required to enhance immunological synapse formation and T-cell functional signaling.

gous patients' T cells enhanced CD8 T-cell effector killing of CLL cells compared to that achieved by untreated cells. Of clinical interest, patients receiving lenalidomide (25 mg/day) exhibited enhanced *ex vivo* autologous T-cell immunological synapse formation and cytoskeletal signaling as early as 2 days after the start of treatment, which was associated with a strong tumor flare reaction and upregulation of CD40 and CD86.[13,14]

Identifying the immunomodulatory mechanisms of lenalidomide in chronic lymphocytic leukemia with functional and correlative science studies

In vitro exposure of primary CLL cells to lenalidomide has already been shown to lead to upregulation of co-stimulatory molecules CD80, CD86 and Fas ligand CD95.[15] It has been postulated that this drug-induced activation of antigen-presenting cell function of CLL cells may aid immune-mediated anti-tumor responses causing the tumor flare reaction.[8] This hypothesis was independently supported by correlative data demonstrating that the lenalidomide-induced CLL-cell activation (including upregulation of CD40 and CD86) corresponded to the degree of tumor flare experienced by patients.[14] Important data were generated showing that lenalidomide did not directly induce apoptosis, proliferation, or cytokine production (IL-6 and tumor necrosis factor-α [TNF-α]) by CLL cells. Moreover, increased proliferation and effector T-cell infiltration (CD3, CD4, CD8 and granzyme B-positive T-cells) were observed in the tonsil of a treated patient suggesting that enhanced immune effector function is an important drug-induced mechanism. CD56-postive NK cells were not increased in the excised tonsil. Furthermore, continuous treatment of relapsed or refractory CLL patients with lenalidomide was associated with long-term stable or increased numbers of T cells in the peripheral blood and early enhanced T-cell activation (day 7) as measured by soluble IL-2 receptor levels.[7] It appears that clinical responses are better in patients who experience a tumor flare reaction than in patients who do not, suggesting that the occurrence of this drug-induced side-effect may be linked to the drug's efficacy.[16]

Lenalidomide has also been shown to activate NK cells and one of its postulated mechanisms of action is increased antibody-dependent cellular cytotoxicity (ADCC). Thus, lenalidomide would seem an attractive therapeutic agent to add to rituximab treatment, which is known to induce ADCC of CD20-expressing CLL cells. However, a recent laboratory study suggested a potential antagonism between these two agents if used simultaneously with CLL cells, since lenalidomide down-regulated CD20 causing a reduction in NK cell-mediated ADCC of rituximab-treated CLL cells. On the other hand, separate pre-treatment of purified allogeneic or autologous NK cells with lenalidomide resulted in subsequent enhanced rituximab-mediated ADCC of CLL cells.[17] This *in vitro* functional work suggests a potential need for alternative sequencing regimes in CLL clinical trials using these two agents and highlights the importance of pre-clinical functional investigations of new agents in CLL using primary patients' T cells. A

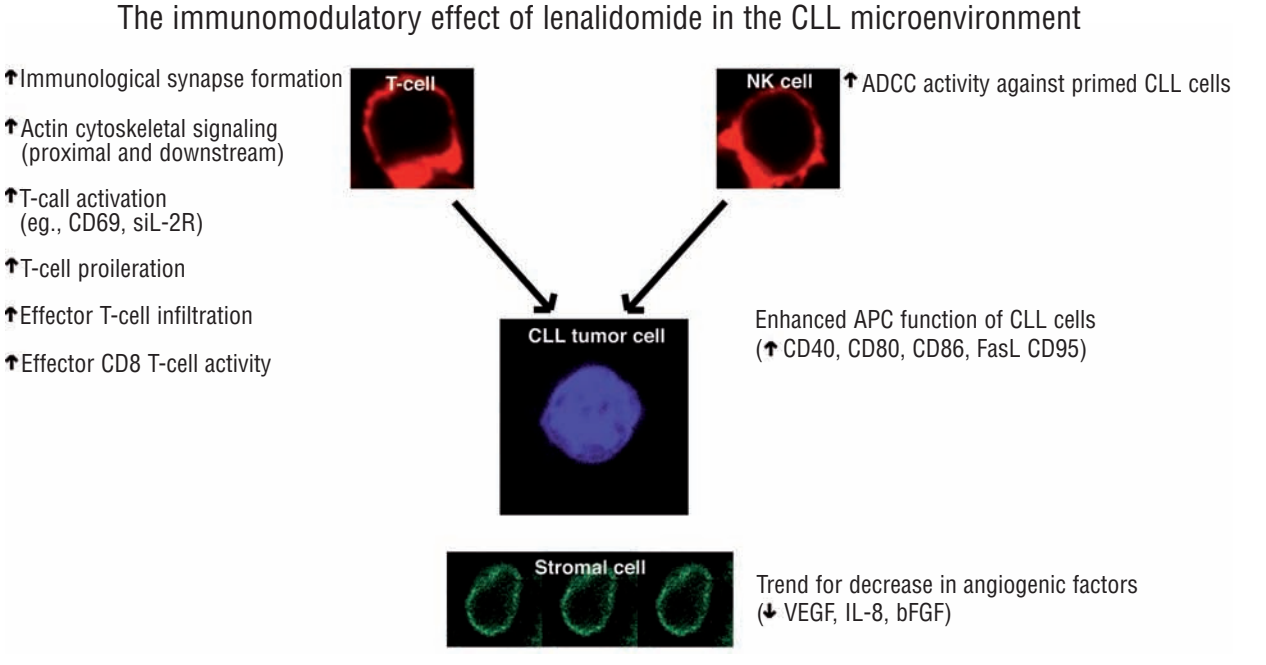


Figure 2. Lenalidomide restores T-cell actin cytoskeletal signaling (synapse formation and functional activity) and enhances antigen-presenting function of chronic lymphocytic leukemia cells (upregulation of co-stimulatory molecules). NK-mediated ADCC of rituximab-treated chronic lymphocytic leukemia cells is enhanced by pre-incubation of NK cells with lenalidomide before exposure to rituximab-exposed chronic lymphocytic leukemia cells. The immunomodulatory effect of lenalidomide on stromal cells and angiogenic status remains to be fully elucidated.

separate study demonstrated that lenalidomide enhanced NK cell-mediated and monocyte-mediated ADCC of rituximab-treated CD20 tumor cells and primary CLL cells.[18] Importantly, NK cells were pretreated separately with lenalidomide before being added to cytotoxicity assays with rituximab-treated CLL cells. Moreover, the use of lenalidomide in these experiments was noted to enhance effector T-cell and monocyte-recruiting chemokines such as monocyte chemoattractant protein-1.[18] Overall, these functional studies have suggested that treatment regimens with rituximab and lenalidomide may act synergistically to enhance immune-mediated anti-CLL responses if the timing and sequencing strategies are optimized. Of interest, the humanized CD40 antibody SGN-40 in combination with lenalidomide has been found to enhance direct apoptosis and predominantly NK-mediated ADCC against primary CLL cells.[19]

The study by Aue *et al.* shows that lenalidomide upregulates the antigen-presenting function of CLL cells (2-fold increase in CD80 and CD95 expression) in agreement with the findings of the studies discussed above. In addition, the authors suggest that lenalidomide induced autologous T-cell activation, as measured by CD69 expression, and that this correlated strongly with CD80 upregulation of CLL cells. Importantly, enhancement of T-cell activity and CLL antigen-presenting cell function by lenalidomide was shown to be CLL-specific with minimal activity when control healthy cells were used. Of note, early into treatment lenalidomide increased inflammatory cytokines, including TNFα, IL-8 and IL-1Rα, in patients' serum. The cytokine release syndrome has been observed previously and is associated with immune activation and tumor flare.[7] Further correlative studies are required to identify the source and effect of these cytokines during CLL treatment. Aue et *al.* raise the question of the role of immune activation in the clinical activity reported in CLL. Analysis of patients' lymph node biopsies on day 8 of treatment showed slightly increased CD3 T-cell counts, compared to pre-treatment levels, in two patients and relatively no change in nine patients. Few NK cells and macrophages were identified with no changes following treatment. They further showed that the tumor flare-associated cytokine release correlated strongly with enhanced upregulation of co-stimulatory molecule CD80 and activation marker CD69 on T cells. Moreover, CD80 upregulation was correlated with rapid clearance of CLL cells from the peripheral blood. However, CD69 upregulation on T cells, the presence of cytokine release factor or tumor flare were not found to correlate with leukemic cell clearance *in vivo*. According to the authors, these data indicate that T-cell activation and anti-CLL activity are separate effects, providing a rationale for the use of immunosuppressive agents such as glucocorticoids and purine analogs in combination with lenalidomide in the clinic. We would recommend caution when basing clinically relevant conclusions on single phenotypic measurements such as serum CD69 levels as a marker for T-cell function. The tumor flare reaction has been linked to better clinical responses and immune activation.[16] Moreover, our own data suggest that it is not just T-cell number or a phenotypic marker that is critical, since the ability of tumor-infiltrated T-cells to regulate and polarize actin cytoskeletal signaling is also functionally important for lenalidomide-induced T-cell anti-CLL activity.

Conclusions and future work

Clearly, lenalidomide has a multitude of biological effects on multiple cell targets that likely contribute to its anti-tumor activity (Figure 2). Management of the tumor flare reaction has been controversial since it has not been clear whether impeding this response might also reduce clinical responses.[16] Our own *ex vivo* functional data show that a lower pharmacological concentration (0.5 μg) of lenalidomide can significantly improve immune synapse formation and effector function, suggesting that lower doses of this agent may result in a more acceptable toxicity while retaining immunomodulatory activity. Rigorous correlative science functional studies, such as the one by Aue *et al.*, will certainly help to identify the mechanism of action of lenalidomide. This immunomodulatory drug has been shown to repair defective T-cell synapse formation and anti-CLL activity *in vitro* and *in vivo*.[11,13] In addition, functional assays with primary cells treated with lenalidomide have shown that this agent has a synergistic anti-CLL activity with rituximab when these treatments are administered sequentially.[17] These findings, together with the data showing that lenalidomide does not directly induce apoptosis of CLL cells, suggest that lenalidomide may be working through an immunostimulatory effect. Future studies should address the effect of lenalidomide on other cells in the CLL microenvironment, including stromal cells, and on other disease factors such as angiogenic status. Collectively, the studies with lenalidomide suggest that concurrently targeting the tumor cell itself with chemotherapy or monoclonal antibodies and targeting the microenvironment with lenalidomide represents an exciting new therapeutic strategy in CLL.

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