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LETTER TO THE EDITOR

Therapeutic Toll-like receptor agonists directly influence mouse and human T cell lymphoma cell viability and cytokine secretion

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Mycosis fungoides (MF) and Sézary syndrome (SS) are the most common forms of cutaneous T cell lymphoma (CTCL), and most frequently manifest as CD4+ CD45RO+ malignant T cells with T cell receptor clonality [1]. MF and SS are often refractory to standard chemotherapeutic treatments, which expose patients to toxicity often without substantial benefit. Because of the limited efficacy of existing treatments, several novel immune-modulating therapies for MF and SS are under investigation, including imiquimod and oligodeoxynucleotides (ODNs), ligands for Toll-like receptor 7 (TLR7) and TLR9, respectively. Imiquimod is already Food and Drug Administration (FDA) approved for the treatment of basal cell carcinoma, actinic keratoses and condyloma.

In a published case report, administration of the synthetic TLR7 ligand imiquimod successfully treated a cancerous skin plaque that was resistant to standard therapy, resulting in disease remission for at least 12 months [2]. Imiquimod treatment resulted in complete clearance of skin plaques in a patient with stage 1A CTCL with a 10-year history of disease [3]. Imiquimod treatment also eliminated skin involvement in a patient with B cell chronic lymphocytic leukemia (B-CLL) [4]. A preliminary study of imiquimod in patients with MF likewise showed a clinical response rate of 50% as measured by the clearance of plaques [5]. Both imiquimod and a synthetic CpG-containing ODN TLR9 agonist have shown the ability to enhance the host immune response in patients with CTCL [6,7]. Kim *et al*. recently reported the results of a phase I clinical trial of ODN 2006 (CpG 7909) in the treatment of a small cohort of patients with CTCL with refractory disease, showing that treatment was well tolerated and demonstrated anti-tumor activity in patients [8]. The ability of imiquimod to alter cell viability has precedent in studies of other skin cancers, where imiquimod has been found to induce apoptosis of malignant skin cells [9].

TLRs are most widely known for their role in pathogen recognition during the innate immune response. Upon detection of TLR agonists, antigen presenting cells (APCs) up-regulate costimulatory molecules and become primed to activate other immune cells. Anti-cancer studies employing TLR agonists are centered on the hypothesis that stimulation through TLR7 or TLR9, respectively, in APCs will enhance APC uptake and presentation of cancer antigens to other cells of the immune system. The alternative hypothesis that TLR ligands may directly affect the cancerous T cells themselves has not been investigated, and may have important implications for continued investigation of TLR ligands as therapeutic agents in the treatment of T cell malignancies. Several studies have demonstrated the ability of TLR ligands to affect malignant B cells with both pro- and anti-cancer outcomes, providing precedent for such a hypothesis [10].

We tested the hypothesis that TLR agonists directly alter T cell lymphoma cell biology, first using mouse T cell lymphoma cell lines as a model system (data not shown), and ultimately confirming these findings on cell lines derived from human patients with CTCL. We focused these studies by stimulating HH, a non-MF/non-SS CTCL cell line, and HuT78, a SS cell line, with ODNs and imiquimod. Cells were stimulated with these ligands for 24, 48 or 72 h and assessed for viability by propidium iodide (PI) stain. By 72 h, the cell line HH demonstrated increased culture viability upon incubation with imiquimod, whereas imiquimod increased cell death in HuT78 cell cultures [Figure 1(A)]. A dose titration of imiquimod revealed maximal viability of HH cells at 5 μg/mL imiquimod, and maximal death induction of HuT78 cells was observed with 10 μg/mL imiquimod [Figure 1(B)]. In contrast, ODNs only minimally altered cell line viability [Figure 1(A)]. These human data, taken together with mouse T cell line viability data, indicate that the TLR7 agonist imiquimod has the greatest potential to influence malignant T cell viability.

We next determined whether TLR ligands alter cytokine secretion by human CTCL cell lines. Human CTCL cell lines were stimulated for 72 h with ODNs or imiquimod, and supernatants were subjected to a bead-based cytokine assay to detect the presence of 51 cytokines, with comparison to TLR ligand-stimulated peripheral blood mononuclear cells (PBMCs) from healthy human blood as a control for ligand activity and assay function (Table I). Soluble vascular cell adhesion molecule 1 (sVCAM-1), regulated upon activation, normal T cell expressed and secreted (RANTES), soluble intercellular adhesion molecule 1 (sICAM-1), interferon inducible protein 10 (IP10) and vascular endothelial growth

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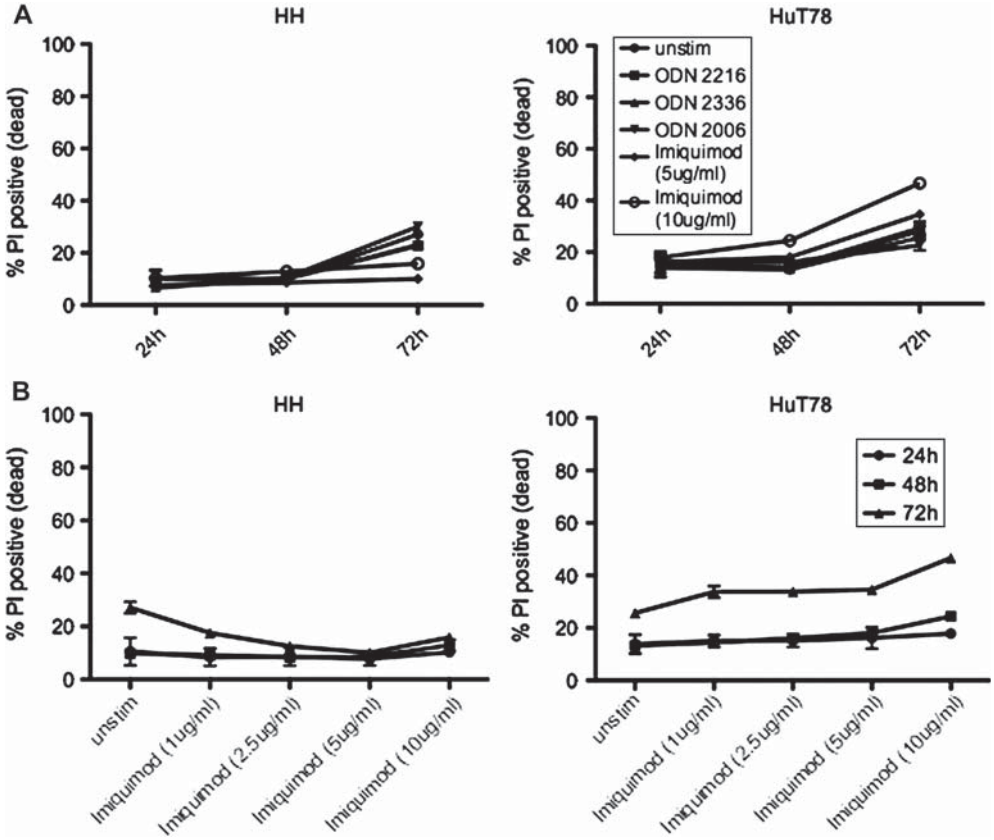

Figure 1. Imiquimod alters human CTCL cell line viability. Cells were stimulated for 24, 48 or 72 h and stained with propidium iodide (PI) to measure the percentage of dead cells by flow cytometry. Error bars represent the standard deviation of values from three independent experiments.

factor (VEGF) were among the cytokines and chemokines secreted at the highest levels by the human CTCL cell lines, with plasminogen activator inhibitor 1 (PAI-1), interleukin 10 (IL-10) and sFas ligand also secreted by the HuT78 cell line but not the HH cell line. ODNs and imiquimod altered secretion of each of these cytokines to varying degrees. As we observed with the mouse cell lines (data not shown), imiquimod lowered the baseline level of VEGF secreted by the human cell lines (HH: 30% decrease, HuT78: 27% decrease). These results are consistent with published findings that imiquimod behaves as an anti-angiogenic agent [11]. The greatest fold change observed was the seven-fold induction of PAI-1 by ODN 2006 in HuT78 cells, which was also observed in PBMCs. This soluble factor is associated with poor prognosis in cancer, where it is thought to promote metastasis and cancer cell growth [12]. This result suggests that clinical treatment of CTCL with ODN 2006 (CpG 7909) may benefit from concurrent treatment with a PAI-1 inhibitor to improve efficacy. ODNs and imiquimod lowered the level of secretion of the adhesion molecules sICAM-1 and sVCAM-1, as well

Table I. Multiplex cytokine analysis of human CTCL cell lines stimulated with TLR ligands*.

	V-CAM-1	RANTES	ICAM-1	IP10	VEGF	PAI-1	IL-10	sFas-L	IL-13	LIF	TRAIL	Resistin	IL-1α	GM-CSF	MIP1α	TNFβ
HH, unstimulated	6603	1213	737	189	199	6	1	23	0	0	15	1	8	10	26	0
HH, ODN 2216	4541	772	598	88	180	5	1	15	0	0	13	1	6	6	19	0
HH, ODN 2336	3570	838	462	135	186	4	0	15	0	0	11	1	8	4	10	0
HH, ODN 2006	4762	467	624	341	189	6	1	18	1	0	16	1	19	7	45	0
HH, imiquimod	4281	807	419	125	139	4	1	18	0	0	10	1	7	9	7	0
HuT78, unstimulated	>70 294	866	426	253	218	168	703	267	84	47	29	50	13	29	12	25
HuT78, ODN 2216	>70 294	409	222	298	193	142	620	199	62	44	23	36	13	19	3	29
HuT78, ODN 2336	>70 294	581	242	381	284	141	780	247	66	48	27	51	18	23	10	32
HuT78, ODN 2006	>70 294	500	267	612	214	1175	283	110	68	51	23	13	26	79	9	21
HuT78, imiquimod	>70 294	672	259	270	158	115	638	178	92	44	22	45	13	26	3	33
PBMC, unstimulated	1	3	79	23	3	148	1	8	0	0	10	36	1	0	5	1
PBMC, ODN 2216	11	4	106	3372	3	216	7	19	0	2	56	37	46	1	17	6
PBMC, ODN 2336	14	5	100	>5000	3	211	10	22	0	3	36	31	50	1	19	7
PBMC, ODN 2006	12	43	249	84	8	1258	50	10	0	4	101	63	6	2	179	36
PBMC, imiquimod	11	4	174	5	27	293	20	8	2	2	187	83	10	4	54	2

CTCL, cutaneous T cell lymphoma; PBMC, peripheral blood mononuclear cell; sVCAM-1, soluble vascular cell adhesion molecule 1; RANTES, regulated upon activation, normal T cell expressed and secreted; sICAM-1, soluble intercellular adhesion molecule 1; IP10, interferon inducible protein 10; VEGF, vascular endothelial growth factor; PAI-1, plasminogen activator inhibitor 1; IL-10, interleukin 10; sFas-L, soluble Fas ligand; LIF, leukemia inhibitory factor; TRAIL, TNF-related apoptosis-inducing ligand; GM-CSF, granulocyte-macrophage colony stimulating factor; MIP1α, macrophage imflammatory protein 1α; TNFβ, tumor necrosis factor β.
*Cells were stimulated with Toll-like receptor (TLR) ligands as shown. After 72 h, supernatants were harvested and analyzed for the presence of 51 soluble factors using a bead-based cytokine assay. Only data for soluble factors scoring as detectable (>15 pg/mL above background) for HH and/or HuT78 cells are shown. Note that HuT78 values for VCAM-1 were elevated beyond the scale of the reference standard curve.

as the chemokine RANTES. The secreted forms of sICAM-1 and sVCAM-1 have been implicated in the loss of epidermotropism seen in advanced cases of CTCL [13]. Malignant cells from patients with CTCL have previously been found to secrete elevated levels of sICAM-1 [14]. RANTES has been implicated in chemoattraction of healthy monocytes to the tumor environment [15]. ODNs and imiquimod modulated IP10 secretion distinctly for HH and HuT78, and all ligands decreased the levels of IL-10 and sFas ligand secreted by the HuT78 cell line, with the exception of ODN 2336 increasing the secretion of IL-10. IP10, increased upon treatment with ODN 2006, is a chemoattractant capable of recruiting immune cells to sites of inflammation, and like sICAM-1 and sVCAM-1 is thought to play a role in CTCL epidermotropism [16]. IL-10, decreased by ODN 2006 and ODN 2216 and increased by ODN 2336, inhibits anti-cancer functions of APCs by promoting their sustained immaturity [15], and is associated with disease progression in cutaneous lymphomas [17]; and sFas ligand, decreased by ODN 2216, ODN 2006 and imiquimod, is elevated in lymphoproliferative disorders where it is thought to inhibit apoptosis, resulting in poor clinical outcome [18]. These results show that TLR agonists under clinical investigation directly influence cytokine secretion patterns by malignant T cells themselves, and therefore may influence disease progression in this manner.

We present the first data demonstrating the ability of malignant mouse T cell lymphoma lines and human CTCL cell lines to respond with functional outcomes to direct TLR ligand stimulation. We show that imiquimod had the greatest impact on human CTCL cell line viability, promoting death of HuT78 cells and survival of HH cells. This differential outcome may be reflective of the variation in the CTCL subtypes of the cell lines assayed: HuT78 cells being of the SS type, and HH cells of a non-MF/non-SS type. Future studies on primary patient samples will be needed to test this hypothesis.

These data have implications for the ongoing investigation of TLR ligands as therapeutic agents in the treatment of human T cell malignancies. Specifically, TLR agonists may promote interaction of cancerous T cells with the anti-tumor infiltrating immune system, and knowledge of this interplay may be leveraged in the design of more effective therapeutic strategies. An enhanced understanding of the effects of TLR agonists on malignant T cells may provide additional targets to block, and may allow better understanding of differing patient responses to therapy. These results highlight the importance of dissecting the relationship between cells expressing TLRs and the mechanisms by which these TLR ligands function. They further demonstrate the complexity of the physiological context, revealing a need for a better understanding of how to fully leverage the immunological environment to promote favorable responses.

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