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## Selective Elimination of a Chemoresistant Side-Population of B-CLL Cells by Cytotoxic T Lymphocytes in Subjects Receiving an Autologous hCD40L/IL-2 Tumor Vaccine

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### Abstract

Side-population (SP) analysis identifies precursor cells in normal and malignant tissues. Cells with this phenotype have increased resistance to many cytotoxic agents, and in malignant disease may represent a primary drug resistant population. To discover whether drug resistant malignant SP cells are nonetheless sensitive to immune-mediated killing, we first established the presence of a malignant CD5<sup>+</sup>CD19<sup>+</sup> SP subset in the blood of 18/21 subjects with B-CLL. We examined the fate of these cells in 6 of these individuals who received autologous hCD40L/IL-2 gene-modified tumor cells as part of a tumor vaccine study. Vaccinated patients showed an increase in B-CLL-reactive T cells followed by a corresponding decline in circulating CD5<sup>+</sup>CD19<sup>+</sup> SP cells. T cell lines and clones generated from vaccinated patients specifically recognized B-CLL SP tumor cells. Elimination of SP cells is likely triggered by their increased expression of target antigens such as RHAMM following stimulation of the malignant cells by hCD40L, since CD8<sup>+</sup> RHAMM-specific T cells could be detected in the peripheral blood of immunized patients and were associated with the decline in B-CLL SP cells. Hence malignant B cells with a primary drug resistant phenotype can be targeted by T cell mediated effector activity following immunization of human subjects.

### Keywords

Immunotherapy; Chemoresistance; Side-Population; B-CLL; Cytotoxic T Lymphocytes

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### AUTHORSHIP

AEF, FVO and MKB designed and performed research, analyzed data and wrote the paper. EB, EY and AL analyzed data. GD, BS and MAG contributed to research analysis and discussion. HEH, GC and MKB contributed to protocol development, clinical correlation and patient care.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

## INTRODUCTION

Differential efflux of fluorescent vital dyes such as Hoechst 33342, can distinguish a distinct side-population (SP) on flow cytometric analysis of animal and human bone marrow cells.<sup>1,2</sup> Cells isolated by this technique can act as multi-lineage, repopulating hematopoietic stem cells in engraftment and serial transplantation studies.<sup>1</sup> SP cells have subsequently been identified in hematopoietic malignancies, including acute myeloid leukemia<sup>3</sup> and myeloma,<sup>4</sup> as well as in many solid tumors including neuroblastoma,<sup>5</sup> and cancers of ovary,<sup>6</sup> breast,<sup>7</sup> gastrointestinal tract<sup>8</sup> and lung.<sup>9</sup> Tumor-derived SP cells display biological similarities to hematopoietic stem cells, including superior proliferative and tumor-initiating capacity,<sup>10</sup> and the ability to produce SP and non-SP progeny.<sup>5</sup> Moreover, their biological characteristics render tumor SP cells more resistant to chemo- and radiotherapy than their non-SP counterparts<sup>11,12</sup>

The SP cell phenotype itself appears to be due an increased activity of transporter proteins including multi-drug resistance protein 1 (MDR1) and other ABC transporters,<sup>13,14</sup> which accelerate the export of Hoechst dyes. These same transporter proteins also reduce the intracellular concentrations, and thus the effectiveness, of many commonly used chemotherapeutic drugs.<sup>4,5,9</sup> Because tumor SP cells may therefore have the ability to resist common chemotherapeutic agents, this subpopulation may contribute to disease persistence or relapse after treatment irrespective of whether or not SP cells play a role as tumor progenitors.

While the SP phenotype may protect cells from many conventional cytotoxic drugs, their unique characteristics may also make them specifically targetable by the immune system.<sup>15,16</sup> If this were so, immunotherapy might be combined with conventional treatments for optimal anti-tumor activity. We now establish that patients with B-CLL have a circulating malignant population with an SP phenotype, and that a T cell immune response generated by these subjects during a course of immunization with hCD40L and interleukin-2 (IL-2)<sup>17</sup> gene-modified autologous tumor cells, leads to the selective disappearance of the SP subset of cells from their circulation, while leaving total circulating B-CLL cell numbers unaffected.

## MATERIALS AND METHODS

### Detection of side-population (SP) cells in peripheral blood samples from B-CLL patients

Blood was obtained from 21 patients with B-CLL who had blood collected for vaccine studies (see Table 1 for disease stage) or healthy donors on IRB approved protocols. Peripheral blood mononuclear cells (PBMC) from B-CLL patients were cultured overnight at 37°C in RPMI supplemented with 10% FBS, 2 mM L-glutamine and 20 mM HEPES (SP buffer). Cells were then counted and diluted to  $1 \times 10^6$  cells/ml in SP buffer and labeled with 5 µg/ml Hoechst 33342 dye (Sigma-Aldrich, St. Louis, MO) and incubated for 90 minutes at 37°C. Following incubation, cells were washed and stained with CD5 FITC and CD19 PE antibodies (BD Biosciences, San Jose, CA) for 30 minutes on ice. Cells were washed again and resuspended in ice cold PBS containing 2 µg/ml propidium iodide (Sigma) to exclude dead cells. We used a MoFlo high-speed cell sorter (MoFlo; Cytomation, Fort Collins, CO)

for cell analysis and sorting, in which SP and non-SP cells were characterized as previously described.<sup>1,2</sup> To validate the SP phenotype, the Ca<sup>2+</sup> channel blocker, Verapamil (50 µM) (Sigma) was used.<sup>1</sup>

### **Analysis of SP cells and anti-tumor immune responses in blood samples from patients receiving autologous B-CLL tumor vaccine**

Between 2002 and 2005, six of the B-CLL patients with an SP subset received 3–8 subcutaneous injections of autologous B-CLL tumor cells that were modified to express CD40L and IL-2 on an IRB, RAC and FDA approved study (CLIMAT).<sup>17</sup> PBMC and B-CLL cells were collected from these patients before, during and after immunization, and viably frozen and stored in liquid nitrogen. We used these samples to measure the fate of the patients' SP B-CLL cells after immunization and to measure T cell mediated immune responses directed to SP and non-SP cells. 15 additional patient samples collected for vaccine manufacture on this study or follow-up studies (CLIPA or PRIMAL) were also analyzed for SP frequency (Table 1).

### **Effect of fludarabine on B-CLL SP cells**

To determine the resistance of B-CLL SP cells to fludarabine, we examined SP frequencies in samples cultured with and without fludarabine.<sup>5</sup> B-CLL PBMC samples were incubated with and without 50 µM fludarabine (Sigma) and then cultured for 48 hours and analyzed by FACS for SP cell frequency and apoptosis/cell death using an Annexin-V/PI kit (BD).

### **RHAMM gene expression analysis of SP and non-SP B-CLL tumor cells**

To examine the effects of CD40L activation on RHAMM expression, B-CLL cells were either cultured overnight at 37°C in media alone or stimulated for 24 hours with 10 µg/ml trimeric recombinant human CD40L and 2 µg/ml trimerization enhancer (Alexis Biochemicals, Lausen, Switzerland). Following stimulation, cells were stained and sorted into CD5<sup>+</sup>CD19<sup>+</sup> SP or non-SP and RNA was extracted using RNeasy Micro Plus Kits (Qiagen, Valencia, CA). Purified RNA was then analyzed by One-Step RT-PCR (Qiagen) using gene-specific primers for RHAMM, survivin (BIRC5), fibromodulin (FMOD), MDM2 and GAPDH (SA Biosciences, Frederick, MD).

### **Generation of B-CLL reactive T cell lines and clones**

**Generation of B-CLL-specific T cell lines and clones from patient blood—**To generate B-CLL-specific T cell lines against autologous tumor cells, CD8<sup>+</sup> T cells were isolated from PBMC by high-speed cell sorting (MoFlo) then stimulated with irradiated (40 Gy), autologous hCD40L-activated B-CLL tumor cells (1:10 responder to stimulator ratio) in 45% RPMI 1640, 45% Click (Eagle Ham amino acids; Irvine Scientific, Santa Anna, CA), 2-mM GlutaMAX-1 and 5% human AB serum (Valley Biomed), referred to hereafter as T cell media (TCM-AB), supplemented with 10 ng/ml of IL-7, IL-12 and IL-21 (R&D Systems, Minneapolis, MN). After 7 days, the cultures were restimulated with hCD40-activated B-CLL tumor cells, and media were supplemented with 50 U/ml IL-2 (Proleukin; Chiron, Emeryville, CA). The T cell lines were expanded by weekly restimulation in the presence of IL-2. CD8<sup>+</sup> T cell clones were generated by limiting dilution, plated at 0.3, 1 or

3 cells/well in 96-well plates and cultured with autologous, CD40-activated B-CLL tumor cells in the presence of 100 U/ml IL-2. After two weeks, wells which possessed proliferating T cells were tested against autologous B-CLL tumor cells for specificity by IFN- $\gamma$  ELIspot. Positive clones were subsequently expanded by weekly stimulation with hCD40L activated, autologous tumor cells.

**Generation of RHAMM-specific T cell lines from HLA-A2<sup>+</sup> B-CLL patients**—To generate RHAMM-specific T cells, T antigen presenting cells (TAPC) were used to stimulate CD8<sup>+</sup> T cells isolated from vaccinated patients using stored samples from weeks 2 through 10 post-immunization. To produce TAPC, PBMC were activated on non-tissue culture treated plates pre-coated with 1  $\mu$ g/ml anti-CD3 (OKT3; Ortho Pharmaceuticals, Raritan, NJ) and anti-CD28 (PharMingen, San Diego, CA) in TCM-AB containing 100 U/ml IL-2.<sup>18</sup> After 3 days, activated T cells were harvested and expanded in IL-2 supplemented media. Prior to use, TAPC were reactivated on anti-CD3/anti-CD28 plates for 48 hours and pulsed with 1  $\mu$ g/ml ILS peptide (ILSLELMKL; Genemed Synthesis, San Antonio, TX), an HLA-A2-restricted peptide derived from RHAMM.<sup>19</sup> CD8<sup>+</sup> T cell responders were then stimulated at a ratio of 1:10, TAPC to responder cells, with irradiated (30 Gy), peptide-pulsed TAPC in media supplemented with IL-7, IL-12 and IL-21, as above. After one week of stimulation, T cell cultures were expanded by restimulation with activated, peptide-pulsed TAPC in TCM-AB supplemented with IL-2.

### Analysis of B-CLL T cell specificity and frequency

The reactivity of T cell lines and clones against B-CLL SP/non-SP target cells and to ILS peptide were assessed using IFN- $\gamma$  ELIspot assays as previously described.<sup>20</sup> For analysis of T cell reactivity against tumor cells, 1 $\times$ 10<sup>5</sup> B-CLL or ILS-specific T cells were plated in duplicate and cocultured with 1 $\times$ 10<sup>4</sup> autologous or allogeneic B-CLL tumor cells and IFN- $\gamma$  production measured. Blocking antibodies W6/32 (anti-MHC class I) and CR3/43 (anti-MHC class II) and isotype controls (Dako) were added to show MHC restriction. To test peptide specificity, ILS-specific T cells were screened using ILS or an irrelevant peptide (HLA-A2-restricted MAGE-3 peptide; FLWGPRALV) as a negative control. To analyze the reactivity of these lines and clones to the SP and non-SP subsets of B-CLL cells, equal numbers of SP and non-SP cells were sorted (10<sup>3</sup> to 10<sup>4</sup> cells) and cocultured with 1 $\times$ 10<sup>5</sup> T cells. The plates were developed and the numbers of IFN- $\gamma$  spots determined in an independent blinded fashion (ZellNet Consulting, Inc., NY, NY). To analyze the frequency of ILS-specific CD8<sup>+</sup> T cells in patient PBMC, these cells were stained with HLA-A2-restricted, ILSLELMKL-specific pentamer (ProImmune, Bradenton, FL) and Fluorotag PE in combination with CD3 PerCP and CD8 FITC (BD), and 1 million events acquired to visualize sufficient CD3<sup>+</sup>CD8<sup>+</sup>pentamer<sup>+</sup> T cells.

### T cell-tumor co-culture assays

We used co-culture assays with ILS-specific T cells generated from the peripheral blood of HLA-A2<sup>+</sup> B-CLL patients to determine whether ILS-specific T cells could eliminate B-CLL SP cells. B-CLL SP cells from peripheral blood were plated in triplicate at 2  $\times$  10<sup>6</sup> cells/well in SP buffer in 24-well plates, and cultured in media alone, with 1 $\times$ 10<sup>6</sup> ILS-specific T cells

or in combination with T cells and soluble CD40L trimerizer as described above. After 48 hours, the cells were harvested and analyzed for SP frequency.

### Statistics

Student's *t* test was used for statistical analyses of functional differences between study groups. P-values of  $<.05$  were considered statistical significant. Error bars in all panels represent standard error of the mean (SEM).

## RESULTS

### Distinct CD5<sup>+</sup>CD19<sup>+</sup> SP phenotype in primary B-CLL tumor samples

PBMC from 21 patients with B-CLL (Table 1) and 5 healthy donors were labeled with Hoechst 33342, and CD5 and CD19 antibodies and then analyzed by flow cytometry for the presence of SP cells (Fig. 1a). A distinct CD5<sup>+</sup>CD19<sup>+</sup> SP phenotype was detectable in the peripheral blood of 18 of 21 (85%) patient samples. We were unable to detect SP cells in ungated PBMC from normal donors (n=5), indicating that SP is confined to malignant B-CLL cells (Fig. 1b). In this patient cohort, the median frequency of SP in the positive samples was 0.22% (range, 0.02% to 2.17%). SP frequency was reduced by co-incubation with the calcium channel blocker Verapamil (n=3;  $p<.001$ ) confirming that the SP phenotype was a result of active dye efflux (Fig. 1c and d). These data show that the peripheral blood of B-CLL patients frequently contains a minor subpopulation of B-CLL tumor cells that are capable of expelling Hoechst dye.

### Enrichment of CD5<sup>+</sup>CD19<sup>+</sup> B-CLL SP cells by fludarabine

The SP phenotype is attributed to the expression of ABC transporter proteins, including ABCG2 and MDR1,<sup>13,14</sup> and can directly contribute to resistance of tumor cells to chemotherapy agents.<sup>5</sup> SP cells are also more resistant to apoptosis through transporter independent mechanisms including increased expression of pro-survival factors<sup>12</sup> or DNA repair enzymes,<sup>21</sup> implying that B-CLL SP cells may be resistant to commonly used agents even when these are not substrates for ABC transporter proteins. Because fludarabine is so commonly used for the treatment of B-CLL, we incubated B-CLL isolated from PBMC with and without fludarabine and looked to see whether SP B-CLL cells became selectively enriched. Figures 2a and b show that incubation of B-CLL cells with a 50  $\mu$ M fludarabine concentration enriches SP cells after 48 hours (n=4;  $p=.02$ ). Additionally, analysis of SP and NSP using Annexin-V in B-CLL samples cultured in media alone or with fludarabine showed that SP cells exhibited less apoptosis than bulk tumor cells (NSP) after 48 hours ( $p=.04$ ) (Fig. 2c and d). Interestingly, SP cells in control cultures also underwent less apoptosis than bulk tumor cells ( $p=.004$ ) suggesting that SP cells are generally more resistant to cell death than NSP. Overall, these data indicate that SP cells are more resistant to apoptosis induced by fludarabine.

### Subcutaneous immunization with CD40L/IL2-expressing B-CLL cells decreases the frequency of tumor SP cells in peripheral blood

Although the SP phenotype is associated with cytotoxic drug resistance, this characteristic should not preclude elimination by immunotherapies that target TAAs. We previously

reported a study of B-CLL patients who received subcutaneous injections of autologous tumor cells expressing transgenic IL-2 and hCD40L over 3 months.<sup>17</sup> In this study, tumor specific Th1 and Th2 cells responded against bulk (i.e. primarily non-SP) autologous tumor, but we observed no significant fall in circulating B-CLL counts. Having detected B-CLL SP cells prior to immunization in 6 patients from this study (Table 1), we re-analyzed these patient samples to determine whether our immunization strategy did in fact have anti-tumor activity that was restricted to the B-CLL SP component, since this would not have been detected in our initial analyses of the effects of the treatment on the bulk tumor population. We did indeed observe a consistent and substantive reduction in CD5<sup>+</sup>CD19<sup>+</sup> SP cells in the peripheral blood of vaccinated patients (Fig. 3a and b), which was associated with a rise in B-CLL-specific immunity during the vaccine treatment (Fig. 3b). Hence grouping of samples into three bins; 1) pre-vaccine (0–2 weeks), 2) during vaccination (2–10 weeks), and 3) post-vaccination (weeks 10+) showed a significant decrease in SP frequency associated with a concomitant significant increase in an anti-SP T cell immune response (Fig. 3c and d). Once the course of immunization ended, however, B-CLL SP cell numbers progressively returned to pre-treatment levels as the T cell response dwindled (Fig. 3b), supporting a causal relationship between the level of the T cell immune response and the frequency of B-CLL SP cells, and suggesting that the anti-tumor effects of the B-CLL directed T cell response are restricted to the SP-subset of cells.

### Recognition of B-CLL tumors by T cells post-vaccination

Since B-CLL-specific T cell immunity generated by hCD40L/IL-2 autologous tumor immunization was associated with a significant decrease in CD5<sup>+</sup>CD19<sup>+</sup> SP cells, we investigated whether tumor-specific T cells isolated from the peripheral blood of vaccinated patients could specifically recognize B-CLL SP cells. We generated CD8<sup>+</sup> B-CLL-specific T cells from PBMC of immunized patients by consecutive stimulation with hCD40L activated autologous tumor cells.<sup>17</sup> We were able to generate B-CLL-specific T cells from PBMC collected post-vaccination (weeks 4 and 10), but not from pre-vaccine samples, indicating that patients had more frequent or more reactive precursors of B-CLL-specific T cells following their vaccine regimen than before (Fig. 4a). IFN- $\gamma$  production by B-CLL stimulated CD8<sup>+</sup> T cell lines was inhibited by the MHC class I blocking antibody W6/32, indicating that specific recognition of autologous B-CLL tumors is mediated through T cell receptor – MHC class I interaction (Fig. 4b). To determine if these B-CLL activated T cells could also recognize SP B-CLL cells, autologous B-CLL tumor cells were sorted into CD5<sup>+</sup>CD19<sup>+</sup> SP and non-SP fractions and used as target cells in IFN- $\gamma$  ELISpot assays. The results in Figure 4c showed that B-CLL-specific T cells generated by stimulation with hCD40L-activated autologous tumor cells were indeed able to recognize both the SP and non-SP subsets (Fig. 4c).

To further characterize the specificity and function of anti-B-CLL activity, we cloned B-CLL-reactive CD8<sup>+</sup> T cells from the T cell lines generated from patient peripheral blood and tested their ability to recognize SP and non-SP tumor cells. 9/13 clones recognized and produced IFN- $\gamma$  in response to both SP and non-SP B-CLL stimulation, but the level of reactivity (measured by IFN- $\gamma$  activity) induced by SP cells was significantly greater than by non-SP ( $p=.005$ ) (Fig. 4d). Four clones recognized *only* SP cells, but no clones recognized



only the non-SP fraction. Importantly, none of the clones showed background reactivity against allogeneic SP or non-SP B-CLL cells. These data suggest that immunization with hCD40L-activated B-CLL tumor cells can induce a T cell response directed to epitopes shared by both SP and bulk tumor cells, and also to antigens differentially expressed by B-CLL SP tumor cells.

### **RHAMM-specific T cells specifically recognize B-CLL SP cells**

Because the antigen specificity of patient-derived T cell lines and T cell clones was unknown, we investigated whether B-CLL patient T cells could recognize previously described B-CLL antigens, including RHAMM,<sup>19</sup> survivin (BIRC5),<sup>22</sup> fibromodulin (FMOD) and MDM2.<sup>23,24</sup> Since CD40 activation has previously been shown to increase antigen presentation<sup>25</sup> and upregulate tumor antigen expression on B-CLL cells,<sup>26</sup> we used RT-PCR amplification to see whether SP and non-SP B-CLL tumor cells express tumor associated antigens (TAA). While survivin and FMOD expression were detected in the SP fraction of B-CLL, RHAMM showed the greatest increase in preferential expression in the SP subset following exposure to soluble hCD40L (Fig. 5a). Since none of our CTL lines or T cell clones in Figure 4 recognized RHAMM (data not shown), and because RHAMM was expressed in B-CLL SP tumor cells, we directly generated CD8<sup>+</sup> CTL cultures against an HLA-A2-restricted RHAMM peptide (**ILSLELMKL**)<sup>27</sup> using T cells from HLA-A2<sup>+</sup> vaccinated patients and autologous APC. We successfully generated ILS-specific T cells from 3 of 4 donors as determined by IFN- $\gamma$  ELISpot using an HLA-A2-restricted peptide from MAGE-3 (FLW) as a negative control (Fig. 5b). To determine whether these patient-derived ILS-specific T cells could also recognize B-CLL SP cells, we separated hCD40 activated and non-activated SP and non-SP tumor cells and used these as the target cells in a IFN- $\gamma$  release ELISpot (Fig. 5c). We found that ILS-specific T cells had increased reactivity toward CD40-activated SP tumor cells compared to non-activated, or CD40-activated non-SP or bulk tumor cells. This is consistent with the observation that RHAMM expression is elevated in SP cells after CD40L stimulation allowing recognition by RHAMM-specific CTL.

To assess whether ILS-specific T cells recognizing RHAMM on B-CLL SP cells could selectively deplete these cells, we co-cultured autologous ILS-specific T cells with B-CLL tumor cells and measured the change in SP frequency after 48 hours. On exposure to autologous ILS-specific T cells, B-CLL SP cells were selectively depleted from the co-culture compared to non-SP cells, and their depletion was greater when the cells had been CD40 activated by CD40L (Fig. 5d). Together, these results suggest that increased expression of antigens such as RHAMM by the SP subset of B-CLL cells allows them to be preferentially recognized and eliminated compared to the bulk tumor population, and that expression of these target antigens by B-CLL SP cells is increased following CD40 exposure.

### **Detection of RHAMM-specific T cells in PBMC from subjects with B-CLL**

The great majority of tumor cells express an array of tumor associated antigens (TAA), so the RHAMM antigen is likely only one of many possible TAA on B-CLL SP cells. Moreover, the ILS peptide is only one of many potential T cell epitopes even within the

single RHAMM antigen. Nonetheless, if we were able to detect T cells specific for the RHAMM ILS peptide in the peripheral blood of patients following immunization with hCD40L/IL-2 tumor cells, and if the appearance of such cells was associated with a decline of SP cells in the circulation, then this would offer support for the concept of a specific anti-tumor immune response to B-CLL SP cells, and would help validate RHAMM as a TAA-target suited to further exploration. We therefore examined PBMC from two HLA-A2 patients (P1393 and P1335) who developed a measurable T cell immune response to B-CLL SP cells that was associated with a decline in this tumor cell subset (Fig 6a and **d**). In association with these anti-tumor effects, ILS-specific T cells that were absent prior to treatment, increased during vaccination, and then subsided after treatment as the malignant SP cells returned (Fig. 6b and **e**). The ILS peptide of RHAMM expressed by b-CLL SP cells can therefore be a target of tumor reactive T cells.

## DISCUSSION

We have identified a distinct CD5<sup>+</sup>CD19<sup>+</sup> SP phenotype in the peripheral blood of subjects with B-CLL. While these B-CLL SP cells are resistant to fludarabine, a commonly used drug for the treatment of B-CLL, we demonstrate that this population can be targeted by T cells following vaccination with autologous tumor cells expressing hCD40L and IL-2. In association with the development of this immune response, there is a loss of malignant SP cells from the circulation, and their resurgence as immunity declines. T cells generated *ex vivo* from autologous CD40L-activated autologous tumor cells or by RHAMM peptide-pulsed TAPC specifically recognized autologous B-CLL SP cells and were able to eliminate this subset in co-culture assays. Moreover, T cells with identical tumor associated epitope specificity and activity were observed *in vivo* following immunization with the tumor vaccine, supporting an immune-mediated mechanism for the depletion of CD5<sup>+</sup>CD19<sup>+</sup> SP cells in these patients.

SP analysis has commonly been used to define normal and malignant cells with stem/progenitor cell-like characteristics.<sup>6-9,11,12</sup> This has been a useful strategy for discriminating precursor cells from more “mature” cells when surface markers are unknown or when antibodies are unavailable. In many cancer types, the association of the SP phenotype with high expression of ABC transporter proteins means that this subset of malignant cells also has primary drug resistance, and may therefore make a significant contribution to disease persistence or relapse. Such a contribution would be independent of any putative activity as a cancer progenitor cell. That a drug resistant subpopulation does indeed exist in B-CLL was first suggested by the detection of ABCG2 expression by a minor subset of B-CLL tumor cells that was also associated with drug resistance.<sup>28,29</sup> Nowakowski and colleagues observed a dramatic increase in ABCG2 expressing B-CLL following treatment with pentostatin, cyclophosphamide and rituximab, suggesting that ABC transporter mechanisms are either directly responsible for drug resistance, or can reveal tumor cells with innate resistance to a variety of anti-cancer agents.<sup>29</sup> de Wolf et al demonstrated that human cell lines transgenically modified to express ABCG2 possessed increased resistance to anti-cancer nucleosides, including fludarabine, cladribine and clofarabine, suggesting that these agents are substrates of ABCG2 transporters.<sup>30</sup> Other groups have shown that the SP phenotype also distinguishes tumor cells that are resistant to



apoptosis through mechanisms not attributed to direct drug efflux.<sup>12,21</sup> Thus, Woodward et al demonstrated that breast cancer tumor SP cells were enriched following radiation treatment,<sup>12</sup> while Bleau and colleagues showed that glioblastoma SP cells expressed higher levels of O6-methylguanine DNA methyltransferase (MGMT), which was protective against temozolomide, which is not a ABCG2 substrate.<sup>21</sup> In our cohort, we found ABCG2 expression to be heterogeneous, with some patient SP cells having clearly elevated expression, while SP cells from others were ABCG2 low (not shown). This may be due to inherent differences between patients, but also suggests that other ABC transporter family members may play a role in Hoechst and drug efflux in B-CLL. Unfortunately, we were unable to obtain sufficient samples to determine the in vivo effects of fludarabine on B-CLL SP cells, or B-CLL cells that express ABCG2. While our data which show that B-CLL SP cells exhibit less apoptosis during fludarabine exposure support the concept that tumor SP cells, including those within the B-CLL population, are inherently drug resistant, further studies will need to be performed to determine whether this rare chemoresistant B-CLL subset persists in vivo during chemotherapy.

SP cells have previously been detected in several other hematopoietic malignancies<sup>3,4</sup> In these disorders, the SP cells often differ in surface phenotype from bulk tumor cells, with higher expression of more primitive lineage markers (e.g. CD34 and CD133).<sup>3,31</sup> However, in primary tumor samples from B-CLL patients, we were unable to discern consistent differences between SP and bulk tumor cells by using a panel of progenitor cell (CD34, CD38, CD133) and B lineage monoclonal antibody markers (CD10, CD19, CD20, CD24, CD40, CD79b and CD127) (data not shown). Others have noted similar heterogeneous expression of progenitor and lineage markers in primary AML SP cells<sup>32</sup> suggesting that a functional assay, such as SP, may more consistently detect rare, but functionally chemoresistant/progenitor tumor cells in heterogeneous human hematological malignancies.

Despite the apparent inability of monoclonal antibody analysis to define a unique surface antigen phenotype on B-CLL SP cells, we were able to detect striking differences in expression of TAAs, including the antigen RHAMM. Analysis of SP and non-SP B-CLL cells before and after hCD40L stimulation showed that RHAMM expression was confined to the SP subset and was enhanced by CD40 activation. Giannopoulos et al have also shown that RHAMM expression is CD40 activation dependent in B-CLL tumor cells,<sup>26</sup> and that expression may correlate with aggressiveness of disease,<sup>27</sup> although they did not analyze expression in B-CLL cell subsets. While expression of RHAMM on tumor SP cells could be detected by patient CTLs (Fig. 5c), we could also isolate T cell clones from vaccinated patients that were not RHAMM-specific, several of these T cell clones could nonetheless selectively recognize B-CLL SP cells rather than non-SP B-CLL (Fig. 4d). This result strongly suggests that other TAAs,<sup>23,24</sup> in addition to RHAMM, are differentially expressed between SP and non-SP cells, and that vaccination of patients with CD40L-expressing B-CLL tumor cells can induce an immune response directed against the minor SP subset of cells that upregulate TAAs such as RHAMM upon CD40 activation. The recognition and elimination of CD40L-activated SP cells by RHAMM-specific T cells in vitro, coupled with the appearance of RHAMM (ILS) peptide-specific T cells in the peripheral blood as the malignant SP declines supports our suggestion even though RHAMM is likely only one of multiple TAAs expressed by SP cells. It is possible that other immunological differences

between SP and NSP could account for the selective elimination of B-CLL SP cells, however we have not found significant differences in cell surface expression of MHC class I, costimulatory molecules (e.g. CD80 and CD86) or expression of immune suppressive cytokines such as IL-10 (not shown).

In this study, the anti-SP B-CLL T cell response we describe is lost 4–6 weeks after vaccine administration ceased. There are a variety of immunological deficiencies in B-CLL patients, including suboptimal antigen presentation and synapse formation<sup>25,33,34</sup> and elevated levels of regulatory T cells<sup>17,35</sup> and direct immune suppression through B-CLL expressed ligands,<sup>36</sup> which likely contribute to the short persistence of T cells after vaccination and only the transient elimination of B-CLL SP tumor cells. Nonetheless, data from this study suggest that a sequential combination of chemotherapy, to debulk the disease, and immunotherapy to target chemoresistant B-CLL tumor cells, may help prolong disease control.

## Acknowledgments

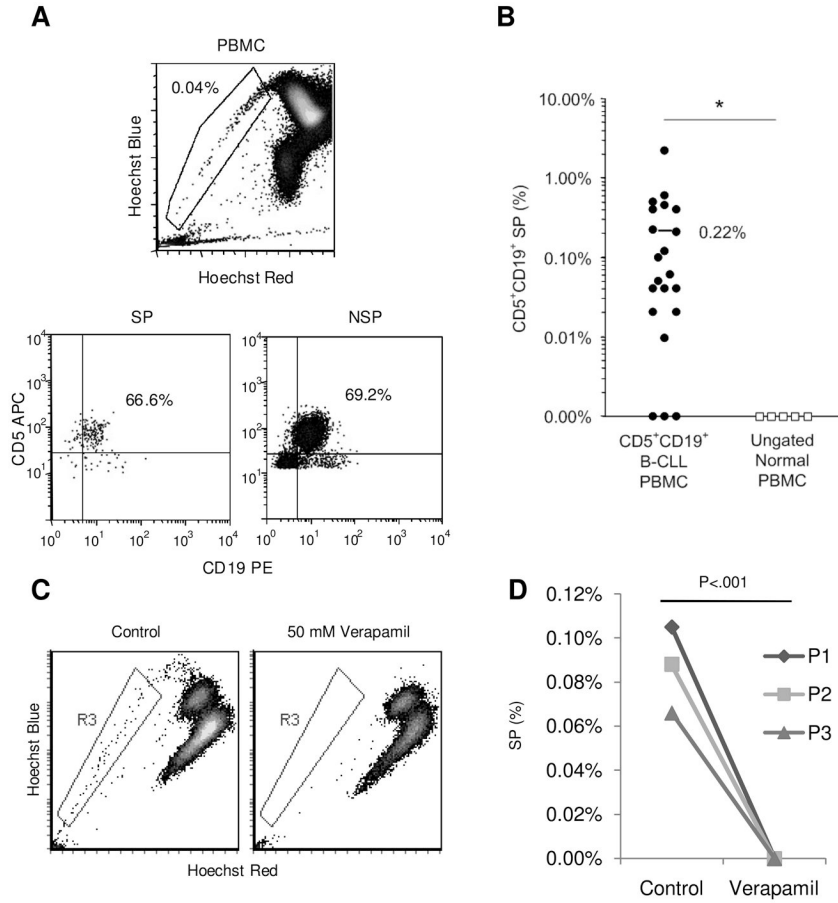
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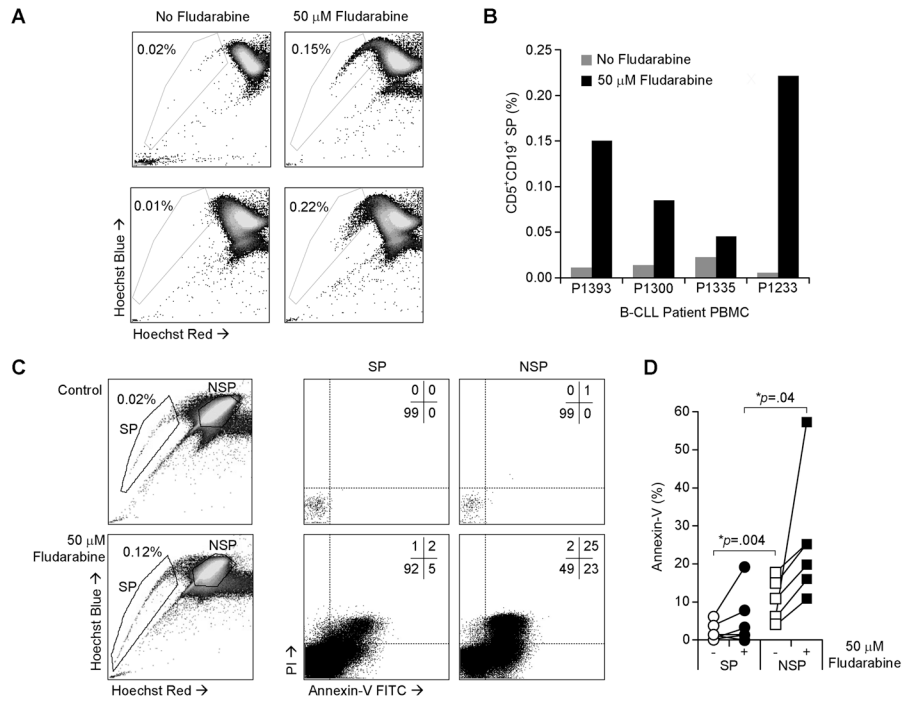
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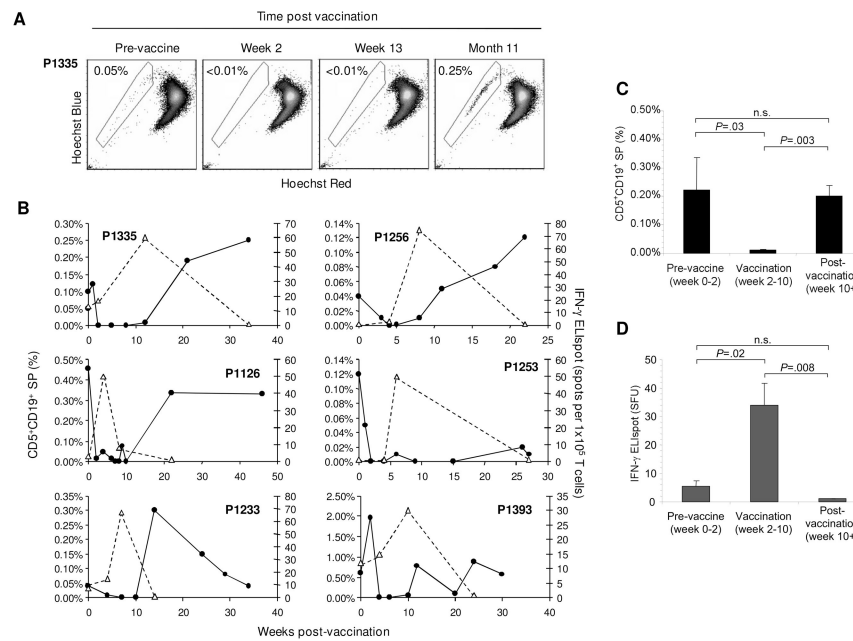
**Figure 1. A distinct CD5<sup>+</sup>CD19<sup>+</sup> SP phenotype is present in B-CLL patient peripheral blood**  
 Samples collected from B-CLL patients were stained with Hoechst 33342 dye followed by antibody and propidium iodide labeling. Cells were gated on either SP or non-SP and examined for expression of CD5 and CD19 showing both populations are positive for CD5 and CD19 in circulating PBMC (**A**). **B**) PBMC collected from 21 B-CLL patients and 5 normal donors were analyzed for the presence of CD5<sup>+</sup>CD19<sup>+</sup> SP cells. 18/21 B-CLL samples contained SP positive cells, whereas none of the normal PBMC had the SP subset ( $p<.05$ ). To verify that the SP phenotype observed in B-CLL tumor samples was produced by efflux of Hoechst dye (**C** and **D**), staining was performed in the presence of 50  $\mu$ M Verapamil, indicated that the SP formation was blocked with Verapamil treatment ( $p=.001$ ).



**Figure 2. B-CLL SP cells are enriched by the presence of fludarabine**

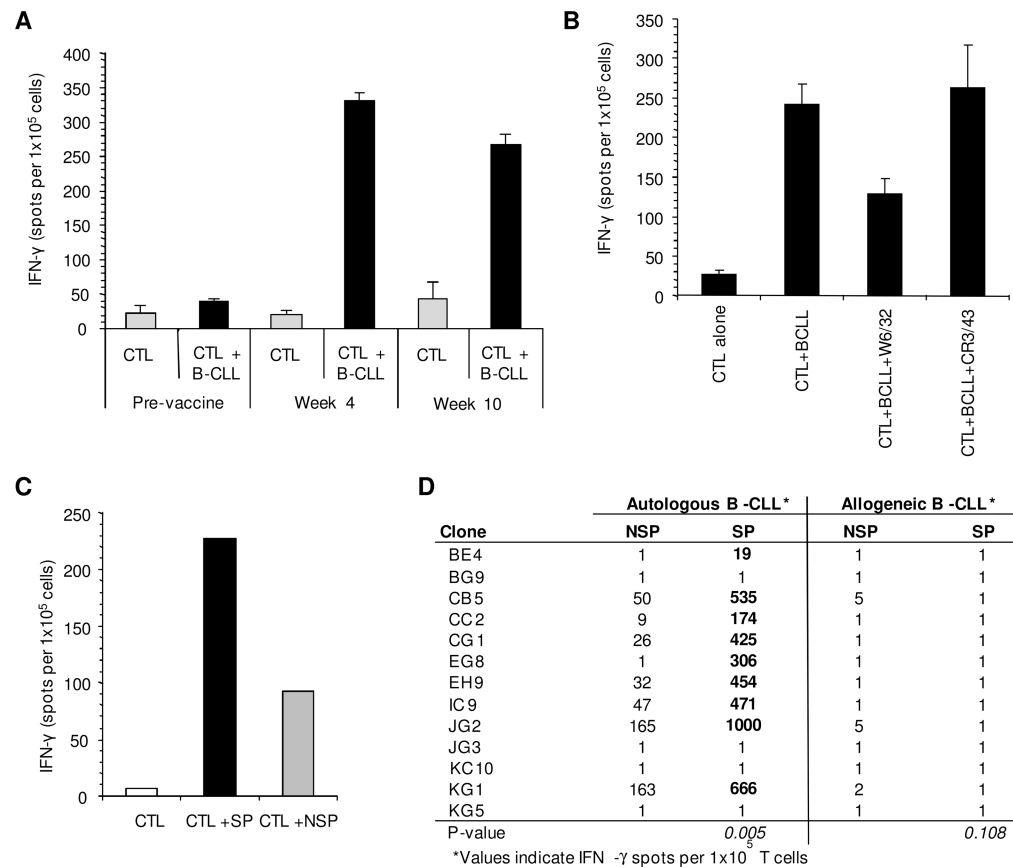
**A)** B-CLL cells from four patients were cultured with or without 50  $\mu$ M fludarabine for 48 hours and then stained with Hoechst dye and CD5/CD19 antibodies. **B)** SP analysis following exposure to fludarabine showed increased frequency of SP cells compared to controls in 4 separate donors, indicating selection over non-SP tumor cells ( $p=.02$ ). **C)** B-CLL SP and NSP cells in media alone or supplemented with 50  $\mu$ M fludarabine for 48 hours were examined for apoptosis and cell death using Annexin V and PI. **D)** B-CLL SP cells from 4 patient samples were compared with and without fludarabine for Annexin-V staining by FACS analysis, where NSP tumor cells expressed significantly higher levels of Annexin-V after fludarabine treatment compared to B-CLL SP cells ( $p=.04$ ).





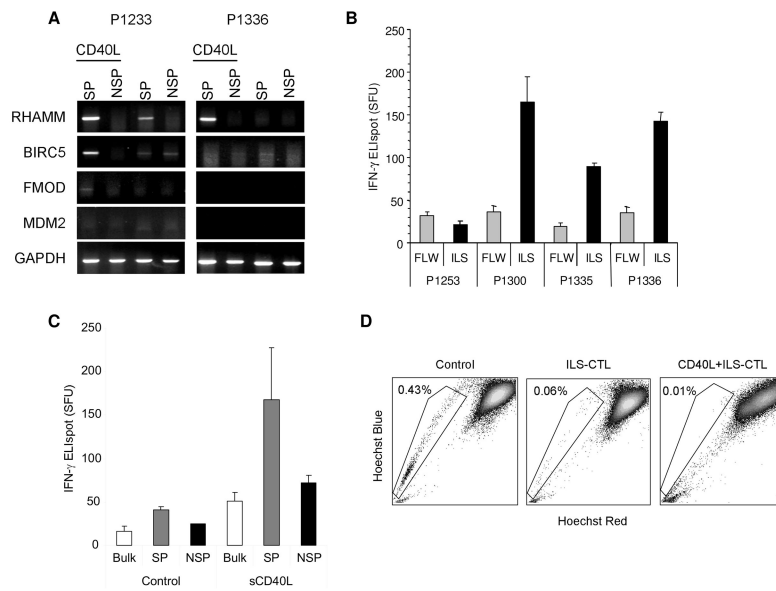
**Figure 3. Vaccination with hCD40L/IL-2 gene-modified B-CLL cells transiently diminishes SP frequency in B-CLL patients**

**A)** Longitudinal analysis of PBMC in B-CLL patients (P1335 shown here) prior, during, and after vaccination shows a transient decrease of CD5<sup>+</sup>CD19<sup>+</sup> SP cells, during vaccination. **B)** Analysis of 6 patients for SP frequency (left y-axis; closed circles) and anti-B-CLL-specific T cell responses as determined by IFN- $\gamma$  ELISpot (right y-axis; open triangles) plotted over time (weeks post 1<sup>st</sup> vaccination with hCD40L/IL-2 gene modified tumor cells). Time point samples from 6 patients treated with hCD40L/IL-2 gene-modified tumor vaccines were separated into 3 groups, pre-vaccination (0–2 weeks), vaccination (2–10 weeks) and post-vaccination (week 10+). **C)** SP frequency was significantly reduced during the vaccination period. **D)** B-CLL-specific T cells, as measured by IFN- $\gamma$  ELISpot, were increased during immunization.



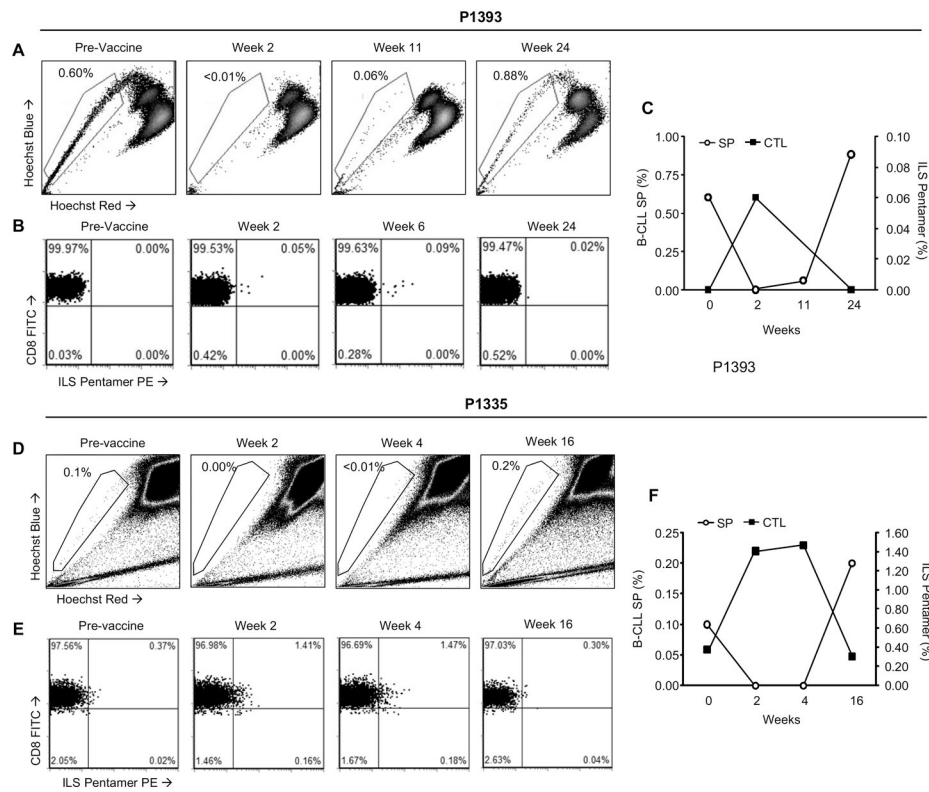
**Figure 4. Isolation and expansion of anti-SP T cells from vaccinated patients**

**A)** T cells from PBMC samples taken pre-vaccine and at week 4 and week 10 after immunization were stimulated with CD40L activated autologous tumor cells. The data show specificity towards autologous tumor cells when analyzed by IFN- $\gamma$  ELISpot. **B)** B-CLL-reactive T cells show specificity towards both B-CLL SP and non-SP (NSP) cells by IFN- $\gamma$  ELISpot when autologous B-CLL samples were sorted and co-cultured with T cell lines. **C)** CD8<sup>+</sup> T cell clones, isolated and expanded by limiting dilution, were tested for reactivity against autologous and allogeneic B-CLL tumor cells sorted into SP and NSP fractions. T cell clones showed enhanced or selective reactivity towards autologous SP tumor cells ( $p=0.005$ ).



**Figure 5. RHAMM-specific T cells recognizes CD40L activated B-CLL SP cells**

**A)** Expression of the TAAs RHAMM, survivin (BIRC5), fibromodullin (FMOD) and MDM2 were examined by RT-PCR in B-CLL tumor cells following CD40L activation and subsequent sorting into SP and non-SP (NSP) fractions. **B)** Using ILS peptide-pulsed TAPC, T cells were generated from the PBMC (week 4) of 4 patients receiving hCD40L/IL-2 vaccines. 3 of the 4 T cell lines were specific for the ILS epitope as determined by IFN- $\gamma$  ELISpot, but failed to recognize the control HLA-A2-restricted MAGE-3 epitope (FLW). **C)** ILS-specific T cell (from P1300) were cultured with autologous B-CLL tumor cells sorted into SP and NSP, either with or without soluble CD40L stimulation (sCD40L). There was enhanced reactivity towards CD40 activated SP tumor cells. **D)** In vitro coculture assays using ILS-specific T cells show specific depletion of SP cells (middle panel), which is enhanced following activation of tumor cells with sCD40L (left panel).



**Figure 6. Detection of circulating ILS-specific T cells in two patients exhibiting selective SP depletion**

**A and D)** CD5<sup>+</sup>CD19<sup>+</sup> SP cells could be detected in PBMC of two HLA-A2<sup>+</sup> patients (P1393 and P1335) prior to vaccination (pre-vaccine), and diminished during immunization (weeks 2 through 11), but returned following the end of treatment (week 24 and 16, respectively). **B and E)** P1393 and P1335 were examined for the presence of ILS-specific CD8<sup>+</sup> T cells in PBMC during immunization and demonstrated an increase in ILS-specific T cells as determined by ILS pentamer staining. **C and F)** CD5<sup>+</sup>CD19<sup>+</sup>SP frequency was plotted against CD3<sup>+</sup>CD8<sup>+</sup> ILS-specific T cells (as measured by pentamer staining) for the same period demonstrating an inverse relationship between SP frequency and induction of B-CLL-specific immunity following vaccination

Table 1

Patient details.

Patient	Protocol	Sex	Age	Disease Stage	Pre-Vaccine Therapy	Disease Status
* P1126	CLIMAT	M	46	III	F	PD
* P1256	CLIMAT	M	49	IV	NT	PD
* P1233	CLIMAT	M	53	IV	NT	PD
* P1335	CLIMAT	F	62	I	NT	PD
* P1393	CLIMAT	M	67	II	NT	PD
* P1253	CLIMAT	M	70	I	NT	PD
P1300	CLIMAT	M	65	II	NT	SD
P1232	CLIMAT	F	51	IV C	F	PD
P1336	CLIMAT	M	54	I	NT	PD
P1190	CLIMAT	F	52	I	F, C, R	NVA; DOD
P1064	CLIMAT	M	68	I	F	NVA; DOD
P1261	CLIMAT	M	63	I	F; F, C, R	NVA; DOD
P1136	CLIMAT	M	61	IV	NT	NVA; DOD
P1370	CLIMAT	M	57	IV	NT	NVA; DOD
P1376	CLIPA	M	69	II	NT	PD
P1477	CLIPA	M	49	IV C	NT	PD
P1954	CLIPA	M	73	IB	NT	SD; 2° Malignancy
P1593	CLIPA	M	69	IV	NT	PD
P2085	CLIPA	M	47	II	NT	SD
P1991	PRIMAL	M	40	IV C	NT	PD
P2090	PRIMAL	M	71	IV C	NT	SD

\* indicates patients analyzed for longitudinal post-vaccine SP and CTL

Treatment: NT, No treatment; F, Fludara; C, Cytosar; R, Rituxan

Disease Status: SD, Stable disease; PD, Progressive disease; DOD; died of disease; NVA, No vaccine administered