Gene immunotherapy of chronic lymphocytic leukemia: a Phase I study of intranodally injected adenovirus expressing a chimeric CD154 molecule

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ABSTRACT

New therapies for chronic lymphocytic leukemia (CLL) are needed, particularly those that can eradicate residual disease and elicit anti-CLL immune responses. CD40 ligation on CLL cells, which can be achieved using adenovirus encoding chimeric CD154 (Ad-ISF35), enhances their ability to function as antigen presenting cells and increases their sensitivity to clearance by immune-effector mechanisms. In this study, we report the results of a first-in-man Phase I trial of intranodal direct injection (IDI) of Ad-ISF35 in patients with CLL to evaluate toxicity, safety, and tolerability. Fifteen patients received a single IDI of 1-33 x 10^{10} Ad-ISF35 viral particles (vp), with a defined maximum tolerated dose as 1x10^{11} vp. Although the most common adverse events were transient grade 1-2 pain at the injection site and flu-like symptoms following IDI, some patients receiving the highest dose had transient, asymptomatic grade 3-4 hypophosphatemia, neutropenia, or transaminitis. Increased expression of death receptor, immune co-stimulatory molecules, and Ad-ISF35 vector DNA was detected in circulating CLL-cells. Notably, we also observed preliminary clinical responses, including reductions in leukemia cell counts, lymphadenopathy, and splenomegaly. Six patients did not require additional therapy for more than 6 months, and 3 achieved a partial remission. In conclusion, Ad-ISF35 IDI was safely delivered in patients with CLL and induced systemic biological and clinical responses. These results provide the rationale for phase II studies in CLL, lymphomas, and CD40-expressing solid tumors.
INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of monoclonal B-cells in the blood, lymphoid tissues, and marrow (1). Although advances in chemoimmunotherapy have resulted in improved response rates and have prolonged survival (2, 3), such treatments also can impair hematopoiesis and immune function and are not well tolerated by all patients, particularly the elderly (4). Furthermore, most treated patients eventually relapse and require additional therapy and the disease still is considered incurable.

It has been reported that the lymph node and bone marrow microenvironments play an important role in protecting CLL cells from apoptosis (5-8). Evidence exists to postulate that proliferating CLL cells in the lymph nodes are the source of the non-proliferating CLL cells present in the peripheral blood (9). However, most therapies used currently in CLL do not target residual niches or leukemia cells that may depend heavily on the microenvironment. As such, relapse after chemotherapy based treatment is inevitable and this argues in favor of the development of novel treatment alternatives, including those that promote immune stimulation and activation of the tumor microenvironment.

We have address this problem by studying in vitro and in vivo mechanism to promote cellular activation and immune recognition in CLL using an approach that involves transduction of CLL-cells with vectors encoding the ligand for CD40 (CD154) (10). Although the leukemia cells express high-levels of HLA-antigens required for presentation of antigen to T-cells, CLL-cells are poor antigen-presenting cells. These
cells lack expression of the immune co-stimulatory molecules required for effective T-cell activation and instead appear to suppress T-cell function (11).

CD40 activation using recombinant antibodies or CD54 ligands have been used in patients with cancer (12) and CLL (13-15) showing objective clinical responses. Activation of B cells through CD40 changes its phenotype and induces immunoglobulin class switching and enhances its antigen-presenting capacity (16). Similar changes are also observed when CLL-cells are activated via ligation of CD40 (17, 18), which can be achieved through transduction of CLL-cells with an adenovirus (Ad) vector encoding CD154 (19). Such transduced and CD40-activated CLL-cells can induce autologous T-cell activation and immune recognition, leading to generation of anti-leukemia immune responses (20, 21).

We previously conducted clinical trials evaluating the safety and clinical activity of this approach. For these trials, patients underwent leukapheresis and CLL-cells were subsequently transduced ex-vivo with an Ad vector encoding either mouse-CD154 or a chimeric-humanized CD154, termed Ad-ISF35 (22, 23). Ad-ISF35 was developed to mitigate generation of immunity against mouse-CD154 and to improve membrane stability. Transduction of CLL-cells with Ad-CD154 or Ad-ISF35 generated transduced-CLL-cells that had phenotypic features of CLL-cells that had been activated by contact with CD154-bearing cells. Moreover, since these transduced CLL-cells expressed a ligand for CD40, they also could activate bystander, non-transduced CLL-cells to undergo such phenotypic changes (24). Clinical studies demonstrated that intravenous infusions of autologous CLL-cells that had been transduced with Ad-CD154 or Ad-ISF35 did not cause unacceptable or long-term toxicity, induced activation of “bystander” non-
transduced CLL-cells similar to that achieved by contact with CD154 / ISF35-bearing cells, almost invariably resulted in acute reductions in leukemia-cell blood counts, lymphadenopathy, and splenomegaly, and could induce anti-leukemia immune responses (22, 23).

However, not all patients have sufficient numbers of circulating neoplastic cells to accommodate this approach, which also requires ex-vivo processing of cells in specialized facilities that are not widely available. Therefore, we considered whether we could achieve similar biological and clinical responses by injecting the Ad-ISF35 vector directly into pathologically enlarged lymph nodes of patients with CLL. This hypothesis is supported by preclinical studies in test animals showing that direct injection of Ad-ISF35 into tumor nodules is safe and capable of inducing anti-tumor responses (25, 26). Thus, we conducted a first in man phase I dose-escalation study to evaluate toxicity, tolerability and safety of Ad-ISF35 intranodal direct injection (IDI) in patients with CLL.
MATERIALS AND METHODS

Patients And Study Design

Patients provided written informed consent in accordance with the Declaration of Helsinki to participate in this study. We enrolled and treated fifteen patients who met eligibility criteria, including diagnosis of CLL and progressive disease requiring treatment according to the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) (27, 28). All patients were offered standard chemoimmunotherapy but declined this treatment in favor of participating in this study. Patients presented at least one single accessible and palpable lymph node in the cervical, supraclavicular, axillary, or inguinal regions. The size of the lymph nodes was larger than 2x2 cm in the horizontal and perpendicular axes. Additional inclusion criteria were hemoglobin $\geq$ 10 g/dL, platelet count $\geq$ 50x10^3/mm^3, total bilirubin, ALT and AST and creatinine $\leq$ 2XULN, ECOG performance status $\leq$ 2 and anticipated survival of at least 3 months. Patients were excluded from participating in this clinical trial if they had received treatment with chemotherapy or monoclonal antibodies within 28 days prior to entering the study. Additional treatments for CLL were not allowed during the time of participation in this trial. In addition, patients with history of secondary malignancies within five years of registration were excluded from the protocol (except for patients with history of treated non-melanoma skin cancer).

The study employed a standard 3+3 dose-escalation scheme. Patients were assigned to one of four dose cohorts to receive one IDI of either 1x10^{10}, 3.3x10^{10}, 1x10^{11} or 3.3x10^{11} viral particles (vp) of Ad-ISF35 (provided by Memgen, LLC -Dallas, TX). The injected node was in the axilla and greater than 2x2 cm in size prior to injection. The study design was reviewed and approved by the Food and Drug
Administration (BB-IND 13046), the Recombinant Advisory Committee (RAC) of the National Institutes of Health, the University of California San Diego (UCSD) Institutional Biosafety Committee (IBC), and the UCSD Institutional Review Board. This clinical trial was registered in the National Institute of Health database (registration NCT00783874) (29).

We evaluated the patient’s white blood cell counts (WBC), absolute neutrophil counts (ANC), absolute lymphocyte counts (ALC), platelet counts (plt), hemoglobin (Hgb), and size of the spleen, liver, and lymph nodes before and at designated times after Ad-ISF35 IDI. The follow up period included frequent clinical and laboratory evaluations on days 0, 1, 2, 7, 14, 21, 30, 60 and 84 after injection and every three months thereafter for a total of one year. Assessment of response to treatment was evaluated following the IWCLL guidelines (28).

Dose limiting toxicity (DLT) was defined as any adverse event (AE) grade 3 or higher that was considered by the investigator to be possibly related or related to Ad-ISF35 treatment. DLTs were evaluated only during the first 21 days of treatment and their assessment determined dose escalation and defined the maximum tolerated dose (MTD). Toxicity was graded according to the NCI-Common Terminology Criteria for Adverse Events (CTCAE) version 3.0, modified for hematologic toxicities according to the IWCLL guidelines (28). Hematological adverse events were considered DLT if they were grade 4 or higher and lasted ≥7 days. We defined the MTD as the highest dose where no more than one patient out of six presented DLT.
Flow Cytometry

Ten million of isolated white blood cells/ml in PBS (+1% FBS) were incubated for 30 min at 4°C with fluorochrome-conjugated mAbs specific for CD5, CD19, CD54, CD80, CD86, CD20, DR5, CD3, CD4, CD8, mCD154 (used for detection of ISF35 protein expression), CD95, CD23 and κ or λ immunoglobulin light chain (Becton Dickinson, San Jose, CA, USA). Fluorescence data was acquired using a FACSCalibur flow cytometer (Becton Dickinson) and analyzed with Flow-Jo software (Tree Star, Ashland, OR, USA).

Measurement Of Cytokines in Patient Sera

We examined serum samples for interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p70), IL-13, IL-15, Monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF-α), granulocyte-monocyte-colony stimulating factor (GM-CSF) and interferon-gamma (IFN-γ) via Luminex xMAP technology following manufacture’s instructions (Millipore, Billerica, MA, USA). Fluorescence intensity was measured in a Luminex 100TM IS machine and BeadView™ Multiplex software v1.0 was used for data analysis by Spline curve-fitting method.

Detection of antibodies against Adenovirus or Human CD154

We measured by ELISA serum antibodies against Ad-ISF35 or recombinant human CD154 (rhCD154) protein. Plates were coated with 4x10⁹ vp/ml of Ad-ISF35 or 2µg/ml of rhCD154 (R&D Systems, Minneapolis, MN) for 2h at 37°C. Plates were blocked with PBS/Tween 0.01%, BSA 1%. Sera samples collected on days 0 and 28 were added at different dilutions and incubated for 1h at 37°C. Anti-human IgG HRP-conjugated antibody (Jackson Laboratories, Bar Harbor, ME) was added at 1/5000
dilution for 1h at room temperature. After 3 washes with PBS-Tween 0.01%, TMB substrate was added and reaction was stopped by adding Phosphoric Acid 1N. Optical densities were obtained in a SpectraMax250 plate reader (Molecular Devices). EC$_{50}$ of each the serum was calculated using GraphPad Prism 4.0.

**Neutralization Assay**

Serial dilutions of serum samples were pre-incubated with $4 \times 10^8$vp/mL of Ad-ISF35 for 10min at 37°C. Then, were added to $2 \times 10^4$ HeLa cells in 96 well plates for 24h at 37°C. HeLa cells were incubated with PBS-EDTA enzyme free dissociation buffer (GIBCO) for 10min at 37°C then incubated with 2µl of anti-mouse CD154 PE labeled (BD Pharmigen San Diego, CA) for 30min at 4°C. Fluorescence data were acquired using a FACSCalibur flow cytometer (Becton Dickinson) and analysis was performed using FlowJo (Tree Star, Ashland, OR, USA). Antibody neutralization activity was assessed by decrease in ISF35 (protein encoded by Ad-ISF35) expression in HeLa cells cultured with Ad-ISF35 pre-incubated with serum dilutions compared to the control cells incubated with Ad-IS35 without serum. Neutralizing activity was defined as at least two-fold increase compared to pre-treatment sample.

**Assay For Ad-ISF35 DNA In Blood Leukocytes**

We used a quantitative PCR (qPCR) assay that could detect $\geq 5$ DNA copies of Ad-ISF35 in 100ng of genomic DNA. All the samples were tested in duplicate with a cycle threshold (Ct) variability that was less than 5% for Ad-ISF35 amplification. The following primers were used for Ad-ISF35 amplification (400nM):

Forward - 5’CCT CTG GCT GAA GCC CAG 3’
Reverse - 5’CTC CCA AGT GAA TGG
ATT GT 3’. MGB-FAM probe 5’TTACTCAAGGCGGCAAA3’ was used at 250nM. The PCR reaction was run using TaqMan qPCR Universal Master Mix w/UNG from Applied Biosystems (Foster City, CA.). The qPCR program employed was 95°C for 3 minutes; 40 cycles: 95°C for 20 seconds, 52°C for 1 minute and 20 seconds in BioRad cycler iQ5 PCR machine. β-actin was used to monitor DNA quality. To calculate the Ad-ISF35 DNA copies present in given sample of DNA or cDNA we used a standard curve with viral DNA ranging from 5x10^7 to 5x10^10 viral copies.

**ELISPOT Assay**

We used the IFN-γ ELISPOT plates from R&D Systems, Inc. T-cells from pre and post treatment (showing the highest number of CD8+ cells) from frozen PBMCs were isolated with dynabeads against CD4 and CD8 (Invitrogen, Carlsbad, CA). CLL cells (5x10^6) from pre treatment samples (depleted of CD4 and CD8) were cultured for 24 hours with HeLa cells expressing CD154 (4x10^5) in 12 well plates. CLL cells were removed from co-cultures and added into empty wells for 2h to allow floating HeLa-CD154 cells to re-attach to the plate. CLL cells were washed and incubated with mitomycin-C (60ug/mL) for 1h at 37°C. Activation of CLL cells was confirmed by flow cytometry measuring the up-regulation of CD54 and CD95 markers. 1x10^5 CLL non-activated (CD4 and CD8 depleted) or CLL CD154-activated cells were cultured for 48 hours with 1x10^5 T cells plus IL2 (25 IU/mL).

**Statistical Analysis**

Descriptive statistics were used to analyze demographic and baseline characteristics, clinical response variables, immunologic variables, and toxicity data.
Univariate analysis was performed using one-way ANOVA with pre and post treatment values. Hazard ratios were calculated by using Kaplan Meier analysis. All analyses were performed using GraphPad Prism v4.0 and JMP v8.0.
RESULTS

Patient Demographics

Five females and ten males with a median age of 56 years (45-73 years) were enrolled in this study. The median leukemia cell doubling time for all patients was 3.3 months (0.9-22.5 months). The median number of prior treatments was 2 (0 to 7). Two patients were refractory to fludarabine-based treatment. CLL-cells expressed unmutated Ig heavy chain variable region genes (IgV_H) in seven patients and ZAP-70 (30) in nine patients. Six patients had leukemia cells with adverse cytogenetics, four with deletions at 11q and two with deletions at 17p (Table 1).

Adverse Events

All patients received Ad-ISF35 IDI. Only two of fifteen patients did not complete the post-injection observation period of 84 days due to disease progression. The most common adverse events were grade 1 or 2 injection site reactions and systemic “flu-like” symptoms. Four patients had transient grade 3 or 4 neutropenia. One of these patients in cohort 2 presented grade 3 neutropenia that lasted for 6 days. This was not considered as DLT since the adverse event was not grade ≥ 4 and the duration was less than 7 days. In cohort 3, the first three patients did not present any DLTs. In cohort 4 three patients were enrolled, two of them presented DLTs including hypophosphatemia (grade 3 and 4) and SGOT elevation (grade 3). Because of this, we proceeded to enroll three additional patients in cohort 3 (3E). One patient presented with neutropenia grade 4 (not considered DLT as it lasted only 5 days) and one patient with hypophosphatemia grade 3 (considered DLT) (Table 2). All adverse effects were subclinical and self-limiting. With these data, we defined cohort 3 dose (1x10^{11} vp) as...
the MTD. In addition, we have not observed long-term adverse effects associated with Ad-ISF35 IDI after a median follow up of 2.5 years.

**Evaluation For Ad-ISF35 DNA presence and ISF35 protein expression In Blood Leukocytes Following IDI of Ad-ISF35**

We monitored the presence Ad-ISF35 DNA and ISF35 (protein encoded by Ad-ISF35 vector) expression in PBMCs collected before and after Ad-ISF35 IDI. For detection of Ad-ISF35 DNA we used a sensitive qPCR assay capable of detecting 5 copies of Ad-ISF35 DNA in 100 ng of genomic DNA (gDNA). Ad-ISF35 was detected in only 3 patients 8-hours after Ad-ISF35 IDI. Each of these patients had PBMCs with less than 9 copies of Ad-ISF35 per 100 ng of gDNA. Ad-ISF35 DNA was not detected at later time points (Figure 1A). ISF35 protein surface expression was measured by flow cytometry. The results showed not detectable expression of ISF35 protein on the PBMCs of any patient at any time point (data not shown).

**Biologic Responses to IDI of Ad-ISF35 and evidence of systemic bystander effect**

Most patients (n=14) experienced transient increases in platelet counts at day 7 and 14. None of the patients developed worsening anemia post-injection (Figure 1B). Also, we observed changes in T cells in the majority of patients enrolled in this study. During the first 48 hours, most patients (n=11) had a transient fall in ALC (Figure 1C) and absolute T cell counts (Figure 1D). The acute drop in T cells counts was significant when compared to baseline levels in both cell subsets (CD3⁺CD8⁺ and CD3⁺CD4⁺ cells) - (p< 0.05). However, the majority of patients (n=10) showed later an increase of T cells ranging from 6-311% during the first four weeks after Ad-ISF35 IDI. We observed that
the majority of patients experienced an increase in ANC during the first two days following Ad-ISF35 IDI (Figure 1D).

We observed systemic biologic responses to Ad-ISF35 IDI in most patients. In particular, we observed statistically significant up regulation of CD95, CD20 and HLAI during the first 48 hours after Ad-ISF35 IDI (Figure 1E). Such phenotypic changes are similar to those of CLL-cells co-cultured with CD154-bearing cells in-vitro (20).

We also noted that levels of IFN-γ and IL-6 in the sera of 11 out of 14 patients increased one-day after Ad-ISF35 IDI relative to pre-treatment levels. The relative increase in serum IFN-γ appeared proportionate to the administered dose of Ad-ISF35 (linear trend analysis \( p=0.0328, r^2=0.35 \)); patients in cohorts 3 and 4 had the highest post-treatment serum levels of IFN-γ (Figure 2A).

We evaluated whether cytokines or other factors present in the serum of patients that received Ad-ISF35 IDI were responsible for the phenotypic changes observed in circulating leukemic cells. CLL-cells were cultured with autologous serum samples collected before and one-day after Ad-ISF35 IDI. As a control, the same CLL-cells were stimulated by co-culture with CD154-bearing cells (HeLa-CD154). The expression levels of surface immune co-stimulatory molecules and death receptors were evaluated via flow cytometry. CLL-cells cultured with sera collected before or after Ad-ISF35 IDI did not induce increased expression of these surface antigens. In contrast, CLL-cells co-cultured with HeLa-CD154 expressed higher levels of CD95 or immune co-stimulatory molecules than CLL-cells co-cultured with wild-type HeLa (Figure 2B).
Samples collected before and after Ad-ISF35 IDI from four patients were evaluated by ELISPOT assay to detect IFN-γ production from T cells in response to co-culture with CD40 stimulated autologous CLL cells. In these patients we did not observe significant increase in the levels of T cell / IFN-γ production after Ad-ISF35 IDI (Supplementary Figure S1).

**Antibody responses against Ad-ISF35 and human-CD154**

We evaluated for antibodies specific for adenovirus or human-CD154 (hCD154). Sera collected from patients prior to Ad-ISF35 IDI had median titer of anti-adenovirus antibodies of 352 (CI 173-531). However, sera collected one month after therapy had significantly higher titers (median 622, CI 151-1093, p<0.05). Except for patient #10, the anti-adenovirus antibodies induced by Ad-ISF35 IDI did not neutralize the capacity of adenovirus to infect HeLa-cells *in-vitro* (Table 3). None of the patients developed detectable antibodies to human-CD154.

**Clinical Response To IDI Of Ad-ISF35**

Although Ad-ISF35 IDI was injected into a single axillary lymph node, we observed systemic clinical responses. The magnitude of these responses did not appear to have a clear dose-response relationship. Considering the best response during 84 days after Ad-ISF35 IDI, 87%, 80%, and 53% of patients had respective reductions in lymphadenopathy, splenomegaly, or ALC by more than 50% (Figure 3A). The response observed in spleen and lymph nodes were durable during the course of the study (Figure 3B). Three patients achieved partial response (PR), seven patients had stable disease (SD), and five had progressive disease (PD) by IWCLL criteria (27, 28) (Table 1). Six patients (patients 1, 2, 5, 7, 11, and 15) had durable responses
(median 8.8 months) and did not require additional treatment for over 6 months. Two of these patients (patients 2 and 5) did not require additional treatment for over a year after Ad-ISF35 IDI. The median time to next treatment (TNTx) for all patients was 5 months.

We performed Kaplan-Meier analysis to determine hazard ratios comparing the duration of response (≥ 6 months), with variables such as number of prior therapies (≤1), ZAP-70 (≤20% positive cells), B2M levels (≤2.5 mg/L), IgG levels (≤500 mg/dL), splenomegaly (≤5 cm), and LNP (total ≤70 cm²). This analysis showed that only the number of prior therapies (≤1) had a statistically significant correlation with the duration of response (Hazard ratio 0.1491; CI=0.04071 to 0.5464; p=0.0041) - (Table 4). Therefore, patients who had no more than one prior treatment for CLL were 6.7 times more likely to have a durable response (TNTx ≥ 6 months).
DISCUSSION

We examined the safety of gene-immunotherapy using Ad-ISF35 IDI in patients with CLL. The injection of Ad-ISF35 into an axillary lymph node was safely delivered and without long-term adverse effects. Most of adverse effects were anticipated and of low-grade (grade 1 or 2). These included “flu-like” symptoms and erythema or pain at the site of injection. Such adverse effects have been observed in patients injected with adenovirus vectors directly into tumor nodules (31-33) and most likely represent an inflammatory response to adenovirus infection / transduction. Many of the adverse events noted in this study also were similar to those observed in patients infused with autologous-CLL-cells transduced ex-vivo with Ad-mCD154 or Ad-ISF35 (22, 23). In these ex-vivo studies, patients who received the highest dose of transduced cells generally had a higher incidence of grade 2 versus grade 1 adverse events. We observed a similar dose-relationship in patients receiving the two higher doses of Ad-ISF35, suggesting that there might be a relationship between the injected dose of Ad-ISF35 and frequency or intensity of adverse events.

We observed grade 3/4 neutropenia, hypophosphatemia and SGOT elevation in six patients treated in cohorts 2, 3E and 4. In three of these patients (2 in cohort 4 and 1 in cohort 3E), hypophosphatemia and SGOT elevation were considered DLTs and thus defined the MTD as $1 \times 10^{11}$ vp (cohort 3 dose). All the adverse events observed were subclinical and transient. The grade 3-4 hypophosphatemia observed could be related to increased serum levels of IFN-γ and IL-6 during the first 24 hours following Ad-ISF35 IDI. Hypophosphatemia has been associated with high serum levels of such
inflammatory cytokines in patients with sepsis and in mice injected with IL-6, TNF-α, or IL-1β (34).

Some patients in the cohorts that received the higher doses also experienced transient elevations in serum hepatic transaminases. Although such transient and asymptomatic elevation of transaminases has been observed in patients who received intravenous injections of adenovirus vectors (22), the patient who had grade 3 elevation in SGOT elevation did not have detectable Ad-ISF35 in the blood. As such, it is conceivable that the transient elevations in hepatic transaminases instead could be associated with high serum levels of inflammatory cytokines (35). These cytokines could have been secreted in response to adenovirus infection as well as in response to CD40-CD154 stimulation (36).

Several patients had decreased ALC and elevated ANC during the first 48 hours after Ad-ISF35 IDI, only to develop neutropenia 2-3 weeks later. Early increases in blood neutrophils associated with decreases in blood lymphocytes have been observed in mice infused with syngeneic dendritic cells stimulated by CD154 and IL-2 and pulsed with lymphoma antigens (37). Changes in ANC could be a direct response to modulation of the CD40-CD154 pathway as it has been observed in our mouse model (25, 26) and in patients with CD154-deficiency and hyper-IgM syndrome (38-40). On the other hand, neutropenia could be secondary to elevated levels of IFN-γ and/or IL-6 (41, 42). Nevertheless, each patient recovered with no evidence of infection or other related complications.

We observed fluctuation in T cell counts after Ad-ISF35 IDI. Initially during the first 48 hours after injection the T cells decreased significantly and this could be in response to viral infection, cytokines, cell migration, or activation induced cell death.
At later time points the majority of patients showed increased in T cells up to 300%, which was similar to what we have observed in previous studies using Ad-CD154 (22). We studied whether T cells isolated after therapy were reactive against autologous CLL cells using ELISPOT assay for IFN-γ. However, in four patients available for evaluation, we did not observe reactivity of T cells against CD40 stimulated-CLL cells. This finding is different than our previous experience in patients receiving ex vivo transduced autologous CLL cells with Ad-CD154 (22). It is unclear the reasons for the lack of evidence of T cell stimulation in response to AD-ISF35 IDI measured by IFN-γ ELISPOT. Potential explanations could be the different route of administration (ex vivo vs. IDI), sample size and time of analysis of the samples. Other immune mediated processes including microenvironment cell activation of macrophages (44) could be responsible of the objective responses observed in some patients in this study. It is conceivable to hypothesize that multiple intranodal injections could enhance immunological responses including T cell reactivity against CLL antigens. We will address these mechanistic questions in future studies.

We did not find evidence for Ad-ISF35 DNA in the PBMCs except in three patients, who had trace levels of detectable Ad-ISF35 DNA (close to the low-limit of sensitivity) 8-hours post-injection. In addition, we did not detect expression of ISF35 protein on circulating CLL-cells at any time following Ad-ISF35 IDI. Nevertheless, all patients experienced a systemic biologic response. We observed phenotypic changes in the circulating CLL-cells that included expression of immune co-stimulatory molecules and death receptors. These changes were similar to those observed in-vitro after leukemia cells are cultured with CD154-expressing cells (19).
It is unlikely that the clinical and biological activity observed after Ad-ISF35 IDI are mediated by direct-transduction of cells distant from the injected lymph node. Our data in an A20-lymphoma mouse model have shown that after intratumoral injections, Ad-ISF35 is primarily found in tumor tissues with no evidence of accumulation or persistence in peripheral organs with a rapid virus clearance 24h after injection (25, 26). In the same mouse model, we have detected the transduction not only lymphoma cells, but also other important cells from the microenvironment such as fibroblasts, macrophages, and epithelial cells (manuscript in preparation). This suggests the possibility that phenotypic changes of microenvironment cells may play a role in the response to intratumoral Ad-ISF35 injection.

Similarly, our data suggest that serum cytokines or other soluble factors are not entirely responsible for the phenotypic changes observed after Ad-ISF35 IDI (Figure 2B). Therefore, the presence of systemic objective clinical response observed in conjunction with phenotypic changes and the lack of detectable Ad-ISF35 in circulating leukemia cells, suggest that these effects are due to the stimulation generated by either contact with ISF35-bearing cells or other non-transduced activated cells, providing the so called “bystander-effect”. Lymph node biopsies before and after Ad-ISF35 injection could have provided additional insight into this process. However, they were not performed in this study in order to avoid additional local toxicities and adverse events that could interfere with the primary endpoint of this phase I study, which was to evaluate safety and tolerability. We are planning to perform these biopsies in subsequent clinical trials.

One concern was whether Ad-ISF35 IDI would induce autoantibodies reactive with hCD154. Such autoantibodies conceivably could induce adverse effects that are
similar to those noted for some patients treated with antibodies specific for CD154 (45, 46). However, none of the patients developed antibodies reactive with hCD154. Nevertheless, 40% of treated patients were induced to increase their titers of antibodies anti-adenovirus, indicating that Ad-ISF35 IDI could elicit an anti-adenovirus antibody response even in patients who have immune deficiency associated with CLL. Moreover, patients with CLL typically have hypogammaglobulinemia (median serum IgG levels in this study was 496 mg/dL) and typically respond poorly to administered vaccines (47). One of the treated patient also developed antibodies to adenovirus that could neutralize Ad-ISF35. It is uncertain whether the development of such antibodies can mitigate the activity of subsequent injections of Ad-ISF35. This will be evaluated in subsequent clinical studies examining the safety and activity of repeated administrations of Ad-ISF35 IDI.

Although the primary purpose of the current study was to examine the safety of Ad-ISF35 IDI, we observed objective clinical responses in the majority of the patients treated. Patients experienced a 50% or more reduction in leukemia cell counts (7 of 15), lymphadenopathy (13 of 15), or splenomegaly (12 of 15). Six patients did not require additional treatment for six months or more and three patients achieved a PR. Moreover, responses achieved in the spleen and lymph nodes were durable and the majority of patients requiring additional treatment did so because of persistent lymphocytosis. This suggests that Ad-ISF35 can promote changes in the microenvironment of lymph nodes and spleen that could translate into clinical benefit. Further studies to corroborate these observations are needed.

Clinical responses in CLL can be compartmentalized. Patients can have excellent reduction of lymphocytosis but no response in large lymph nodes like in the
case of patients treated with alemtuzumab (48), or good reduction in lymphadenopathy and spleen but lack of response or lymphocytosis progression like in patients receiving tyrosine kinase inhibitors (49, 50). In the patients reported in this phase I study, we observed better and more durable responses in reduction of lymphadenopathy and splenomegaly. Despite of the fact that most patients had some degree of response (Figure 3), only three of them fulfill all the criteria needed to achieve a clinical response based on the international guidelines.

Previously untreated patients or those with less than one previous treatment showed the best clinical responses to Ad-ISF35 IDI (almost seven times more likely to have durable responses). This supports the notion that early immunological intervention in patients with CLL could result in better clinical outcomes. Although whether prior treatment with drugs or agents that suppress immune function mitigates the activity of Ad-ISF35 IDI needs to be evaluated in subsequent clinical trials.

In summary, this first in man phase I clinical study demonstrates that gene-immunotherapy using Ad-ISF35 IDI is feasible and safe. We defined the dose of $1 \times 10^{11}$ vp per injection as the MTD. Even though the study was designed to determine safety and tolerability of a single Ad-ISF35 IDI, we also observed systemic biologic and clinical effects. This is despite the fact that we could not detect expression of ISF35 protein in mononuclear cells isolated from the blood of patients following Ad-ISF35 IDI. This suggests that such responses were due to a bystander-effect, in which leukemia cells are induced to undergo phenotypic changes when in contact with ISF35-bearing cells or other non-transduced activated cells. Our results also indicate that Ad-ISF-35 IDI induced antibody, cytokine responses and changes in the phenotype of circulating CLL-cells that are consistent with immune activation similar to that induced by intravenous
administration of autologous Ad-ISF35-transduced CLL-cells. This suggests a greater interchange of cells between the blood, spleen and lymphoid compartments of patients with CLL.

To our knowledge this is the first clinical study conducted in CLL patients showing that direct intratumoral / intranodal modulation by an immune based strategy can result in clinical activity. This argues that the tumor microenvironment is a relevant target in this disease.

Additional studies of the safety and potential activity of multiple Ad-ISF35 intranodal injections for patients with CLL, related B-cell malignancies and solid tumors expressing CD40 are warranted.
Acknowledgments

We thank Jose Sandoval-Sus and Theresa Bishop for data preparation, Laura Rassenti and CRC Tissue core staff for technical support and Karen Messer for helping with the statistical analysis of our data. We also want to thank the Alliance for Cancer Gene Therapy (TJK, JEC, JMC, MU, JSB, RSP), P01-CA81534 grant - CLL Research Consortium (TJK, JEC), FDA-OOPD- R01-3427 grant (JEC, TJK) and Memgen LLC for providing Ad-ISF35.
REFERENCES


Table 1. Patient demographics

<table>
<thead>
<tr>
<th>Cohort (dose)</th>
<th>Pt #</th>
<th>Age</th>
<th>Sex</th>
<th>IgVH (% Hom)</th>
<th>%ZAP70+</th>
<th>%CD38+</th>
<th>Prior Treatments</th>
<th>Cytogenetics</th>
<th>FISH</th>
<th>ECOG</th>
<th>Rai Stage</th>
<th>β2M (g/dL)</th>
<th>Response†</th>
<th>TNTx</th>
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<td>49</td>
<td>M</td>
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<td>29.8</td>
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<td>Idiotype vaccine conjugated with KLH</td>
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<td>del13q</td>
<td>0</td>
<td>II</td>
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<td>PD</td>
<td>6.8</td>
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<td>55</td>
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<td>96.8</td>
<td>36.7</td>
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<td>46,XY</td>
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<td>4</td>
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<td>PR</td>
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<tr>
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<td>3</td>
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<td>M</td>
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<td>28.1</td>
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<td>AT-101, HDMP+R, A</td>
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<td>61</td>
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<td>98.7</td>
<td>98.7</td>
<td>FR, R</td>
<td>47,XY</td>
<td>Tri12</td>
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<td>SD</td>
<td>2.7</td>
</tr>
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<td>tri12, del17p</td>
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<td>PD</td>
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</tr>
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<td>92.5</td>
<td>19.3</td>
<td>HDMP+R</td>
<td>46,XY</td>
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<td>2.9</td>
<td>SD</td>
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<td>SD</td>
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<td>0</td>
<td>II</td>
<td>2.8</td>
<td>SD</td>
<td>7.7</td>
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</tbody>
</table>

*Patient fludarabine refractory based on IWCLL 2008 definition.
†Response assessment was performed based on IWCLL 2008 guidelines criteria.
A = Alemtuzumab; AT-101 = BCL-2 family member inhibitor; C = Chlorambucil; CP = Chlorambucil and Prednisone; F = fludarabine; FCR = Fludarabine, Cyclophosphamide and Rituximab; FM = Fludarabine and Mitoxantrone; FR = Fludarabine and Rituximab; GT = gene therapy (CD154); HDMP = High-Dose Methylprednisolone; Hsp90 = Heat Shock Protein 90 Inhibitor; NA = not available; O = Ofatumumab; R = Rituximab; X-cyte = T-Cell Therapy. % Hom =Percentage of gene homology. PD = Progressive disease, PR = Partial response, SD = Stable disease. TNTx = Time to next treatment in months.
<table>
<thead>
<tr>
<th>Event</th>
<th>Grade 1 / 2</th>
<th>Grade 3 / 4</th>
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<td>Cohort 1</td>
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<td>Cohort 2</td>
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<td>Neutropenia(^1)</td>
<td>1 (33%)</td>
<td>2 (67%)</td>
<td>2 (33%)</td>
<td>1 (33%)(^3)</td>
<td>1 (17%)(^4)</td>
<td>2 (67%)(^5)</td>
<td></td>
</tr>
<tr>
<td>Hypophosphatemia</td>
<td>1 (33%)</td>
<td>1 (33%)</td>
<td>1 (17%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGOT elevation</td>
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<td>0</td>
<td>1 (17%)</td>
<td>1 (33%)</td>
<td></td>
<td></td>
<td>1 (17%)(^*)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>3 (100%)</td>
<td>3 (100%)</td>
<td>6 (100%)</td>
<td>3 (100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection site reaction</td>
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<td>3 (100%)</td>
<td>6 (100%)</td>
<td>3 (100%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Flu-like symptoms</td>
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<td>3 (100%)</td>
<td>6 (100%)</td>
<td>3 (100%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hyperglycemia(^2)</td>
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<td>3 (100%)</td>
<td>5 (83%)</td>
<td>3 (100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombocytopenia(^1)</td>
<td>1 (33%)</td>
<td>3 (100%)</td>
<td>5 (83%)</td>
<td>2 (67%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperuricemia</td>
<td>2 (67%)</td>
<td>2 (67%)</td>
<td>4 (67%)</td>
<td>1 (33%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweating</td>
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<td>4 (67%)</td>
<td>2 (67%)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Hypocalcemia</td>
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<td>2 (67%)</td>
<td>3 (50%)</td>
<td>1 (33%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
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<td>1 (17%)</td>
<td>2 (67%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGPT elevation</td>
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<td>2 (67%)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Constipation</td>
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<td>1 (33%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Insomnia</td>
<td>1 (17%)</td>
<td>2 (67%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Anorexia</td>
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<td></td>
<td></td>
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<td>Arthritis (non-septic)</td>
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<td>1 (17%)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Diarrhea</td>
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<td></td>
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<tr>
<td>Hypotension</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>TOTAL Events / Patient</strong></td>
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<td><strong>8.6</strong></td>
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<tr>
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<td><strong>16</strong></td>
<td><strong>26</strong></td>
<td><strong>52</strong></td>
<td><strong>28</strong></td>
<td>0</td>
<td>1</td>
<td>2</td>
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</tbody>
</table>

\(^1\) Based on IWCLL 2008 criteria

\(^2\) Patient with history of insulin dependent diabetes mellitus (ID-DM) was not included

\(^3\) Patient presented neutropenia grade III (not considered DLT)

\(^4\) Patient presented neutropenia grade IV that lasted for 5 days (not considered DLT)

\(^5\) Patients presented neutropenia grade III (not considered DLTs)

\(^*\) Adverse events considered DLTs
Table 3. Anti-adenovirus and neutralizing antibodies titer before (Pre) and after (Post) Ad-ISF35 injection.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Patient ID</th>
<th>Pre</th>
<th>Post$^1$</th>
<th>Pre</th>
<th>Post$^1$</th>
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<tbody>
<tr>
<td>1 1(\times)10^{10} vp</td>
<td>1</td>
<td>244 ± 51</td>
<td>664 ± 87</td>
<td>&lt;50</td>
<td>60</td>
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<tr>
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<td>2</td>
<td>635 ± 157</td>
<td>458 ± 89</td>
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<td>ND</td>
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<tr>
<td></td>
<td>3</td>
<td>352 ± 60</td>
<td>317 ± 52</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2 3.3(\times)10^{10} vp</td>
<td>4</td>
<td>1060 ± 94</td>
<td>1619 ± 138</td>
<td>289</td>
<td>281</td>
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<tr>
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<td>5</td>
<td>32 ± 16</td>
<td>30 ± 23</td>
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<tr>
<td></td>
<td>6</td>
<td>944 ± 145</td>
<td>622 ± 104</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3 1(\times)10^{11} vp</td>
<td>7</td>
<td>891 ± 95</td>
<td>1282 ± 152</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<tr>
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<td>8</td>
<td>113 ± 29</td>
<td>186 ± 43</td>
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<tr>
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<td>470 ± 114</td>
<td>475 ± 158</td>
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<tr>
<td>4 3.3(\times)10^{11} vp</td>
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<td>1888 ± 273</td>
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<td>12</td>
<td>178 ± 34</td>
<td>243 ± 51</td>
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<tr>
<td>3E 1(\times)10^{10} vp</td>
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<td>21 ± 88</td>
<td>21 ± 88</td>
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<td>ND</td>
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<tr>
<td></td>
<td>14</td>
<td>173 ± 34</td>
<td>776 ± 123</td>
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<td>15</td>
<td>837 ± 160</td>
<td>752 ± 123</td>
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$^1$ Post treatment evaluation performed 1 month after Ad-ISF35 injection. ND: Not determined. Neutralizing antibodies assay was only performed in samples showing increased titer after Ad-ISF35 IDI.
Table 4. Factors influencing duration of response (TNTx ≥ 6 months) after Ad-ISF35 IDI.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Hazard Ratio*</th>
<th>95% CI of ratio</th>
<th>p value</th>
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<tbody>
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<td># of previous Tx (≤1)</td>
<td>0.1491</td>
<td>0.04071 to 0.5464</td>
<td>0.0041</td>
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<tr>
<td>ZAP-70 (≤20% positive cells)</td>
<td>0.6217</td>
<td>0.1900 to 1.941</td>
<td>0.4001</td>
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<tr>
<td>B2M (≤2.5 mg/L)</td>
<td>1.029</td>
<td>0.3536 to 3.011</td>
<td>0.9543</td>
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<tr>
<td>IgG levels (≤500 mg/dL)</td>
<td>1.799</td>
<td>0.6586 to 5.830</td>
<td>0.2266</td>
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<tr>
<td>Splenomegaly (≤5 cm)</td>
<td>1.974</td>
<td>0.7330 to 6.671</td>
<td>0.1589</td>
</tr>
<tr>
<td>LNP (total ≤70 cm²)</td>
<td>2.104</td>
<td>0.6982 to 9.480</td>
<td>0.1556</td>
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</table>

*Hazard ratios calculated with Kaplan Meier test. Splenomegaly measured in cm below the right costal margin. LNP – bidimensional lymph node product. B2M – Beta 2 microglobulin. TNTx: Time to next treatment.
FIGURE LEGENDS

**Figure 1. Laboratory parameters in patients that received Ad-ISF35 IDI.**

A. DNA isolated from blood leukocytes of patients that received Ad-ISF35 IDI was evaluated for the presence of virus DNA by qPCR. The dotted line indicates the limit of detection of this assay (5 copies of Ad-ISF35 in 100ng of genomic DNA). B. Hemoglobin levels and platelet counts, C. absolute lymphocyte counts (ALC), D. T-cell counts (CD3⁺CD4⁺ and CD3⁺CD8⁺ subsets) and neutrophil absolute counts (ANC) of all patients over time following IDI of Ad-ISF35 are represented in the graphs. E. Expression of cell surface markers in CLL-cells obtained pre and post (1-2 days) Ad-ISF35 IDI. Error bars represent the standard deviation (SD). * Denotes statistical significant difference by t-test when compared to baseline level.

**Figure 2. Cytokine levels in serum and evidence of bystander effect in leukemic cells from peripheral blood after Ad-ISF35 IDI.**

A. Serum cytokine levels measured over time were assessed using the Luminex™ assay on each cohort of patients that received Ad-ISF35 IDI. B. CLL-cell surface expression of CD95, CD54, CD86 and CD80 was assessed by flow cytometry in vivo and in vitro. Histograms on the left show in-vivo data obtained from blood samples collected before (Day 0) and 2 days after Ad-ISF35 IDI. Histograms on the right represent the in vitro data obtained after CLL-cells were cultured with autologous serum collected before (pre) and 1 day (highest levels of IFN-γ and IL-6) after Ad-ISF35 IDI. As a control, CLL-cells were co-cultured with Hela cells expressing CD154 (CD154 stimulation).
**Figure 3. Clinical parameters of patients that received Ad-ISF35 IDI.**

CLL patients were enrolled in 4 cohorts using a 3+3 dose escalation design with doses of Ad-ISF35 ranging from $1 - 33 \times 10^{10}$. **A.** Absolute lymphocyte counts (ALC), bidimensional lymph node product (LNP) of measurable and palpable lymph nodes and palpable spleen size (below the left costal margin) were recorded from Day 0 (Ad-ISF35 IDI) to Day 90. Data was normalized to day 0 and the best response change is defined as the maximum reduction/minimum increase from baseline. The dotted line indicates 50% reduction. **B.** ALC, LNP and splenomegaly are shown over time. Cohort 3E correspond to the extension of cohort 3 after toxicities in cohort 4 were observed. The dotted line indicates 0% change from baseline.
**Figure 1.**

(A) Ad-ISF35 copy number per 100ng gDNA.

(B) Platelet count and Hb levels over time.

(C) Absolute lymphocyte count over time.

(D) ANC, CD3+CD4+, and CD3+CD8+ cells per mL over time.

(E) Absolute MFI levels for various markers over time.
Figure 2.

A

<table>
<thead>
<tr>
<th>Cohort 1</th>
<th>Cohort 2</th>
<th>Cohort 3</th>
<th>Cohort 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMCSF</td>
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<tr>
<td>IFNγ</td>
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<tr>
<td>TNFα</td>
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<tr>
<td>IL-4</td>
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<td>IL-8</td>
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<td>IL-10</td>
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<tr>
<td>IL-12p70</td>
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</tbody>
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B

<table>
<thead>
<tr>
<th>In-vivo</th>
<th>In-vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Day 2</td>
</tr>
</tbody>
</table>

- CD95
- CD54
- CD86
- CD80

% of Max:
- CD95: 16%, 31.8%
- CD54: 79.4%, 90.9%
- CD86: 6.94%, 11.1%
- CD80: 4.63%, 6.64%

Isotype Sample

Days after injection

Figure 3.

**A.**

- **ALC**
- **LNP**
- **Splenomegaly**

% change

Patient ID

<table>
<thead>
<tr>
<th>Cohort 1</th>
<th>Cohort 2</th>
<th>Cohort 3</th>
<th>Cohort 4</th>
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<tbody>
<tr>
<td>Subject 1</td>
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<td>Subject 3</td>
<td>Subject 4</td>
</tr>
<tr>
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<tr>
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<td>Subject 12</td>
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<tr>
<td>Subject 13</td>
<td>Subject 14</td>
<td>Subject 15</td>
<td></td>
</tr>
</tbody>
</table>

**B.**

% change from baseline

Days after Ad-ISF35 injection

**Cohort 1**
- Subject 1
- Subject 2
- Subject 3

**Cohort 2**
- Subject 4
- Subject 5
- Subject 6

**Cohort 3**
- Subject 7
- Subject 8
- Subject 9

**Cohort 4**
- Subject 10
- Subject 11
- Subject 12

**Cohort 3E**
- Subject 13
- Subject 14
- Subject 15