Persistence of Long-Lived Plasma Cells and Humoral Immunity in Individuals Responding to CD19-Directed CAR T cell Therapy

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Running Title: Humoral Immunity following CAR-T cell Therapy
Key Points

• CD19-targeted T cell immunotherapy reveals that a population of plasma cells lacking CD19 expression survives long-term, independent of B cells.

• Pre-existing humoral immunity to vaccine-related antigens can persist in patients despite marked B-cell aplasia following CTL019 immunotherapy.
Abstract
The mechanisms underlying the maintenance of long-lasting humoral immunity are not well understood. Studies in mice indicate that plasma cells (PCs) can survive up to a lifetime, even in the absence of regeneration by B-cells, implying the presence of long-lived PCs as a mechanism for long-lasting immunity. Evidence from humans treated with anti-CD20, which depletes circulating B-cells, also suggests B-cell independent long-term survival of some PCs. On the other hand, antibody responses may be sustained solely by short-lived PCs with repopulation from clonally-related memory B-cells. To explore PC longevity and humoral immunity in humans, we investigated the fate of PCs and their antibodies in adult and pediatric patients who received chimeric antigen receptor (CAR)-based adoptive T-cell immunotherapy targeting CD19 to treat B-cell lineage malignancies (CTL019). Treatment with CTL019 is frequently associated with B-cell aplasia that can persist for years. Serum antibody titers to vaccine-related antigens were measured and quantitative assessment of B-cells and PCs in blood and bone marrow were performed at various time points before and after CTL019 therapy. While total serum immunoglobulin concentrations decline following CTL019-induced B-cell aplasia, several vaccine/pathogen-specific serum IgG and IgA titers remain relatively stable for at least 6 and 12 months post treatment, respectively. Analysis of bone marrow biopsies after CTL019 revealed 8 patients with persistence of antibody-secreting PCs at least 25 months post-CTL019 infusion despite absence of CD19+CD20+ B-cells. These results provide strong evidence for the existence of memory B-cell-independent, long-lived PCs in humans that contribute to long-lasting humoral immunity.
Introduction

Antibodies are relatively short-lived proteins with serum half-lives ranging from approximately one week to one month. However, antigen-specific antibody responses can last as long as a lifetime. Thus, the PCs that produce them must be maintained long-term. Upon antigen encounter, a B-cell proliferates and gives rise to clonally-related PCs and memory B-cells, the latter giving rise to additional PCs upon antigen re-encounter. Long-lived humoral immunity may theoretically be maintained by PCs that are long-lived or replenished from long-lived memory B-cells, or both. Cell-labeling studies in rodents show that a fraction of newly generated PCs survive for at least 6 months in mice, supporting the existence of long-lived plasma cells. Additionally, B-cell depletion studies in mice suggest that at least some PCs are maintained independently of regeneration from B-cells. Whether these observations also apply to PC longevity in humans is not well known. In patients with rheumatoid arthritis or immune-thrombocytopenic purpura, PCs have been demonstrated for 3-6 months post treatment with anti-CD20. We addressed this question by studying PCs in patients experiencing B-cell aplasia induced by CD19-targeted adoptive T-cell immunotherapy.

CTL019, a CD19-specific chimeric antigen receptor (CAR)-based T-cell therapy, has resulted in long-term disease remissions in some patients with chemotherapy-resistant B-lineage malignancies including chronic lymphocytic leukemia (CLL) and B-lymphoblastic leukemia (B-ALL). CD19 is a pan-B-cell surface protein with expression that spans the development of B-cells from early pre-B-cells to mature, fully-differentiated B-cells. Thus, successful therapy with CTL019 is often accompanied by profound and persistent B-cell aplasia. Following differentiation of B-cells to PCs,
CD19 expression is thought to decline. Immunophenotypic analyses of bone marrow-derived PCs demonstrate both CD19+ and CD19-neg populations. Little is known about the ontogeny, functions, and fate of these two immunophenotypically-distinct PC populations. Recent data on human PCs suggest that CD19-neg PCs are enriched in bone marrow and may include long-lived cells that give rise to long-lasting humoral immunity.

We hypothesized that CTL019 would spare the population of CD19-neg PCs, leaving previously established humoral immunity relatively intact. Furthermore, we anticipated that tracking the fate of the PCs in the context of CTL019-induced B-cell aplasia would shed light on the question of CD19-neg PC lifespan and maintenance. In the present study (Figure 1), we use multiple methods to examine the fate of PCs and humoral immunity in the context of CTL019 therapy. We show that CD19-neg bone marrow PCs are indeed resistant to direct elimination by CTL019 and persist independent of B-cell repopulation for at least 25 months. We further show that a variety of humoral responses established prior to CTL019 infusion are retained even as total immunoglobulin levels decline. These results support the hypothesis that human CD19-neg PCs can be long-lived to maintain long-lasting humoral immunity.

Materials and Methods

Human Subjects

The present study included 4 subjects from adult CTL019 trials at the University of Pennsylvania (Penn) (ClinicalTrials.gov number, NCT01029366 and NCT02030834) and 12 from a pediatric CTL019 trial at the Children’s Hospital of Philadelphia (CHOP)
Written informed consent for participation was obtained from patients or their guardians according to the Declaration of Helsinki and protocols were approved by the institutional review boards of Penn and CHOP.

B-cell aplasia was defined as <1% CD19+CD20+ B-cells of PBMC as determined by flow cytometry. In the vast majority of time-points, the frequency of circulating B-cells was <0.1% (Supplementary Figure 1). For samples from UPN-1 and UPN-2 in particular, in the post-treatment samples with CD19+CD20+ frequencies above 0.1%, kappa and lambda light-chain staining was performed and showed that events in the CD19+C20+ gate were light-chain negative, thus, likely representing technical artifact.

In the adult and pediatric trials, administration of immunoglobulin as replacement therapy was left to the discretion of the treating physician. As such, there was variation in treatment but, in most cases, it was given when serum IgG was below the normal range.

For UPN-1, UPN-3 and CHP-7 for whom antigen-specific titers are shown, vaccination history could not be obtained due to retrospective nature of serum sample testing.

**Flow cytometry and cell sorting**

Flow cytometry for assessment of peripheral blood B-cells was done by the Translational Correlative Studies Laboratory of the Translational Research Program at the University of Pennsylvania as previously described.12
Flow cytometry of bone marrow aspirate cells was performed using approximately 1-5x10^6 total cells per condition. Surface staining was performed using anti-CD3APC-Cy7 (SP34-2), anti-CD14APC-H7 (M5E2), anti-CD16-APC-H7 (3G8), anti-CD20PerCP (L27), anti-CD38BV605 (HB7), anti-CD45FITC (HI30), anti-CD138BV421 (MI15) all from BD Biosciences, anti-CD19PE-Cy7 (J3-119, Beckman Coulter) and anti-CD27VB711 (O323, BioLegend). Samples were stained for viability using eFluor780 (eBioscience). Following permeabilization using Permeabilization Medium-B (Invitrogen), intracellular-staining was performed using anti-lambda-PE (MHL-38) and anti-kappa-APC (MHK-49) from BioLegend. Samples were analyzed using an LSR II (BD Biosciences). For cell sorting, mononuclear cells isolated by Ficoll density centrifugation were stained with surface antibodies and eFluor780 and sorted using a FACS Aria (BD Biosciences).

**Elispot**

To enumerate total IgG, IgA and IgM secreting cells, bone marrow aspirate mononuclear cells or sorted cells were plated at 100 to 2.5 x 10^5 cells per well of elispot plates. Wells Elispots for total IgG, IgM and IgA secreting cells were performed using elispot kits (Mabtech, Cincinnati, OH) according to the manufacturer’s instructions.

Elispots for antigen-specific antibody-secreting cells were performed by modifications of the Mabtech elispot protocol. Tetanus toxoid (2ug/ml, Santa Cruz Biotechnology, Dallas, TX), Measles (10 ug/ml, AbD Serotec, Raleigh, NC) or Mumps (10ug/ml, AbD Serotec),
each diluted in phosphate buffered saline, were used to coat wells overnight at 4°C. Wells were then washed and blocked with culture medium followed by addition of cells for overnight culture. Spots were then developed using detection reagents in the Mabtech IgG elispot kit according to the manufacturer’s instructions.

**Elisa**

For measurement of antigen-specific-IgG levels, only patients with available serum, who also had protective antibody levels prior to CTL019 therapy, were tested. Additionally, for specific-IgG levels, only patients who had not been treated with IVIg for at least 3 months post CTL019 infusion were included and only serum samples from time points prior to IVIg initiation were analyzed.

Antibodies were measured using the following kits according to manufacturer’s instructions: anti-measles IgG (Serion Immunodiagnostica GmbH, Würzburg, Germany), anti-mumps IgG (Calbiotech, Spring Valley, CA), anti-rubella IgG (Phoenix Pharmaceuticals Inc., Burlingame, CA), anti-*Streptococcus pneumoniae* capsular polysaccharide IgG (TheBindingSite, Birmingham, UK), anti-*Haemophilus influenzae* type b capsular polysaccharide IgG (TheBindingSite), anti-tetanus toxoid IgG (TheBindingSite), and anti-HSV1/2 IgA (Serion Immunodiagnostica GmbH). Protective levels were defined as >0.15 IU/ml for anti-tetanus toxoid IgG\(^\text{19}\), ≥120 mIU/ml for anti-measles IgG\(^\text{20}\), >15 IU/ml for anti-rubella IgG\(^\text{21}\), and >0.15 mg/ml for anti-*Haemophilus influenzae* type b capsular polysaccharide IgG\(^\text{22}\). For anti-mumps IgG and anti-
Streptococcus pneumoniae capsular polysaccharide IgG, the limits of antibody positivity were an index of 1.1 and 5 mg/L, respectively.

**Statistical Analysis**

Due to the exploratory nature of the study, statistical analyses on subjects were primarily descriptive. Overall and subject-specific patterns over time were described qualitatively. Wilcoxon signed-rank test was used to analyze data in Figure 6. Statistical analysis was performed using GraphPad Prism v.5.00 (San Diego, CA).

**Results**

**Patient Demographics**

In the present study, we investigated the fate of humoral immunity in adult and pediatric patients with either CLL, ALL, or follicular lymphoma (FL) who were treated with CD19-specific CAR T-cells on one of three clinical trials (NCT01029366, NCT02030834, and NCT01626495) (Table 1). Our goal was to understand the fate of pre-existing humoral immunity after successful CTL019 treatment and to explore a fundamental question about the maintenance of long-lived plasma cells.

Study subjects were selected based on availability of samples from a cohort of 16 patients, all of whom achieved a complete response (CR) at the 1 month time point following CTL019 therapy. Among these patients, the median time of B-cell aplasia onset was day -1 (range -2 to +29, mean=3). The median duration of B-cell aplasia was 589 days (range 94-1803, mean=571). Four of these 16 subjects experienced disease relapses. In three of these cases, CHP-1, CHP-5 and CHP-9, relapse was surface-CD19
negative and B-cell aplasia remained; the fourth subject, UPN-2, was subsequently considered a partial responder based on radiology findings although CLL and B cells were undetectable in the blood and bone marrow. Finally, autopsy findings are presented from UPN-4, a patient treated for FL who achieved and remained in CR until death.

CTL019 therapy, which specifically targets CD19, would be expected to eliminate the CD19+PC subset, but spare the CD19-neg fraction. Using bone marrow aspirates from healthy donors, we confirmed by flow cytometry that normal CD138+CD38+PCs are comprised of CD19+ and CD19-neg fractions (Figure 2A). The CD19+ fraction varied from approximately 54% to 92% (mean 71%, median 72.5%, n=7). We evaluated pre- and post-infusion bone marrow aspirates on patients selected from our cohort (Table 1) based upon the availability of fresh bone marrow aspirate material, which was only available from the pediatric CTL019 trial (NCT01626495). PCs were not detectable in all patients or at all time points. This may be attributable to sampling biases inherent with the low frequency of PCs and their non-homogeneous distribution within the bone marrow (Supplementary Table 1). We were able to identify 3 CTL019-complete responders (CHP-3, CHP-7, CHP-11) with absence of CD19+ cells, including B-cells, in their bone marrow, but with a distinct population of CD138+CD38+ PCs with a normal ratio of kappa:lambda light chain expression and lacking CD19 (Figure 2B and Supplementary Table 1). ELISPOT analysis of cells from these samples also confirmed the presence of antibody-secreting PCs (Figure 2C). These results show that CTL019 effectively eliminates both B-cells and CD19+ PCs, but spares a CD19-neg population of PCs.
Given the inaccuracy of enumerating PCs in bone marrow aspirates by flow cytometry and since hemodilution and cell viability may additionally affect the analysis of bone marrow aspirate specimens, we also performed immunohistochemistry (IHC) analysis of bone marrow core biopsies that were available from both the adult and pediatric CTL019 trials. Stains for CD20, CD138 and CD19 were performed to identify B-cells and PCs. Overall, consistent with previous observations, bone marrow plasma cells generally declined in frequency over time after CTL019 treatment. However, similar to results by flow cytometry, we noted several cases in which CD138+ cells remained despite peripheral B-cell aplasia as well as absence of CD19+CD20+ cells by IHC in the biopsy (Figure 3 and Supplementary Table 2). In UPN-2, the subject with the longest post-CTL019 follow-up, we found that PCs persisted for at least 746 days in the absence of B cells (Figure 3A). Interestingly, although the number of patients studied is limited, we noted that, in the adult cohort (UPN), CD138+ cells remained above the approximate limit of detection (0.001%) in all 4 subjects while, in contrast, plasma cells remained above this level in only 4 of the 12 pediatric subjects (Figure 3B). With 1 exception (CHP-1), CD20 and CD19 IHC (Figures 3C-D) confirmed post-infusion mature B-cell aplasia with the occasional presence of rare CD20+ or CD19+ cells likely indicative of early B cell development not proceeding to full B-cell maturation. CHP-1 experienced surface-CD19-neg leukemia relapse with continued normal B cell aplasia; the subject continued to demonstrate CD138+ PCs at day 261. Overall, the presence of CD138+ cells in biopsies with absence or extremely low level CD19 staining was consistent with findings by flow cytometry indicating preservation of CD19-neg PCs.
While conducting these studies, which were limited to blood and bone marrow, autopsy material from a patient with follicular lymphoma (UPN-4) who was treated with CTL019 (NCT02030834, clinicaltrials.gov) became available. At the time of death on day 234 (clinical details of this case have been reported\textsuperscript{23}, the patient had persistent B-cell aplasia. Extensive clinical testing failed to show any signs of infection around the time of death. IHC analysis of bone marrow, spleen, lymph nodes, small and large intestine revealed absence of CD20+ (Figure 4), Pax5+ or CD19+ (data not shown) cells. CD138 staining was weak, likely related to its well-described instability ex-vivo\textsuperscript{24}. However, morphologic assessment in addition to kappa and lambda light chain staining clearly demonstrated PCs in bone marrow, lymph nodes, and small and large intestine (Figure 4). In this patient, PCs were rare but evident in the bone marrow and most abundant in the lamina-propria of the colon. In contrast, sections of the spleen showed no evidence of PCs. IgG, IgA and IgM staining revealed that the majority of the PCs were IgG or IgA positive although all three subsets were detected in the gastrointestinal tract (Supplementary Figure 2). The weak staining observed in scattered cells by CD138, CD20 and CD19 IHC was confirmed to represent an artifact due to residual endogenous myeloperoxidase activity in maturing myeloid cells (Supplementary Figure 3). Mei et. al. previously described a population of CD19+ plasmablasts circulating in blood in Rituximab treated patients. These cells, suggested to derive from Rituximab-resistant mucosal B-cells, were mostly positive for IgA and Ki67 and appeared to have a mucosal phenotype\textsuperscript{25}. In contrast, we did not see any CD19+ cells in circulation following CTL019. Moreover, dual staining for Ki67 and IgA/kappa/lambda in the gastrointestinal tract showed no dual-positive cells; more than 200 cells of each type (IgA+, kappa+,}
lambda+) were analyzed per section (Supplementary Figure 4). This supports the notion that the CD19-neg PCs observed represent a terminally differentiated, non-proliferating, long-lived population.

The ontogeny and role of CD19+ and CD19-neg PC in humoral immunity has yet to be fully defined. However, recently, Mei et. al. ⁸ suggested that the CD19-neg PC fraction represents a long-lived population of PCs capable of producing antibodies to vaccine antigens. We hypothesized, therefore, that a long-lived CD19-neg PC might play such a role also in CTL019-treated patients. In order to evaluate this further, we quantitatively measured vaccine/pathogen-specific IgG antibodies in subjects prior to and after receiving CTL019. We restricted antibody testing by several criteria to ensure measurements were made during deep, sustained CD20+CD19+ B-cell aplasia, which would be most informative regarding the question of B-cell-dependence of the antibody-specific PCs. Additionally, only subjects with baseline antigen-specific IgG levels at or above commonly accepted protective thresholds (Tetanus, measles, rubella, Hemophilus influenza) or above the limit of detection (mumps, Streptococcus pneumoniae), and only time points prior to immunoglobulin-replacement therapy with IVIg were assessed. Due to these stringent criteria and limited serum sample availability, data presented in Figure 5 is limited to UPN-1 and UPN-4, but is nonetheless informative.

UPN-1 experienced B-cell aplasia from day 24 (first post-infusion time point measured) which has been sustained to at least day 1827 and began IVIg replacement from day 259. This subject remains in CR. In UPN-3, B-cell aplasia was documented from day 29 to at least day 741. This subject, who also remains in CR, did not receive
IVIg until day 223. For both subjects, antigen-specific titers were measured at baseline, 90 and 180 days post-CTL019 infusion.

Serum concentrations of IgG specific to the T-cell-dependent protein antigens from tetanus toxoid, measles virus, mumps virus, and rubella virus remained relatively stable and within the protective range (Figure 5C-F) with the exception of anti-rubella virus IgG in UPN-3. This titer declined by approximately 53% of baseline level over 180 days but remained in the protective range. Evaluation of IgG responses to capsular polysaccharide antigens from *Streptococcus pneumoniae* and *Hemophilus influenzae* type-B (Figure 5A-B) showed differing results between UPN-1 and UPN-3. Whereas anti-*Streptococcus pneumoniae* IgG was stable in UPN-1, antibodies to both *Streptococcus pneumoniae* and *Hemophilus influenzae* type-B declined substantially in UPN-3.

Since IgA titers may also be sustained following natural infection, we evaluated IgA titers to HSV-1/2. We identified two subjects with detectable anti-HSV1/2 IgA at baseline (Figure 5G). IgA to HSV-1/2 remained detectable and largely stable for up to 360 days following CTL019-induced B-cell aplasia. In UPN-3, the initial decline in titer may reflect a loss of CD19+ HSV-specific PCs or may represent normal decline following an active immune response although no evidence of active infection was noted. IgA levels were tested even during periods of Ig replacement therapy as IV and subcutaneous Ig preparations contain a relatively small amount of IgA that is diluted in the recipient and is expected to decline rapidly with a half-life of about one week. As controls, two subjects without detectable anti-HSV1/2 IgA at baseline, but who began
receiving IVIg due to hypogammaglobulinemia were also tested (Figure 5G; subjects CHP-1 and CHP-3). Both continued to demonstrate undetectable anti-HSV1/2 IgA.

In addition to antigen-specific antibody concentrations measured in UPN-1, UPN-3, and CHP-7, total serum IgG, IgA and IgM concentrations were measured (Supplementary Figure 5). Except for IgG in CHP-7, who received IVIg from day 18 onward, total serum IgG, IgA and IgM concentrations declined after CTL019.

ABO blood group antibodies were also evaluated at baseline and after CTL019-induced B-cell aplasia. Anti-A and anti-B titers were maintained in the setting of continued B-cell aplasia and, to the best of our knowledge, absence of transfusion (Supplementary Table 3).

To evaluate the specificity and function of CD19neg PCs that remain following CTL019 therapy, we performed flow cytometry-based sorting of bone marrow PCs into CD19+ and CD19-neg populations (Figure 6A), and assessed their total and antigen-specific IgG secretion by ELISPOT. Due to limitations of sample quantity, these studies were performed using bone marrow from healthy donors. Similar to data reported by others, we observe that the CD19-neg PC fraction contains PCs capable of producing tetanus toxoid-, mumps virus-, and measles virus-specific IgG (Figure 6B). Overall, these results confirm the presence of vaccine-specific plasma cells within the CD19-neg fraction of plasma cells that remain following CTL019 therapy.

Discussion

Recent evidence in humans suggests that long-lived humoral immunity resides within the CD19-neg PC fraction\textsuperscript{8,18}. Phenotypic analysis in mice suggests that loss of
CD19 expression represents progressive differentiation, marking long-lived PCs\textsuperscript{28}. Similar to depletion studies in mice\textsuperscript{5-7}, significant reductions in the number of CD19+ PCs in patients’ bone marrows have been observed following anti-CD20 therapy without a significant change in the CD19-neg subset suggesting that the CD19+ PC subset represents a short-lived population requiring replenishment from CD20+ B cells\textsuperscript{8}. While total IgG may decline, stability of pre-existing antigen-specific IgG following Rituximab-induced B cell depletion has been demonstrated\textsuperscript{29-33}. However, several studies have shown that rituximab therapy effectively eliminates B-cells from the peripheral blood but fails to effectively deplete all B-cells within secondary lymphoid tissues\textsuperscript{34-39}. Thus, whether PCs remaining in this setting are long-lived or dependent on replenishment by the residual B-cells is unknown.

Overall, our results indicate that a population of PCs lacking CD19 can persist in the bone marrow following an effective CTL019–mediated B-cell depletion. IHC studies performed on autopsy material from one subject with a complete tumor response suggest highly effective B cell depletion in secondary lymphoid organs by CTL019. Serology results from 3 subjects indicate that the remaining CD19-neg PCs are capable of secreting antibodies to vaccine and pathogen-related antigens. Our results further suggest that CD19-neg PCs are likely long-lived given the absence of B-cells to replenish this pool, and these cells most likely contribute to stable serum antibody titers observed in patients with CTL019-induced B-cell aplasia. Our analysis of PCs following CTL019-treatment allowed assessment of CD19-neg PCs only. It is possible that some CD19+ PCs may also have the capacity to survive long-term; in fact, we and Mei et. al. found that ex-vivo sorted CD19+ PCs could also contribute to antibody responses that are
relatively long-lived such as responses to tetanus toxoid, and measles and mumps viruses\(^8\). In contrast, for unknown reasons, Halliley et. al. did not detect these antibody responses in the CD19+ PC fraction\(^{18}\).

Several CD19-directed antibody-based immunotherapeutic approaches are currently in clinical trials for the treatment of B-cell lineage malignancies\(^{40-42}\). Neither preclinical nor clinical studies employing such approaches have addressed the fate of PC or pre-existing antigen-specific antibodies\(^7,^{40,43}\). The recent introduction of CD19-directed CAR T-cell-based therapy has provided a unique opportunity to study the impact of prolonged B-cell aplasia on the CD19-neg plasma cell population. The duration of continuous B-cell aplasia in the cohort presented here, which was observed for at least 1,827 days in one subject, depends on the duration of CAR T-cell persistence \(^{13}\). Thus, B-cell aplasia is likely maintained by the continuous action of CAR T-cells. The pharmacokinetic profile of CAR T-cells is vastly different from that of passively transferred B-cell depleting antibodies and may translate to more robust B-cell depletion. Consistent with this notion, autopsy material from UPN-4 demonstrated total B-cell elimination from all tissues analyzed (Figures 3 and 4). In this context of marked and sustained B-cell aplasia, we found that some previously generated antigen-specific antibody responses are preserved for at least 6 months (IgG) and up to approximately a year (IgA) despite absence of normal B cells. Given the 3-4 week half-life of IgG and much shorter half-life of IgA, antigen-specific IgG and IgA concentrations would be expected to be reduced by greater than 10-fold without their continued synthesis. This stability strongly argues for the existence of a long-lived pool of antibody-producing CD19-neg plasma cells that do not require replenishment by CD19+ precursors. Declines
in some antibody levels (e.g. anti-rubella virus IgG and anti-HSV1/2 IgA in UPN-3) were noted but these did not occur at the rate expected if the antibody producing cells were directly targeted by CTL019 cells. Thus, their decline may represent a combination of initial CTL019-mediated loss of antigen-specific CD19+ PCs as well as the turnover of CD19-neg PCs. Longer follow-up in CTL019-treated patients in the absence of IVIg therapy would be interesting to determine the long-term kinetics of these responses.

Results of our IHC studies raise interesting questions regarding long-lived PCs. Although the lack of identified PCs in some patients may simply reflect their rarity and limitations of tissue sampling, it may be that not all CTL019-treated patients maintain bone marrow PCs. This may be due to differences in PC-intrinsic properties as well as PC-extrinsic factors that constitute the PC-niche. Although not yet precisely understood, the PC niche is thought to include factors such as IL-6, APRIL, BAFF and CXCL12, and cell-surface molecules such as VCAM1 provided by stromal cells, eosinophils, and possibly, others. In our cohort, serum BAFF, but not APRIL or IL-6, was increased post-CTL019 compared to pre-treatment but we did not see a difference in individual changes between those in whom we did and did not detect PCs post treatment (data not shown). However, we cannot rule out the fact that loss of PCs in the context of CTL019, and differences between subjects, may be the result of indirect effects. Differences noted between subjects could also be related to age, disease, or prior therapy. The demonstration of CD19-neg PCs in compartments outside the bone marrow (lymph node and gastrointestinal tract, Figure 4 and Supplementary Figure 4) in a patient approximately 8 months after CTL019 infusion and in the context of deep B-cell aplasia suggests that PCs at these sites may also be relatively long-lived.
This is the first report, to our knowledge, of the effect of CD19+ cell depletion on plasma cells and specific humoral immunity in humans. Our findings contribute to a growing body of evidence that suggests B-cell-independent longevity of some human PCs. The maintenance of pre-existing humoral immunity observed in our study challenges assumptions regarding the degree of immunosuppression induced by CTL019. Assessment of mucosal antibodies, which constitute a substantial portion of total body immunoglobulin and an important part of host defense, is also needed to understand the breadth of humoral immune deficiency induced by CTL019 therapy.

Our findings focus on normal PCs that remain following CTL019 treatment. Garfall et. al. recently reported application of CTL019 for the treatment of multiple myeloma where the vast majority of malignant PCs do not express CD1945. While the mechanism of response to CTL019 in this context is unclear, the findings suggest that maintenance of malignant PCs maybe different from that of normal PCs analyzed in this study.

Finally, our results have also raised important questions regarding the application of B-cell directed therapies for the eradication of pathogenic autoimmune or allograft antibodies. The durability of pre-existing humoral immunity in CTL019-treated subjects suggests that therapies directed at the CD19neg long-lived PCs may be required to fully eradicate these types of pathogenic humoral immunity.

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Authorship Contributions


Disclosure of Conflict of Interest

M.C.M., C.J. and D.P. are inventors on patents related to CTL019 that have been licensed to Novartis Pharmaceuticals. These financial conflicts of interest are managed in accordance with policies established by the University of Pennsylvania.
References


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<tr>
<td>CHP-2</td>
<td>9</td>
<td>ALL</td>
<td>CR</td>
<td>Day 0</td>
<td>Fludarabine/Cyclophosphamide</td>
<td>IVIg Day+2, then monthly, then switched to weekly subQ from Day+255</td>
<td></td>
</tr>
<tr>
<td>CHP-3</td>
<td>15</td>
<td>ALL</td>
<td>CR</td>
<td>Day -1</td>
<td>Fludarabine/Cyclophosphamide</td>
<td>IVIg Day+35, then monthly, then switched to Q2weeks subQ from Day+400</td>
<td></td>
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<tr>
<td>CHP-4</td>
<td>9</td>
<td>ALL</td>
<td>CR</td>
<td>Day -1</td>
<td>Fludarabine/Cyclophosphamide</td>
<td>IVIg Day+30, then monthly, then switched to weekly subQ from Month+16</td>
<td></td>
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<tr>
<td>CHP-5</td>
<td>22</td>
<td>ALL</td>
<td>CR-&gt; relapse</td>
<td>Day -2</td>
<td>Fludarabine/Cyclophosphamide</td>
<td>IVIg Day+2, then monthly</td>
<td></td>
</tr>
<tr>
<td>CHP-6</td>
<td>16</td>
<td>ALL</td>
<td>CR</td>
<td>Day -1</td>
<td>Fludarabine/Cyclophosphamide</td>
<td>IVIg Day+7, then monthly, then switched to weekly subQ from Day+548</td>
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<tr>
<td>CHP-7</td>
<td>21</td>
<td>ALL</td>
<td>CR</td>
<td>Day -1</td>
<td>Fludarabine/Cyclophosphamide</td>
<td>IVIg Day+18, then monthly</td>
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<td>timepoint tested</td>
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<tr>
<td>CHP-8</td>
<td>5</td>
<td>ALL</td>
<td>CR</td>
<td>Day+1</td>
<td>To day+647, last timepoint tested</td>
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<td>Fludarabine/Cyclophosphamide</td>
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<td></td>
<td></td>
<td>IVIg Day+17, then monthly, then switched to weekly subQ from Day+630</td>
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<td>CHP-9</td>
<td>13</td>
<td>ALL</td>
<td>CR-&gt; relapse (CD19 neg)</td>
<td>Day -1</td>
<td>To day+268, (time of relapse)</td>
<td></td>
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<td>Fludarabine/Cyclophosphamide</td>
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<td></td>
<td></td>
<td>IVIg Day+8, then monthly</td>
<td></td>
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<tr>
<td>CHP-10</td>
<td>19</td>
<td>ALL</td>
<td>CR</td>
<td>Day -2</td>
<td>To day+545, last timepoint tested</td>
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<td></td>
<td></td>
<td>IVIg Day+146, then monthly</td>
<td></td>
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<tr>
<td>CHP-11</td>
<td>9</td>
<td>ALL</td>
<td>CR-&gt; Allo Txp in CR</td>
<td>Day -1</td>
<td>To day+93 --&gt; Allo Txp in CR</td>
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<td>Fludarabine/Cyclophosphamide</td>
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<td></td>
<td></td>
<td>IVIg Day+24, then monthly</td>
<td></td>
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<tr>
<td>CHP-12</td>
<td>17</td>
<td>ALL</td>
<td>CR</td>
<td>Day -1</td>
<td>To day+500, last timepoint tested</td>
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<td>Fludarabine/Cyclophosphamide</td>
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<td></td>
<td></td>
<td>IVIg Day+7, then monthly</td>
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</table>
Table 1. Footnote: *B-cell aplasia was defined as CD19+ B-cells < 1% of PBMC. In the majority of samples, the percentage was 0-0.1%. †Chemotherapy aimed at lymphodepletion was given within 1 week prior to CTL019 infusion, timed so that the last dose was given 2-6 days prior to infusion. ‡First post-infusion flow cytometry assessment, true onset of B-cell aplasia may be earlier. §UPN-2 was considered CR at month 6, then PR at month 14 based on radiology findings (at month 14, neither B-cells nor CLL was detected in blood and bone marrow).

Figure Legends

Figure 1. Scheme of subject selection and testing. Samples from patients enrolled in CTL019 clinical trials, UPCC04409, UPCC13413, and CHP959, were tested for the presence of B-cells, plasma cells and serum antibodies to evaluate the state of humoral immunity post treatment.

Figure 2. CD19-neg bone marrow plasma cells resist elimination by CTL019. (A) Bone marrow aspirate from healthy donors were stained and analyzed by flow cytometry. The bottom right panel shows CD19 expression on CD138+CD38+ plasma cells (red) and CD20+CD19+ cells (blue); the green histogram represents CD138+CD38+ cells with fluorescence-minus-one(CD19) staining. Representative data from 7 donors in shown. The "dump" channel contains stains for viability, CD3, CD14 and CD16. (B) Bone marrow aspirate cells from CHP-11 on day 28 post-CTL019 infusion were analyzed as in (A). Gating for B-cells (top left), plasma cells (top right) is shown. The bottom left panel shows CD19 expression on CD38+CD138+ cells (red histogram) and on all singlet
events in the forward and side scatter gate (blue histogram). In both (A) and (B),
expression of light-chains was assessed by intracellular staining. (C) 200,000 cells from
the aspirate material shown in (B) were plated in ELISPOT wells, which were stained for
total IgG, IgM, and IgA after overnight incubation.

Figure 3. Immunohistochemical analysis demonstrates long-term presence of bone
marrow PCs and B-cell aplasia after CTL019 treatment. (A) Bone marrow core
biopsies from subject UPN-1 at baseline and serial time-points post CTL019 infusion
were stained, separately, for CD138, CD20, and CD19 by IHC. Representative regions at
20x magnification are shown. (B-E) The frequency of CD138+ (B), CD20+ (C), and
CD19+ cells (D) among total nucleated cells in bone marrow biopsies from adult (red
curves) and pediatric (blue curves) patients was determined by digital image analysis for
the indicated time-points pre- and post-CTL019.

Figure 4. CTL019 results in B cell depletion in primary and secondary lymphoid
tissues. Sections of bone marrow, spleen, lymph nodes, terminal ileum and colon,
obtained at autopsy from a CTL019-treated subject (day 234), were analyzed by H&E
and IHC (CD20, kappa, lambda). Representative sections are shown at 20x (all spleen
and CD20 images) and 40x magnification (all other images). H&E insets are shown at
100x magnification.

Figure 5. Antigen-specific antibody titers at baseline and after CTL019 infusion.
Streptococcus pneumoniae- (A), Hemophilus influenza type-B- (B), tetanus toxoid- (C),
measles- (D), mumps- (E), and rubella- (F) specific IgG, and HSV1/2-specific IgA (G) levels were measured in CTL019-treated patients who experienced persistent B-cell aplasia. For anti-Hemophilus influenzae, -tetanus toxoid, -rubella, and anti-measles, thresholds of protective IgG levels are indicated by the dashed lines. For anti-Streptococcus pneumoniae, anti-mumps, and anti-HSV1/2 IgA, the dashed lines indicate the lower limit of detection. Error bars indicate standard deviations.

Figure 6. CD19-neg PCs produce vaccine/pathogen specific antibodies. (A) Bone marrow aspirate cells from healthy donors were sorted into four populations (CD19+ PCs, CD19-neg PCs, B-cells, and Dump+) and were analyzed by ELISPOT. (B) Total-IgG, Tetanus toxoid-, Measles- or Mumps-specific IgG producing cells were enumerated by ELISPOT. Each symbol represents an individual donor. Means and standard deviations are indicated. For total-IgG, all pair-wise comparisons demonstrated a p-value of 0.0625 except CD19+PC vs. CD19-neg PC and B cells vs. Dump. For Tetanus toxoid, all pair-wise comparisons demonstrated a p-value of 0.0625 except B cells vs. Dump. For Measles, only CD19+PC vs. B cells and CD19+ vs. Dump comparisons demonstrated a p-value of 0.0625. p-values for all other pair-wise comparisons were 0.1088 or greater (Wilcoxon signed-rank test).
Availability of baseline and at least 3 month post-treatment vaccine titers (n=30 for IgG, n=12 for IgA)

Exclusion of subjects with undetectable antigen-specific antibody titers at baseline. (n=0 for IgG, n=10 for IgA)

Exclusion due to transfusion with plasma-containing blood products or IVIg administration within 3 months following CTL019 treatment (n=28 for IgG, n=0 for IgA)

Measurement of serum antigen-specific antibodies (n=2 for IgG, n=2 for IgA)

Availability of fresh bone marrow aspirate (n=26)

Exclusion of subjects with inadequate aspirate cellularity (n=8)

Flow cytometry (n=18) +/- ELISPOT (IgG, IgM, IgA) analysis if adequate cellularity (n=8)

Availability of bone marrow core biopsy sample (n=16)

H&E and immunohistochemistry (n=16)
Figure 2

A

B

C

IgG

IgM

IgA

CD138

CD38

Kappa

Lambda

CD19

FSC-A

FSC-H

SSC-A

Dump

CD19

CD20

CD138

Kappa

Lambda
Figure 4

Bone Marrow | Spleen | Lymph Node (Hilar) | Terminal Ileum | Colon

H&E

CD20

Kappa

Lambda
Figure 5

A) Anti-Streptococcus pneumoniae IgG

B) Anti-Hemophilus influenzae IgG

C) Anti-Tetanus Toxoid IgG

D) Anti-Measles IgG

E) Anti-Mumps IgG

F) Anti-Rubella IgG

G) Anti-HSV1/2 IgA
Figure 6

A

- Dump+ (CD3+/CD14+/CD16+)
- CD19- PC (CD38+CD138+CD19-)
- CD19+ PC (CD38+CD138+CD19+)
- B cells (CD20+CD19+)

B

- Total IgG ASC
- T.Toxoid IgG ASC
- Measles IgG ASC
- Mumps IgG ASC
Persistance of long-lived plasma cells and humoral immunity in individuals responding to CD19-directed CAR T cell therapy