Expansion of a CD8<sup>+</sup>PD-1<sup>+</sup> Replicative Senescence Phenotype in Early Stage CLL Patients Is Associated with Inverted CD4:CD8 Ratios and Disease Progression

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**Abstract**

**Purpose:** Patients with chronic lymphocytic leukemia (CLL) display immune deficiency that is most obvious in advanced stage disease. Here we investigated whether this immune dysfunction plays a pathologic role in the progression of early stage disease patients.

**Experimental Design:** We carried out eight-color immunophenotyping analysis in a cohort of 110 untreated early stage CLL patients and 22 age-matched healthy donors and correlated our findings with clinical outcome data.

**Results:** We found a significant reduction in naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells in CLL patients. Only the CD4<sup>+</sup> subset showed significantly increased effector memory cells (TEM and TEMRA) in the whole cohort (P = 0.004 and P = 0.04, respectively). However, patients with inverted CD4:CD8 ratios (52 of 110) showed preferential expansion of the CD8 compartment, with a skewing of CD8<sup>+</sup> TEMRA (P = 0.03) coupled with increased percentage of CD57<sup>+</sup>CD28<sup>-</sup>CD27<sup>-</sup> T cells (P = 0.008) and PD-1 positivity (P = 0.027), consistent with a replicative senescence phenotype. Furthermore, inverted CD4:CD8 ratios were associated with shorter lymphocyte doubling time (P = 0.03), shorter time to first treatment (P = 0.03), and reduced progression-free survival (P = 0.005).

**Conclusions:** Our data show that the emergence of CD8<sup>+</sup>PD-1<sup>+</sup> replicative senescence phenotype in early stage CLL patients is associated with more aggressive clinical disease. Importantly, these findings were independent of tumor cell prognostic markers and could not be accounted for by patient age, changes in regulatory T-cell frequency, or cytomegalovirus serostatus (n = 217). *Clin Cancer Res*; 18(3); 1–10. ©2011 AACR.

**Introduction**

Chronic lymphocytic leukemia (CLL) is characterized by immunodeficiency of which hypogammaglobulinemia is the most clinically obvious (1). However, profound defects in cell-mediated immunity are also a feature of this disease and patients usually have abnormalities in T-cell numbers and function (2–5). Treatment with chemotherapy seems to exacerbate this problem by inducing T lymphopenia, thereby rendering patients more susceptible to infection (6). A number of recent studies have shown abnormalities in the phenotype of CD4 and CD8 T cells, including inversion of the normal CD4:CD8 ratio (2–5) and the accumulation of terminally differentiated effector memory T cells with relative absence of naive precursors (7, 8). By definition, these memory T cells are derived from antigen exposure and it would seem that repeated or chronic antigen stimulation is a feature of this disease. In keeping with this notion, markers of chronic activation are increased on both the CD8<sup>+</sup> and CD4<sup>+</sup> T-cell subsets in CLL patients compared with normal controls (9). Precisely what antigen(s) are involved in this process remains unresolved, but a number of groups have suggested that cytomegalovirus (CMV) may play a role in driving CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells (5, 10, 11). However, CMV accounts for a relatively small number of the total T-cell count and CMV-seronegative individuals still manifest T-cell expansion (5).

Although the exact mechanism by which T cells accumulate in CLL is not well defined, T cells derived from patients with advanced disease have shorter telomeres, suggesting that these T cells are in some way reacting to the growth of the CLL clone (12). Consistent with this idea, a number of groups have identified a population of antileukemic T cells, these have been the subject of interest especially in the context of immunotherapy (13–15).
Translational Relevance

This article represents the most in-depth, large-scale analysis of T-cell subsets in early stage CLL patients yet to be conducted. We show, for the first time, that inverted CD4:CD8 ratios are associated with shorter lymphocyte doubling time, shorter time to first treatment, and reduced progression-free survival in this disease, suggesting that T-cell dysfunction contributes toward disease progression. We went on to show that patients with inverted CD4:CD8 ratios showed a preferential expansion of CD8+ terminal effector memory cells, coupled with a replicative senescence phenotype. Taken together, our data show that the emergence of a CD8+ PD-1++ “exhaustion” phenotype in early stage CLL patients is associated with a loss of immunologic control and more aggressive clinical disease (n = 113).

Here we describe the most detailed, large-scale, immunophenotypic analysis ever carried out on treatment-naive, early stage CLL patients. We showed a skewed distribution of T-cell subsets and a preferential expansion of terminally differentiated CD8+ T cells in CLL. Strikingly when patients were stratified into those with inverted (CLLIR) and normal (CLLNR) CD4:CD8 ratios, we observed that the CLLIR group had a preferential expansion of CD8+ PD-1+ T cells expressing other markers consistent with replicative senescence. These same patients showed significantly poorer prognosis suggesting that a loss of immunologic control has the capacity to alter the pathology of CLL.

Materials and Methods

Blood samples from healthy volunteers and CLL patients

A total of 110 CLL patients were recruited from clinics at the University Hospital of Wales and Llandough Hospital (age range: 48–93 years). Blood samples were collected with informed consent in accordance with the ethical approval obtained from South East Wales Research Ethics Committee (02/4806). A control group of 22 age-matched healthy donors (age range: 42–77 years). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Histopaque-1077; Sigma) density gradient centrifugation. A total of 95 of 110 (86.4%) of the CLL patients were diagnosed in Binet stage A, and none had received treatment prior to sample analysis. The median follow-up for the cohort was 4.6 years and during this time 23 of 110 (21%) required treatment.

Antibodies

The following pretitrated antibodies were used in this study: anti-CD56-FITC (fluorescein isothiocyanate), CD16 APC, CD57-FITC (Serotec), CD5-PE-Cy5.5, CD45RO-APC (Invitrogen), CD19-PE-Cy7, CD27-PE-Cy7, FoxP3-FTTC (eBioscience), CD8-APC-Cy7, CD4-Pacific blue, CD3-AmCyan, TCRγδ-APC, CD28-percp-cy5.5 (BD Biosciences), CCR7-PE (RnD Systems), CD25-PE (Miltenyi Biotec), and PD-1-FITC (BD Biosciences).

Immunofluorescent staining and flow cytometric analysis

Direct immunofluorescent staining was carried out by adding conjugated antibodies to cells for 15 minutes at 4°C in the dark, before being washed twice with 1% fetal calf serum (FCS)-PBS. Between 5 × 10^6 to 1.5 × 10^6 cells were used per tube and were analyzed immediately after staining without fixation. For the Treg panel, cells were resuspended in 100 μL of 1% FCS-PBS and stained for 30 minutes on ice with the surface cell markers: CD25-PE, CD45RA-APC, CD4-Pacific blue, and CD3-AmCyan. Cells were washed twice with 500 μL cold 1% FCS-PBS. FOXP3 staining was done using the FITC anti-human FOXP3 staining kit (eBioscience). Compensation was done automatically using an anti-Mouse Ig/Negative control compensation particles set (BD) and FACSDiva software. Fluorochrome-conjugated monoclonal antibodies (with the same concentration added to the cell samples) were used as single-color compensation controls. Cells were analyzed using a BD FACSCanto II Cytometer (BD) and analysis was done using Flowjo analysis software. Analysis of PBMCs by flow cytometry involved the construction of a live lymphocyte gate that was based upon the forward and side scatter profiles of these cells. CD4:CD8 ratios were considered inverted if the ratio was 1.0 or less. This threshold was selected based on the median CD4:CD8 ratio of the cohort but also represents the point at which the numbers of CD8+ cells outnumber CD4+ cells.

CMV serostatus

CMV (IgG) serostatus was determined in a blinded fashion by the local Public Health Laboratory Service in a subset of 40 of the patient cohort, 20 inverted ratio samples (CLLIR) and 20 normal ratio samples (CLLNR).

Statistics

Nonparametric Mann-Whitney U test was used for comparison of 2 independent groups. Analysis of prognostic subsets was done using Fisher’s exact test or χ2. Time to first treatment and progression-free survival time were calculated from date of diagnosis and curves were constructed using the method of Kaplan and Meier. Statistical analysis was carried out using Prism 5.0 (GraphPad Software).

Results

Evaluation of peripheral blood lymphocyte populations

As a first step, we compared the frequency of peripheral blood lymphocyte populations in CLL patient samples and age-matched healthy donors (Supplementary Table S1). The percentages of B cells (CD19+), malignant CLL B cells (CD19+ CD5+), T cells (CD3+ CD4+ and CD8+), innate natural killer (NK) cells (CD56+ CD16+), γδ T cells (CD3+...
healthy donors, along with significantly decreased percent-

age (P = 0.0004), whereas the percentage of CD4+ T cells among the CD3+ T cells was significantly reduced (P < 0.0001). On closer evaluation, there was a marked differential expansion of CD8+ T cells in 52 patients that resulted in the inversion of their CD4:CD8 ratio (CLLIR). Absolute counts were available for 25 of 52 of the CLLIR patients. Comparison with 25 normal CD4:CD8 ratio (CLLNR) patients and 10 age-
matched healthy controls revealed that, consistent with previous findings (5), the numbers of both CD4+ and CD8+ T cells were increased in CLL patients compared with normal age-matched controls. Furthermore, the inverted CD4:CD8 ratio patients showed no evidence of a decline in CD4+ T-cell numbers, rather the inversion of the CD4:CD8 ratio was caused by the preferential expansion of CD8+ T cells (Supplementary Table S2).

Comprehensive analysis of T-cell subsets in CLL

It is now accepted that a skewed maturation of T cells occurs with ageing and disease, including chronic viral infection (16–18). In the first instance, we evaluated whether the frequency of CD4+ and CD8+ memory T-cell subsets differed between CLL patients and age-matched controls. The T-cell subsets were defined by dividing both the CD4 and CD8 compartments on the basis of CD45RO and CCR7 expression into naive (CCR7+ CD45RO+), central memory (TEM; CCR7+CD45RO+), and terminally differentiated effector cells (TEMRA; CCR7+CD45RO−). Supplementary Fig. S1 shows the gating strategy employed in this study to define T-cell subsets. Figure 1A shows that there was a significant reduction in the percentage of naive CD8+ subsets in CLL patient samples (P = 0.0001), with a trend toward an increased frequency of TEM and TEMRA in the CD8+ compartment. Figure 1B shows a similar pattern in the CD4+ compartment; reduced frequency of naive (P = 0.03) and central memory (P = 0.04) subsets as well as an increase in TEM (P = 0.004) and TEMRA subsets (P = 0.04). On the basis of these results, the greatest differences in T-cell subsets between CLL patients and healthy age-matched controls seemed to be found in the CD4+ compartment.

CD8+ cells from patients with inverted CD4:CD8 ratios have an increased terminal differentiation effector phenotype

A more detailed analysis of our cohort revealed that 47% (52 of 110) showed a marked preferential expansion of CD8+ cells, which resulted in an inversion of the normal CD4:CD8 ratio. We therefore divided our cohort into CLLNR and CLLIR subsets based on a CD4:CD8 ratio threshold of 1.0. This threshold was derived from the median CD4:CD8 ratio for the entire cohort (Fig. 2A) but a ratio of 1.0 or less also represents the point at which the CD8 compartment becomes larger than the CD4 compartment. We subsequently reanalyzed the T-cell subsets in these groups. Figure 2B shows significant reductions in the naive and central memory CD8+ subsets in CLLIR patients (P = 0.002 and P = 0.004, respectively) when compared with CLLNR patients. Furthermore, there was a significant increase in the TEMRA subset in the CLLIR group (P = 0.03). Figure 2C shows that naïve CD4 cells were also significantly reduced in the CLLIR subset (P = 0.0007) with a concomitant increase in T EM (P = 0.0005), but not TEMRA (P = 0.32) in this compartment. Figure 2D and E show the skewing of the CD8 and CD4 subsets in CLLIR and CLLNR samples compared with normal age-matched controls. Most notably, we observed a preferential relative expansion in CD8+ EMRA (P = 0.03) and a concomitant decrease in CD8+ naive and central memory subsets (P = 0.002 and P = 0.004, respectively) in the CLLIR subset. Consistent with
previous findings (19), the inversion of the CD4:CD8 ratio was not associated with the age of the patients because the median age of the CLLNR and CLLIR subsets was not significantly different ($P = 0.47$).

**CLLIR is associated with inferior prognosis in CLL**

Intuitively, the increased percentage of effector T-cell subsets seen in CLL, particularly in the CLLIR group, might be beneficial either because of increased ability to provide protection against pathogens or, perhaps, increased immunologic control over the tumor cells. Therefore, we evaluated whether an inverted CD4:CD8 ratio had any prognostic relevance in CLL. Contrary to our expectations, the CLLIR group had significantly shorter time to first treatment (Fig. 3A; $P = 0.03$) and progression-free survival (Fig. 3B; $P = 0.005$) when compared with the CLLNR group. Therefore, an inverted CD4:CD8 ratio seemed to confer an inferior clinical prognosis in this early stage cohort of CLL patients.

**CLLIR patients show increased frequency of a CD57$^+$CD28$^+$CD27$^-$ phenotype**

In an attempt to rationalize the poor prognosis associated with an inverted CD4:CD8 ratio, we carried out a more detailed analysis of the T-cell subsets in our cohort. The
The expression of PD-1 in 77 of 110 (70%) of our patient cohort. PD-1 expression was significantly lower in CD4 and CD8 T-cell subsets in normal age-matched controls when compared with CLNR patients (Fig. 5A). We found no significant difference in the frequency of Tregs in CLNR patients when compared with CLNR patients (Fig. 6A). We found no significant difference in the frequency of Tregs in CLNR patients when compared with CLNR patients (Fig. 6A).

In this study, we found a significant increase in Tregs in CLIR patients when compared with healthy donors (P = 0.03). This is in accordance with previous reports that show that the frequency of Tregs is higher in CLIR patients and is further increased in patients with advanced stage disease (25, 26). We therefore analyzed whether there was a significant difference in the frequency of Tregs in CLIR patients when compared with CLIR patients (Fig. 6A). We found no significant difference in the frequency of Tregs in CLIR patients when compared with CLIR patients (Fig. 6A).

To establish whether there were confounding factors that could explain the prognostic significance of inverted CD4:CD8 ratios in our CLIR cohort, we assessed whether there was an association between this phenomenon and known tumor cell prognostic markers (Fig. 6B–F). We found no significant difference in CD38 expression (P = 0.15) or IGHV mutation status (P = 0.55), or Binet stage at diagnosis (P = 0.51) between the CLIR and CLIR groups. However, we did show an association with replicative senescence and has been observed with age-matched healthy donors. Figure 4A shows that a CD57CD28CD27 phenotype was significantly enhanced in CD8+ T cells of CLIR patients compared with healthy donors (P = 0.019). Furthermore, when we split the cohort into CLINR and CLIR subsets (Fig. 4B), we showed a significant increase in CD57CD28CD27+ expressing cells in both the TEM (P = 0.0007) and TEMRA subsets (P = 0.008) in the CLIR group. The CD4+ T cells also showed an increase in CD57CD28CD27+ phenotype in the TEM subset (Fig. 4C; P = 0.004) and again this was most noticeable in the CLIR group (Fig. 4D; P = 0.02). Differences in CD4+ TEMRA were not evaluated due to the very low percentage of this subset in CD4+ T cells. In accordance with previous findings, this suggested that there was an accumulation of terminally differentiated T cells, particularly in the CLIR group, supporting the concept of chronic activation of the T-cell compartment (9, 22, 23). A previous report suggested that the accumulation of CD8+ CD57+ CD27+ T cells in CLIR was caused by the expansion of CMV-specific T cells in CMV-seropositive patients (10). We therefore serotyped 20 CLIR and 20 CLINR patients to assess whether this phenotype was linked to CMV serostatus. A total of 17 of 20 of the CLIR group were CMV seropositive. However, 15 of 20 of the CLINR group were also CMV seropositive. This indicated that CMV is not solely responsible for the preferential expansion of CD8+ CD57+ CD28− CD27+ T cells in the CLIR group. In keeping with this notion, there was no significant percentage increase in either CD8+ cells (P = 0.75) or CD8+ CD57+ CD28− CD27+ T cells (P = 0.43) in the CMV-seropositive subset when compared with the seronegative subset (Fig. 4E).

### CD8+ PD-1 expression is associated with inverted CD4:CD8 ratios

Given that the differential expansion of CD8+ T cells in the inverted CD4:CD8 ratio group was characterized by a replicative senescence phenotype, we went on to analyze the expression of PD-1 in 77 of 110 (70%) of our patient cohort. PD-1 expression has been associated with a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia (24), we therefore investigated whether this marker was differentially expressed in the CLIR group. Figure 5A shows that there were significantly more PD-1+ CD8+ T cells in the CLIR group when compared with the CLINR group (P = 0.027). Furthermore, the percentage of PD-1+ CD8+ T cells was not associated with CMV serostatus (Fig. 5B; P = 0.63). It is important to note that PD-1 expression was significantly lower in CD4 and CD8 T-cell subsets in normal age-matched controls when compared with CLIR samples (Supplementary Fig. S3).

### CLIR is not associated with preferential reduction in Tregs

In this study, we found a significant increase in Tregs in CLIR patients when compared with healthy donors (P = 0.03). This is in accordance with previous reports that show that the frequency of Tregs is higher in CLIR patients and is further increased in patients with advanced stage disease (25, 26). We therefore analyzed whether there was a significant difference in the frequency of Tregs in CLIR patients when compared with CLINR patients (Fig. 6A). We found no significant difference in the frequency of Tregs in the inverted ratio group (P = 0.46), suggesting that changes in Tregs cannot explain the prognostic significance of inverted CD4:CD8 ratios.

### CLIR is not associated with tumor cell phenotype or Binet stage at diagnosis

To establish whether there were confounding factors that could explain the prognostic significance of inverted CD4:CD8 ratios in our CLIR cohort, we assessed whether there was an association between this phenomenon and known tumor cell prognostic markers (Fig. 6B–F). We found no significant difference in CD38 expression (P = 0.8), ZAP-70 expression (P = 0.15) IGHV mutation status (P = 0.55), or Binet stage at diagnosis (P = 0.51) between the CLINR and CLIR groups. However, we did show an association...
between inverted CD4:CD8 ratio and lymphocyte doubling time less than 12 months ($P = 0.03$), suggesting a role for T cells in the immunologic control of tumor cell expansion in this disease.

**Discussion**

In this study, we carried out a comprehensive analysis of T-cell subsets in a large cohort of 110 untreated CLL patients. Most of our analysis was based on percentage expression of specific phenotypic subsets, but this comparative analysis was also supported by absolute counting data in a subset of the cohort. In keeping with previous studies (27–31), we found that the T-cell subset composition was skewed toward a memory phenotype with a reduced CD4:CD8 ratio in 52% of the patients. Inverted CD4:CD8 ratios have been previously documented in CLL (2), but here we show for the first time that inverted CD4:CD8 ratios are associated with a shorter lymphocyte doubling time and confer an inferior prognosis in early stage disease patients. Importantly, this was independent of patient age, tumor-associated prognostic markers, percentage of Tregs and CMV serostatus. A recent paper by Gonzalez-Rodriguez and colleagues (4) seems to contradict our findings in terms of the prognostic impact of CD8 T cells. However, this study did not assess the clinical impact of comparative changes in CD4 and CD8 subsets. Instead, they evaluated the CD8:tumor B cell ratio and concluded that higher ratios conferred a good prognosis. This is not surprising given that this ratio is influenced predominantly by the tumor burden, so their findings are not directly comparable with the ones reported in our study.

As reported previously (32), CD4$^+$ T-cell subsets were significantly skewed in our CLL cohort when compared with normal age-matched controls, and this was most apparent in the CLL$_{IR}$ subset. However, absolute T-cell count analysis of the CLL$_{IR}$ group revealed that the inversion was caused by the preferential expansion of the CD8$^+$ compartment, rather than a relative reduction in CD4$^+$ cells (Supplementary...
**Table S2.** Further examination of CD8^+^ T-cell subsets revealed a significant skewing toward TEMRA cells in the CLL^{IR} subset; a phenomenon that has been previously reported in a very small cohort of 11 CLL patients (30). Our study confirms and extends those observations in a cohort of 110 patients and shows that expansion in CD8^+^ T cells in CLL^{IR} and CLL^{NR} patients and CMV-seropositive samples.

Downregulation of CD28 and increased expression of CD57 is associated with replicative senescence that can lead to the inability of T cells to proliferate and an enhanced tendency to apoptosis (9, 20, 21, 33, 34). The development of this phenotype can occur in response to repeated/chronic antigenic stimulation and has been associated with ageing. Several lines of evidence suggest that the alteration in the T-cell subsets in both CLL patients and healthy older individuals can be associated with chronic antigenic stimulation by viral infections, such as CMV (10, 11, 35). Therefore, the expansion of TEM and TEMRA cells might be related to the fact that subclinical CMV infection is common in CLL, and this can drive chronic stimulation and exhaustion in the T-cell compartment (10). However, here we present evidence that the preferential expansion of the CD8^+^ compartment in CLL^{IR} patients cannot be solely explained by CMV infection. Our data confirm that CMV seropositivity is a common phenomenon in CLL, but it was almost as prevalent in the CLL^{NR} group and so the preferential expansion of CD8^+^ T cells and the increase in CD57^+^ and CD28^+^ and CD27^+^ TEMRA in the CLL^{IR} subset cannot be attributed to CMV alone. These findings are in keeping with a recent report that showed CD8^+^ expansion in CMV-seronegative individuals (5). Further evidence for a CMV-independent process driving the emergence of replicative senescence phenotype was derived from our assessment of PD-1 expression in CD8^+^ T cells. PD-1 was significantly increased in CLL^{IR} patients but was not associated with CMV serostatus.

Intuitively, large numbers of effector T-cell subsets should be beneficial because of their ability to provide immediate immunity. Thus our findings associating increased CD8^+^ T-cell numbers with inferior clinical prognosis may seem somewhat paradoxical. However, the depletion of naive and central memory CD8^+^ T cells and the expansion of TEM and TEMRA cells may hamper long-term immunoprotection, as there is a reduction in the diversity and functional integrity of the T-cell subsets. The increase of CD57^+^, CD28^+^, and CD27^+^ effector T cells, coupled with increased PD-1 expression, indicates that a large proportion of the effector cells are highly differentiated and in a state of replicative senescence and thus have the least proliferative potential among the T-cell subsets. This contributes to a decrease competence to effectively respond to reinfection or maintain memory for tumor antigens expressed by relapsing tumor cells. Moreover, a decrease in the relative proportion of naive T cells will inevitably decrease the diversity of the naive T-cell receptor repertoire. This can affect the capacity of CLL patients to resist de novo infections or to respond to the appearance of new tumor antigens.

Tumor antigens might drive the preferential expansion of CD8^+^ T cells in the CLL^{IR} patients. Proliferation of T cells against autologous CLL tumor cells has been shown in vitro (12, 14, 36, 37), and this is likely to reflect a composite response against multiple tumor antigens, rather than a response restricted to a single immunodominant antigen. This concept of T-cell expansion driven by CLL tumor cells seems to be at odds with the suppressive properties of CLL cells. Laboratory studies have shown a defective immune synapse between T cells and CLL cells (38) and the ability of CLL cells to manipulate the gene expression profile of T cells through cell–cell contact (8). However, tolerance of CD8^+^ T cells can be accompanied by proliferation (39, 40), and expanded populations of anergic or nonfunctional CD8^+^ T cells can be found in human cancer (41) and chronic infection (42, 43). This suggests the possibility that CLL tumor cells can induce the proliferation of CD8^+^ T cells that become incapable of initiating, continuing, or completing an immune response against the leukemic B cells and other antigens, and thus may be involved directly in sustaining the tumor. However, the ability to mount a response against CMV seems to be maintained in CLL patients as CMV reactivation is not common, except for those treated with alemtuzumab (44).
Taken together, our results suggest that the inversion of the CD4:CD8 ratio in treatment-naive, CLL patients is associated with the preferential expansion of CD8⁺ PD-1⁺ highly differentiated memory cells with a replicative senescence phenotype. Crucially, the emergence of these cells seem to signify a loss of immunologic control against the leukemia because patients with CLLIR have shorter lymphocyte doubling times, more progressive disease, and require earlier treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

C. Nunes and R. Wong carried out research and analyzed data; M. Mason contributed vital new reagents and revised the paper; C. Fegan designed research, contributed vital reagents, and revised the paper; and S. Man and C. Pepper designed research, analyzed data, and wrote the paper.

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