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PEGylated IL-10 (Pegilodecakin) Induces Systemic Immune Activation, CD8+ T Cell Invigoration and Polyclonal T Cell Expansion in Cancer Patients

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SUMMARY

Tumor-reactive T cell exhaustion prevents the success of immune therapies. Pegilodecakin activates intratumoral CD8+ T cells in mice and induces objective tumor responses in patients. Here we report that pegilodecakin induces hallmarks of CD8+ T cell immunity in cancer patients,

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AUTHOR CONTRIBUTIONS

Conceptualization and Investigation, A.N., J.R.I., K.P.P., I.H.C., K.A.A., D.J.W., M.R.P., P.A.O., G.S.F., S.P., A.H., J.B.M., C.S., V.W., N.P.R., K.L.P., S.McC., B.R., M.A., P.W., P.V.V., J.L., N.M.T., and M.O.; Methodology, I.H.C., C.S., N.P.R., K.L.P., S.McC., B.R., and M.A.; Writing – Original Draft, M.O.; Writing – Review & Editing, A.N., J.R.I., K.A.A., P.V.V., J.L., and M.O. Funding Acquisition, P.V.V., J.B.M., J.L., and M.O.; Supervision, A.N., J.R.I., N.M.T., P.V.V., J.L., and M.O.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at https://doi.org/10.1016/j.ccell.2018.10.007.

DECLARATION OF INTERESTS

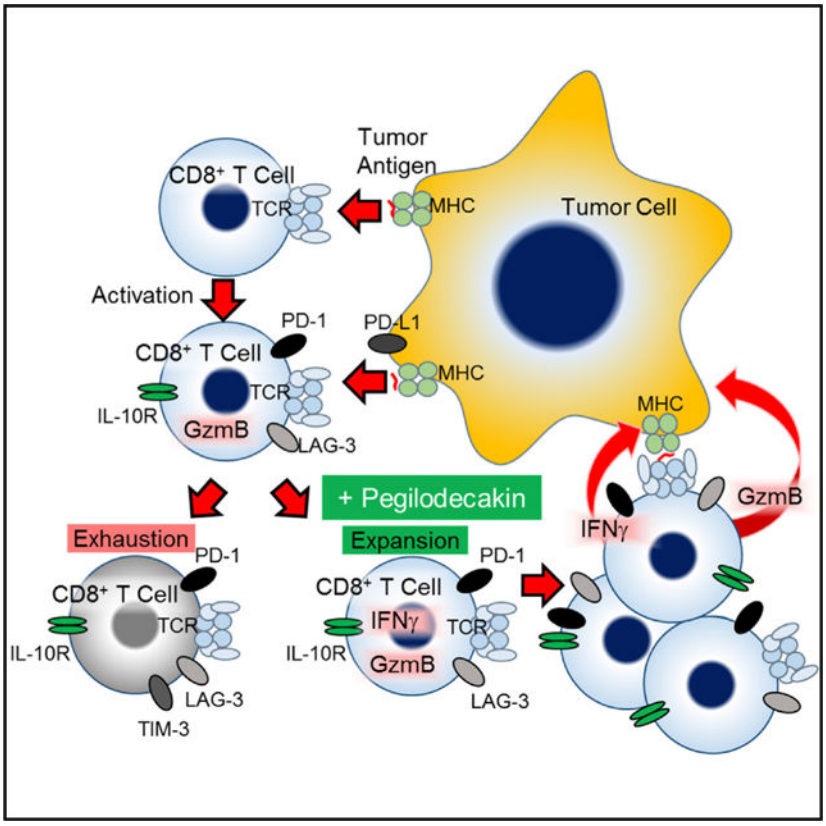
N.P., A.H., S.M., and M.O. are current employees of ARMO BioSciences, a wholly owned subsidiary of Eli Lilly and Company.

including elevation of interferon-γ and GranzymeB, expansion and activation of intratumoral CD8$^+$ T cells, and proliferation and expansion of LAG-3$^+$ PD-1$^+$ CD8$^+$ T cells. On pegilodecakin, newly expanded T cell clones, undetectable at baseline, become 1%–10% of the total T cell repertoire in the blood. Elevation of interleukin-18, expansion of LAG-3$^+$ PD-1$^+$ T cells and novel T cell clones each correlated with objective tumor responses. Combined pegilodecakin with anti-PD-1 increased the expansion of LAG-3$^+$ PD-1$^+$ CD8$^+$ T cells.

In Brief

Naing et al. report that pegilodecakin, PEGylated IL-10, which achieves objective tumor responses in patients, induces hallmarks of CD8$^+$ T cell immunity in cancer patients. Pegilodecakin promotes expansion of underrepresented T cell clones as well as LAG-3$^+$ PD-1$^+$ CD8$^+$ T cells, which are further induced by anti-PD-1.

Graphical Abstract



INTRODUCTION

The expansion of activated tumor-specific CD8$^+$ T cells and their activation in the tumor is essential for the success and durability of immune-oncology approaches (Rosenberg and Dudley, 2009; Tumeh et al., 2014). Long-term success of immune-oncology strategies depends on the safe amplification and activation of a tumor-specific CD8$^+$ T cell memory (Apetoh et al., 2015). Clinical efficacy of immune-oncology therapies such as PD-1/PD-L1

inhibitors is dependent on a high density of preexistent tumor-infiltrating $CD8^+$ T cells (Tumeh et al., 2014). Therapy with multiple immune checkpoint inhibitors increases the clinical efficacy (Larkin et al., 2015), but systemic activation of the T cell repertoire is also associated with dose-limiting autoimmunity (Subudhi et al., 2016). T cells recognizing tumor neo-antigens can be found in most cancer patients, albeit at very low numbers (Cohen et al., 2015; Knuth et al., 1989; Tran et al., 2015). These preexisting, tumor neo-antigen-specific $CD8^+$ T cells within the patient's tumor or blood have an exhausted phenotype and elevated expression of immune checkpoint molecules (e.g., PD-1, LAG-3, and TIM-3), indicating prior antigen recognition (Gros et al., 2014, 2016). However, patients with increased numbers of checkpoint-positive $CD8^+$ T cells respond better to checkpoint inhibition therapy (Daud et al., 2016), and immune checkpoint inhibition only leads to a transient re-invigoration of exhausted T cells in models of chronic virus infection (Pauken et al., 2016; Wherry and Kurachi, 2015). In melanoma patients, treatment with anti-PD-1 leads to the invigoration of exhausted $PD\text{-}1^+$ $CD8^+$ T cells, lasting for several weeks (Huang et al., 2017). This reactivation is transient, returning to baseline proliferation at around 9 weeks of treatment. Strategies to activate, invigorate, and expand this preexisting but exhausted tumor-specific T cell repertoire are needed. In addition, the presence of inflammatory rather than cytotoxic $CD8^+$ T cells may promote tumor progression (Oft, 2014). Activated T cells and dendritic cells produce interleukin-10 (IL-10), which is well known for its anti-inflammatory function, but, at higher concentrations, IL-10 and PEGylated IL-10 activate the cytotoxicity and proliferation of $CD8^+$ T cells (Emmerich et al., 2012; Fujii et al., 2001; MacNeil et al., 1990; Mumm et al., 2011). Elevation of IL-10 in experimental tumors leads to T cell-mediated tumor rejection (Moore et al., 2001). In animal studies, sustained elevated serum concentrations of IL-10 as achieved with PEGylated IL-10 (pegilodecakin) enhanced cytotoxicity and expansion of tumor-specific $CD8^+$ T cells resulted in cure from tumors (Mumm et al., 2011). Importantly, pegilodecakin induced amplification of tumor-specific activated $CD8^+$ T cells, increased proliferation of intratumoral IL-10 receptor (IL-10Ra)-expressing $CD8^+$ T cells and $CD8^+$-mediated rejection of tumors in mouse models of cancer (Emmerich et al., 2012; Mumm et al., 2011). Moreover, mice and humans deficient for IL-10 or the IL-10 receptor develop inflammatory bowel disease and cancer (Berg et al., 1996; Neven et al., 2013). B cell lymphomas that develop in IL-10R-deficient children lack infiltration by cytotoxic T cells (Neven et al., 2013). We recently reported objective tumor responses in 4 of 15 patients with intermediate- to poor-risk renal cell cancer (RCC) treated with pegilodecakin monotherapy (20 μg/kg) in median fourth line of treatment (LOT) (range 1–8) without inducing autoimmune toxicities (Naing et al., 2016). In addition, 15 of the total 41 patients with advanced disease receiving pegilodecakin in the third to fifth LOT had durable disease stabilization. Here we investigate the immunological underpinnings of pegilodecakin-induced tumor responses in cancer patients.

RESULTS

Pegilodecakin Induces Sustained Elevation of Th1 and Th2 Cytokines in the Serum

Pegylated IL-10 induces objective tumor responses as monotherapy (Naing et al., 2016). To understand the immune response in pegilodecakin-treated patients and identify immune correlates to objective tumor responses, 83 immune-related cytokines, chemokines, and

serum proteins were measured in patients who self-administered 20 μg/kg pegilodecakin daily by subcutaneous injection for 28 days (Figure 1A; Table S1). IL-10 was elevated to 18.9 ng/mL, which represents both the endogenous IL-10 and the PEGylated IL-10, and indicates the serum trough of pegilodecakin. Pegilodecakin induced an immune cytokine profile biased toward Th1 and Th2 upregulation and products of activated $CD8^+$ T cells (Figure 1A). Four of 16 patients with RCC had a partial tumor response (PR) (Figure 1B). Th1 cytokines (interferon-γ [IFN-γ], interleukin-18 [IL-18], and tumor necrosis factor alpha [TNF-α]), as well as IL-4, which are products of activated Th2 $CD4^+$ and $CD8^+$ T cells were moderately but consistently increased (Figures 1C and S1A). IL-7, which is essential for the proliferation of $CD8^+$ T cells (Mackall et al., 2011), was also significantly induced after 1 month of treatment (Figures 1A and S1B). Patients treated with pegilodecakin had also elevated serum concentrations of cytotoxic effector molecules (FasL and lymphotoxin β; Figure 1D). Simultaneously, pegilodecakin led to a reduction of the immune suppressive cytokine transforming growth factor β (TGF-β) and Th17-related cytokines IL-23 and IL-17, and the homodimeric IL-12p40, which mediate tumor-associated inflammation (Langowski et al., 2007), were reduced by approximately 40% (Figure 1E), while IL-6 was not consistently altered (data not shown). To further understand the kinetics of the immune activation, we performed a 12-day, single-followed by multiple-dose time course in normal healthy volunteers. Pegilodecakin was elevated above 1 ng/mL in the serum throughout the study (Figure 1F). This led to a significant increase of IFN-γ, IL-18, FasL, and GranzymeB after 6 days (Figures 1F and S1C). In cancer patients, the systemic increase in immune-stimulating cytokines and cytotoxic effector molecules, including IFN-γ, IL-18, FasL, and IL-4, was durably sustained throughout the treatment (Figures 1G and S1D) and for periods up to at least 400 days. Notably, pegilodecakin induced the same consistent changes of the immune milieu in every patient, spanning multiple tumor types including colorectal cancer and pancreatic cancer (Figure 1H), demonstrating that pegilodecakin engaged its mechanism of action in immune-sensitive and insensitive tumor types. To further understand the relationship between the induction of cytokine, and objective tumor responses, the absolute serum concentration of IL-18 and the magnitude of serum IL-18 induction were correlated with the degree of tumor response. The magnitude of IL-18 correlated best with the tumor response (Figure 1I) and IL-18 induction was higher in patients with a PR and with stable disease than in patients with progressive disease (Figure 1J).

Invigoration of Exhausted $CD8^+$ T Cells by Pegilodecakin

IL-18 is produced by dendritic cells in response to T cell-derived IFN-γ and reciprocally enhances the expansion of effector memory $CD8^+$ T cells and their secretion of IFN-γ (Iwai et al., 2008). Since the observed cytokine profile suggested an activation of $CD8^+$ T cells in pegilodecakin-treated patients, we further tested this hypothesis by analyzing $CD8^+$ and $CD4^+$ T cells in the blood. Immune checkpoints such as PD-1, LAG-3, or TIM-3 have been reported to be expressed on T cells at elevated levels upon activation (Freeman et al., 2010; Keir et al., 2008). In addition, $CD8^+$ T cells with increased immune checkpoint expression isolated from the tumor or the blood of cancer patients were found to contain increased percentages of T cells recognizing tumor antigens (Gros et al., 2014, 2016). However, expression of immune checkpoints has also been associated with the functional exhaustion of T cells in models of chronic virus infection or in cancer patients (Wherry and Kurachi,

2015), while immune checkpoint blockade can transiently reactivate proliferation of immune checkpoint-positive $CD8^+$ T cells (Huang et al., 2017). We therefore investigated phenotypic changes with respect to checkpoint expression in T cells from the peripheral blood of patients in response to pegilodecakin treatment. In a patient with RCC who had a durable tumor response to pegilodecakin treatment (Figure 2A), $LAG\text{-}3^+$ $CD8^+$ T cells were rare prior to treatment in the blood, but were significantly increased and maintained for at least 20 weeks in this patient in response to pegilodecakin treatment (Figure 2B). A similar increase was observed in a cohort of 22 cancer patients on pegilodecakin (Figure 2C). In addition, these patients also showed co-expression of PD-1 on more than half of the expanding $LAG\text{-}3^+$ $CD8^+$ T cells (Figures 2D and 2E). Prior to pegilodecakin treatment, the $CD8^+$ T cells displayed the characteristic exhausted T cell phenotype expressing LAG-3, PD-1 with a low proliferative index as shown here by the $Ki\text{-}67^+$ subset among the $LAG\text{-}3^+$ $PD\text{-}1^+$ $CD8^+$ T cells. This is consistent with the exhausted phenotype described for T cells expressing multiple checkpoint molecules (Wherry and Kurachi, 2015). Pegilodecakin treatment significantly increased the percentage of proliferating $Ki\text{-}67^+$ $LAG\text{-}3^+$ $PD\text{-}1^+$ $CD8^+$ T cells (Figures 2F, 2G, and S2A), indicating that pegilodecakin induced the activation and proliferation of $LAG\text{-}3^+$ $PD\text{-}1^+$ $CD8^+$ T cells, positive for multiple immune checkpoints. The timing of this increase in proliferative $PD\text{-}1^+$ cells correlated with the observation of the reduction in tumor burden in patients with renal cancer (Figure S2B).

In untreated cancer patients, only 1.5% of $CD8^+$ T cells were $LAG\text{-}3^+$ $PD\text{-}1^+$ double-positive. The total number of $LAG\text{-}3^+$ $PD\text{-}1^+$ $CD8^+$ T cells increased during treatment with pegilodecakin to a mean of 12% of $CD8^+$ T cells in the blood (range 1.8–28.7) (Figure 2E). Importantly, the numeric increase of $LAG\text{-}3^+$ $PD\text{-}1^+$ $CD8^+$ T cells with increased proliferative index was sustained throughout pegilodecakin treatment for at least 20 weeks (Figures 2D–2F). In contrast, anti-PD-1 antibody treatment induces a transient re-invigoration of exhausted $CD8^+$ T cells (Huang et al., 2017).

The sustained expansion of immune checkpoint-positive effector $CD8^+$ T cells is also consistent with the observation that serum cytokines and cytotoxicity-mediating factors, such as FasL were sustainably induced throughout the treatment (Figure 1G). TIM-3 expression was not increased on $CD8^+$ T cells during pegilodecakin treatment (Figures 2H and 2I). $CD8^+$ T cell exhaustion is characterized by the expression of several inhibitory co-stimulatory signals, including CTLA-4 and TIM-3 (Wherry and Kurachi, 2015). TIM-3 induces T cell apoptosis (Freeman et al., 2010) and is expressed on exhausted T cells in cancer patients (Afanasiev et al., 2013). In line with the strongly inhibitory function of TIM-3, $TIM\text{-}3^+$ $CD8^+$ T cells, only a small proportion of $TIM\text{-}3^+$ $PD\text{-}1^+$ $CD8^+$ T cells in cancer patients were proliferating (Figure 2H) and the number of $TIM\text{-}3^+$ $PD\text{-}1^+$ $CD8^+$ T cells or $TIM\text{-}3^+$ $CD8^+$ T cells did not increase with pegilodecakin (Figures 2I and S3A). Interestingly, prior to treatment, activated $PD\text{-}1^+$ $CD8^+$ T cells expressed higher levels of the IL-10Ra (Figures 2J and 2K). With persistent increase in the $PD\text{-}1^+$ $CD4^+$ T cells similar to the $PD\text{-}1^+$ $CD8^+$ T cell pool (Figure S3B), the total number of $Foxp3^+$-regulatory $CD4^+$ T cells (Tregs) did not change in cancer patients on pegilodecakin (Figure S3C), and the proliferation of $FoxP3^+$ Tregs was reduced (Figure S3D), suggesting a reduced activation of Tregs. In contrast to $LAG\text{-}3^+$ $PD\text{-}1^+$ $CD8^+$ T cells, $LAG\text{-}3^+$ $PD\text{-}1^+$ $CD4^+$ T cells did not

increase (not shown), indicating that pegilodecakin primarily activated $CD8^+$ T cell responses.

Pegilodecakin-Induced $CD8^+$ T Cell Reactivation Correlates with Tumor Response

The activation of LAG-3^+ PD-1^+ $CD8^+$ T cells coincided timely with the occurrence of clinical responses. An RCC patient on pegilodecakin monotherapy had initial increase of tumor burden, followed by an objective tumor response at 20 weeks of treatment (Figures S2A and S2B). While LAG-3^+ PD-1^+ $CD8^+$ T cells had subtle increase in proliferation within the first months of treatment, a strong burst in proliferation and expansion of LAG-3^+ PD-1^+ $CD8^+$ T cells occurred at the time of the tumor response, suggesting a functional involvement of the T cells in the response. In a cohort of 22 cancer patients on pegilodecakin, the percentage of LAG-3^+ $CD8^+$ T cells after 2 months of treatment correlated with tumor response, with patients who had a larger percentage of LAG-3^+ $CD8^+$ T cells during pegilodecakin treatment showing a larger reduction in tumor burden (Figure 3A). In contrast, the number of LAG-3^+ $CD8^+$ T cells prior to pegilodecakin treatment did not correlate with the response (Figure 3B). Patients with progressive disease had an increase of LAG-3^+ T cells, however this increase was less pronounced than in patients with stable disease or PR (Figure S4A). The percentage of proliferating or total LAG-3^+ PD-1^+ $CD8^+$ T cells on treatment correlated with tumor response, both directly or when patient with progressive disease were compared with responding patients (Figures 3C–3E and S4B). Similar results were observed for the total PD-1^+ $CD8^+$ T cells, Ki-67^+ PD-1^+ $CD8^+$ T cells and Ki-67^+ LAG-3^+ $CD8^+$ T cells but not for PD-1^+ $ICOS^+$ $CD8^+$ T cells (Figures S4C–S4F). TIM-3 marks a deeply exhausted T cell population in models of chronic virus infection and in cancer patients (Anderson et al., 2016). Interestingly, PD-1^+ TIM-3^+ $CD8^+$ T cells remained rare among patients with 2 months of pegilodecakin treatment, and their presence was lower in patients with partial tumor responses (Figures 3F and 3G) suggesting that the absence of TIM-3 expression on $CD8^+$ T cells correlated with a tumor response. TIM-3^+ expression of PD-1^+ $CD8^+$ T cells did negatively correlate with tumor reduction (Figures 3F and 3G). Moreover the ratio of proliferating LAG-3^+ PD-1^+ to TIM-3^+ PD-1^+ $CD8^+$ T cells revealed a stronger correlation with tumor response than either marker alone (Figure 3H). This may suggest a mechanism wherein the functional activation and expansion of LAG-3^+ PD-1^+ $CD8^+$ T cells at the expense of a reduction of exhausted TIM-3^+ $CD8^+$ T cell populations tips the immune balance toward the objective reduction in tumor size in response to pegilodecakin treatment.

Pegilodecakin Increases Phospho-$STAT3^+$ and LAG-3^+ Intratumoral $CD8^+$ T Cells

The increased prevalence of activated $CD8^+$ T cells in the blood raised the question of whether pegilodecakin would also increase the number of activated $CD8^+$ T cells in the patients' tumor. We therefore analyzed tumor tissue before and during pegilodecakin treatment. Prior to treatment, the prevalence of $CD8^+$ T cells in the tumor was low and increased significantly during pegilodecakin treatment (Figure 4A). Pegilodecakin also led to a more than 3-fold increase in the number of $GranzymeB^+$ $CD8^+$ T cells in the tumor tissue (Figures 4B–4D). IL-10-activated IL-10 receptor leads to the phosphorylation of STAT3 (Moore et al., 2001), a transcription factor which induces the transcription of anti-apoptotic and proliferation-mediating genes (Bromberg and Darnell, 2000). We had

previously shown that activation of STAT3 is important for the increased functional activity of $CD8^+$ T cells in response to pegilodecakin (Mumm et al., 2011). Prior to pegilodecakin treatment the majority of intratumoral $CD8^+$ T cells did not express activated, phosphorylated STAT3. On treatment, 80% of $CD8^+$ T cells were phospho-STAT3-positive (Figures 4E–4G). Similar to the expansion of LAG-3^+ $CD8^+$ T cells in the blood, pegilodecakin treatment increased the proportion of the $CD8^+$ T cells in tumor tissue that were positive for LAG-3 (Figures 4H–4J). The increase of STAT3 signaling in antigen-exposed tumor-infiltrating $CD8^+$ T cells may provide a rationale for the expansion of intratumoral LAG-3^+ $CD8^+$ T cells. Since we observed increased IFN-γ in the serum and GranzymeB-expressing $CD8^+$ T cells in the tumors of patients on pegilodecakin, we further explored the transcription factor T-bet, which is essential for the expression of both molecules (Kallies and Good-Jacobson, 2017). Prior to treatment, most tumor-infiltrating T cells were T-bet-negative, while 76% of $CD3^+$ T cells were positive on pegilodecakin (Figure 4K). While IL-10 can reduce MHC expression *in vitro* (Koppelman et al., 1997), treatment of mouse tumor models with PEG-IL-10 led to IFN-γ-mediated induction of MHC I and II expression in the tumor (Mumm et al., 2011). Pegilodecakin treatment of cancer patients led to an increase of HLA-A expression in tumor cells (Figures 4L–4N).

Pegilodecakin Enhances T Cell Receptor-Mediated $CD8^+$ T Cell Activation

To exclude that the pegylation may alter the signaling or target cell characteristics of IL-10, we analyzed IL-1b and IL-6, two cytokines repressed by IL-10. Both were strongly induced by bacterial lipopolysaccharide and dose-dependently suppressed by pegilodecakin (Figures S5A and S5B). To understand why pegilodecakin increases the activation, proliferation, and expansion of immune checkpoint-positive, exhausted $CD8^+$ T cells, we used an *in vitro* re-stimulation model of human $CD8^+$ T cells isolated from peripheral blood. Purified $CD8^+$ T cells were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (mAb) for 3 days, followed by addition of anti-CD3 mAb in the presence or absence of pegilodecakin. Under these conditions, repeated anti-CD3 mAb stimulation leads to decreased viability or activation-induced cell death (AICD) (Green et al., 2003), which has mechanistic similarities with the overstimulation and exhaustion of tumor antigen-specific T cells in cancer. Addition of pegilodecakin significantly increased the T cell survival (Figure 5A). To understand the mechanism, we first investigated the expression of the IL-10 receptors (IL-10Ra and IL-10Rb, respectively) on the surface of T cells in response to T cell receptor (TCR) activation. Confirming results previously reported for murine $CD8^+$ T cells (Emmerich et al., 2012; Foulds et al., 2006), the IL-10Ra was upregulated for several days (day 3 shown) following anti-CD3 or anti-CD3/CD28 stimulation (Figure 5B). The surface expression of IL-10Rb was not significantly increased (Figure S5C). Pegilodecakin treatment induces phosphorylation of STAT3 (p-STAT3) in $CD8^+$ T cells (Moore et al., 2001; Mumm et al., 2011). P-STAT3 mediates anti-apoptotic and proliferation signals in most cells (Bromberg and Darnell, 2000), and p-STAT3 is essential for the functional maturation of $CD8^+$ T cells (Cui et al., 2011). Pegilodecakin increased STAT3 phosphorylation in all $CD8^+$ T cells, with or without anti-CD3 ± anti-CD28 stimulation; however, $CD8^+$ T cells pretreated with anti-CD3 mAb had a significantly higher level of p-STAT3 in response to pegilodecakin (Figure 5C). This may be explained by the higher expression of the IL-10 receptor on anti-CD3 mAb-stimulated $CD8^+$ T cells. Anti-CD28 mAb did not further increase the surface

expression of the IL-10R or the phosphorylation of STAT3 (Figures 5B and 5C). This may support the concept that $CD8^+$ T cells with recent antigen exposure may be more susceptible to receiving IL-10-mediated proliferation induction through p-STAT3.

Direct Activation of PD-1^+ $CD8^+$ T Cells by Pegilodecakin

Co-inhibitory or immune checkpoint receptors are upregulated upon T cell activation to ensure immune homeostasis (Anderson et al., 2016). Expression of immune checkpoints can lead to functional impairment of T cells and is also increased on exhausted $CD8^+$ T cells (Wherry and Kurachi, 2015). Upon anti-CD3 mAb stimulation, donor-derived $CD8^+$ T cells upregulated PD-1 and LAG-3 (Figures 5D and 5E). The persistence of this activated population was maintained in the presence of anti-CD3 mAb stimulation but rapidly disappeared upon anti-CD3 mAb withdrawal (Figure 5E, upper versus lower left panel). Pegilodecakin treatment partially rescued the progressive loss of LAG-3^+ PD-1^+ $CD8^+$ T cells in the absence of anti-CD3 mAb and also led to further increase in LAG-3^+ PD-1^+ $CD8^+$ T cells in the presence of anti-CD3 mAb (Figure 5E). We further investigated if pegilodecakin treatment would directly increase the functional activation of PD-1^+ $CD8^+$ T cells, as observed in the patients. In anti-CD3/CD28 mAb pre-activated $CD8^+$ T cells, pegilodecakin treatment led to an increase in proliferation of PD-1^+ $CD8^+$ T cells, with an average of a 3-fold increase in Ki-67^+ proliferating cells (Figure 5F). While the majority of $CD8^+$ T cells expressed low levels of GranzymeB after anti-CD3/CD28 mAb activation (Figure 5G), pegilodecakin increased the expression level of GranzymeB, both in PD-1^{lo} and in PD-1^{hi} $CD8^+$ T cells (Figure 5G), and the secretion of GranzymeB from $CD8^+$ T cells (Figure 5H). Collectively, these data suggest that pegilodecakin induces sustained proliferation of a functionally maturated $CD8^+$ T cell effector population and prevents the loss of anti-CD3-stimulated, LAG-3^+ PD-1^+ $CD8^+$ T cells.

Oligoclonal T Cell Expansion in Response to Pegilodecakin Treatment

The observed increase of the proliferation and expansion of LAG-3^+ PD-1^+ $CD8^+$ T cells in patients may be a reflection of the expansion of a distinct, antigen-challenged clonal T cell population or the functional maturation of an existing subset of the peripheral T cells. To distinguish between these possibilities we analyzed the composition of the T cell repertoire of pegilodecakin-treated patients by TCR-deep sequencing from the peripheral blood (Robert et al., 2014). Comparison of the clonal T cell repertoire before and on treatment revealed that patients on pegilodecakin had a strong expansion of a distinct subset of T cell clones, while the majority of T cells remained unchanged (Figures 6A–6D). The T cell expansion included clones that were detectable in the pretreatment or pre-existing repertoire, but most expanding clones were not detectable or underrepresented in the patients before treatment (“novel clones”). Surprisingly, the *de novo* expansion was observed in patients with a wide variety of cancer types (Figures 6A–6D). In a previously described RCC patient with a delayed objective tumor response (Figure 6E), only very few T cell clones expanded at 5 weeks of treatment preceding the tumor response (Figure 6F), while strong clonal T cell expansion occurred at the time of tumor size reduction at 21 weeks (Figures 6G and 6H). At the time of tumor rejection expanded clones largely exceeded contracted clones (Figures 6G and 6H).

Pegilodecakin-Induced Clonal T Cell Expansion Correlates with Tumor Response

We next asked if the expansion of "novel" T cell clones was more succinct in patients with objective tumor response. RCC patients with progressive disease (PD) (Figure 6I), stable disease (SD) (Figure 6J), or PRs (Figure 6K) were analyzed for the clonal composition of the peripheral T cell repertoire. The expansion of previously underrepresented T cell clones was most apparent in the patient with a PR, while the patient with PD had only limited expansion of novel T cell clones. To further understand the clonal T cell response, we analyzed the number of T cell clones which changed more than 10-fold from baseline during pegilodecakin treatment. Pegilodecakin led to a more than 10-fold expansion of a median of 240 T cell clones per patient (range 17–786), while only a median of 18 T cell clones per patient (range 0–150) contracted more than 10-fold (Figure 6L). Importantly, the number of expanding peripheral T cell clones (more than 10-fold) correlated with the tumor response. Patients with an objective tumor response on pegilodecakin alone had a median of 761 (range 524–786) expanding individual clones, compared with 194 clones (81–519) in patients with SD and 164 clones (17–328) in patients with PD (Figure 6M). To understand how the pegilodecakin treatment contributed to the re-shaping of the peripheral T cell repertoire in patients we analyzed the relative size of the expanding T cell compartment in relation to all peripheral T cells. T cells from expanding clones (the sum of T cells in expanding clones) represented an average of 0.06% of a patient's peripheral T cell repertoire prior to treatment. T cells from those clones expanded during treatment to occupy on average 6% of the total peripheral T cell repertoire after 3 months of treatment (Figure 6N). It is important to note that pegilodecakin led to a continued expansion of those T cell clones throughout the treatment period. We next analyzed if the sum of T cells in expanding clones in patients correlates with the objective tumor response to pegilodecakin. Indeed, the sum of T cells in expanding T cell clones correlated with the best tumor response (percent change from initial tumor burden) in patients on pegilodecakin (Figure 6O).

Treatment with Pegilodecakin + Anti-PD-1 Increases Immune Response

Since pegilodecakin led to the expansion and increased proliferation of PD-1^+ $CD8^+$ T cells, we treated a cohort of heavily pretreated patients with melanoma, non-squamous cell lung cancer or RCC with a combination of pegilodecakin and pembrolizumab (2 mg/kg, every 3 weeks). Nineteen patients were evaluable for tumor response and eight had an objective response (42% overall response rate) (Figure 7A). LAG-3^+ PD-1^+ $CD8^+$ T cells expanded in the patients to become ~15% after 3 weeks of treatment (Figure 7B), and proliferating LAG-3^+ PD-1^+ $CD8^+$ T cells were 3.2% of all $CD8^+$ T cells after 3 weeks (Figure 7C). This suggests an additional independent proliferation stimulation with PD-1 inhibition as reported previously. Anti-PD-1 treatment alone induced a transient proliferation of T cells 2–4 weeks after the start of the anti-PD-1 treatment, which reverted to pretreatment levels in subsequent time points (Huang et al., 2017). In our study, on pegilodecakin + anti-PD-1, the proliferation and expansion of LAG-3^+ PD-1^+ $CD8^+$ T cells at 4 weeks was 2-fold larger when compared with pegilodecakin monotherapy, but LAG-3^+ PD-1^+ $CD8^+$ T cells constituted similar percentage of all $CD8^+$ T cells in both cohorts after 2 months of treatment (Figures 2E and 7B). Similar to pegilodecakin alone, combination with anti-PD-1 induced the expansion of newly detectable, previously rare T cell clones in the blood (Figure 7D). The number of newly expanding T cell clones detectable in patients on the combination

treatment (mean of 457 expanding clones/patient) were slightly but not significantly higher than on pegilodecakin alone (mean of 324 expanding clones) (Figure 7E). Expanding T cell clones had a similar expansion kinetic on the combination treatment (Figure 7F) to pegilodecakin alone (Figure 6N), and patients with a PR again had a higher number of expanding T cell clones than patients with SD (Figure 7G).

DISCUSSION

IL-10 has anti-inflammatory properties and stimulates antigen-specific $CD8^+$ T cell cytotoxicity and anti-tumor immune responses. The contribution of the treatment with pegilodecakin induced immune-activating cytokine cascades, which may promote antigen-specific or $CD8^+$ T cell-mediated immunity. This included the Th1 and Th2 lineage-defining cytokines IFN-γ and IL-4, and also cytotoxic effector molecules. IL-10 increased IFN-γ and FasL in purified, TCR-stimulated $CD8^+$ T cells *in vitro* (Mumm et al., 2011), suggesting a direct activation of these cytokines by PEG-IL-10 in the T cells of cancer patients. T cell-derived IFN-γ has been shown to increase IL-18 secretion (Iwai et al., 2008), and T cell-derived GranzymeB cleaves pro-IL-18 into active IL-18 (van de Veerdonk et al., 2011). IL-18 in turn increases the secretion of IFN-γ by $CD8^+$ T cells (Iwai et al., 2008), and also prevents AICD in $CD8^+$ T cells (Li et al., 2007). The observed systemic increase of IFN-γ, IL-18, and effector molecules of $CD8^+$ T cells is consistent with the sustained activation of $CD8^+$ T cells observed in patients. The concentration of IL-18 observed in patients on pegilodecakin is expected to be an immunologically active concentration. Intriguingly, several groups recently reported that the expression of IL-18 in chimeric antigen receptor-expressing $CD8^+$ T cells enhances their therapeutic efficacy and durability in the host (Chmielewski and Abken, 2017; Hu et al., 2017). Pegilodecakin treatment also increased IL-7. The levels reached appear to be at a therapeutically active level (Mackall et al., 2011). IL-7 is a growth factor for memory $CD8^+$ T cells and has an anti-apoptotic function on $CD8^+$ and $CD4^+$ T cells. IL-7 increases the expression of the TCR co-receptor CD8 to promote TCR engagement of $CD8^+$ T cells (Park et al., 2007). In mice, tumor regression in response to anti-CTLA-4 plus anti-PD-1 is dependent on both IL-7 and IFN-γ signaling (Shi et al., 2016). The combined activation of those cytokines may contribute to the observed activation and expansion of $CD8^+$ T cells in pegilodecakin-treated patients. At the same time, reduction of cytokines related to tumor-promoting, chronic inflammation and Th17 confirms the anti-inflammatory properties of IL-10. The reduction in TGF-β levels and the reduction of Treg proliferation may further support the immune-activating role of pegilodecakin.

The reduction of both TGF-β and several inflammatory cytokines may point to an additional benefit of pegilodecakin in ameliorating the immune suppressive but pro-inflammatory milieu in the tumor. IL-23 and IL-17 are associated with increased tumor incidence (Langowski et al., 2006).

Tumor responses to anti-PD-1 correlate with a high mutational burden in the tumor, suggesting that the pre-existing T cell response to the resulting neoantigens may facilitate the tumor response (Rizvi et al., 2015). In support of this notion, melanoma with a high number of tumor-infiltrating $CD8^+$ T cells (more than 1,000 cells/mm^2) had a response to

anti-PD-1, while tumors with less intratumoral T cells progressed. In this study, we observed a clonal expansion of rare or novel T cells in all tumor types—with high or low predicted mutational burden. In addition, the majority of patients had a low number of preexisting $CD8^+$ T cells in their pretreatment tumor samples. The magnitude of the *de novo* T cell expansion was correlated with tumor responses in patients on pegilodecakin monotherapy, while autoimmune-related AEs were not observed (Naing et al., 2016). In prostate cancer patients receiving anti-CTLA-4 therapy the expansion of more than 55 $CD8^+$ T cell clones per patients preceded severe immune-related adverse events, suggesting self-reactivity of these expanding T cell clones (Subudhi et al., 2016).

$CD8^+$ T cells expressing multiple co-inhibitory receptors are thought to represent an exhausted population with reduced cytotoxic and proliferative capacity (Apetoh et al., 2015). However, LAG-3^+ TIM-3^+ PD-1^+ $CD8^+$ T cells in tumors and the blood of cancer patients represent T cells with recent TCR activation and a high percentage of tumor-specific T cells (Gros et al., 2016). It is therefore very intriguing that pegilodecakin increased the proliferation and the expression of cytotoxic molecules in LAG-3^+ PD-1^+ $CD8^+$ T cell and that the extent of the expansion of those cells correlated with the tumor response. LAG-3 is upregulated on $CD8^+$ T cells upon antigen recognition, and limits the expansion of antigen-specific $CD8^+$ T cells and infiltration into tumors (Grosso et al., 2007). Interestingly, treatment with pegilodecakin led to increased numbers of tumor-infiltrating GranzymeB$^+$ $CD8^+$ T cells and expansion of LAG-3^+ $CD8^+$ T cells. In contrast to the exhausted phenotype described in untreated patients, LAG-3^+ PD-1^+ Ki-67^+ $CD8^+$ T cells, which expand in pegilodecakin-treated patients, appear to represent an activated T cell population. This may be explained by the absence of TIM-3 or CTLA-4 on the expanding LAG-3^+ PD-1^+ $CD8^+$ T cells. TIM-3 has pro-apoptotic functions (Freeman et al., 2010), and CTLA-4 inhibits $CD8^+$ memory T cell formation (Pedicord et al., 2011). In addition, in the LCMV model of chronic virus infection, virus persistence is associated with an exhausted phenotype of $CD8^+$ T cells, characterized by high expression of PD-1, LAG-3, and TIM-3. Anti-PD-1 transiently rescues T cell exhaustion by inducing the expansion of TIM-3^- $CD8^+$ T cells, which is followed by an expedited conversion of those cells to exhausted TIM-3^+ $CD8^+$ T cells (Im et al., 2016). In contrast to the transient invigoration of exhausted $CD8^+$ T cells by anti-PD-1, the treatment with pegilodecakin led to sustained increase in the proliferation and expansion of LAG-3^+ PD-1^+ $CD8^+$ T cells. The sustained systemic $CD8^+$ T cell activation is further supported by the notion that T cell-derived IFN-γ, IL-4, and FasL are elevated throughout the treatment period and at least up to 400 days.

Pegilodecakin induced an expansion of previously undetectable T cells in the systemic circulation of patients. These cells occupied in average 6% of the peripheral T cell repertoire of patients. This is reminiscent of the T cell burst in response to a viral infection controlled by the immune response (He et al., 2003; Heidema et al., 2008). Importantly, PEG-IL-10 induced a significant expansion of tumor-reactive $CD8^+$ T cells in the blood and tumors of murine tumor models (Mumm et al., 2011). This and the fact that immune checkpoint-positive $CD8^+$ T cells in cancer patients contain an increased percentage of tumor-reactive $CD8^+$ T cells (Gros et al., 2016) may suggest that pegilodecakin expands tumor-reactive or -specific T cells in cancer patients. Importantly, immune checkpoint inhibition appears to provide no expansion or only a transient burst in clonal T cell responses in patients, raising

the possibility that the two mechanisms could be combined for additional therapeutic benefit.

In summary, we observed a systemic Th1/Th2 immune activation and a reduction of Th17 inflammation in all pegilodecakin-treated patients, regardless of objective response. In contrast, the magnitude of the LAG-3^+ PD-1^+ $CD8^+$ T cell expansion and the number of novel T cell clones correlated with radiographic tumor response of the patient. It is possible that patients with a PR to pegilodecakin had a higher number of tumor-reactive T cells at the start of the therapy, or that immune suppressive mechanisms, including immune checkpoints, limit the response in patients with PD. This mechanism of action may provide an opportunity for combinations of PEG-IL-10 with other immune therapies such as checkpoint inhibitors or emerging immune-oncology therapies, but also with standard chemotherapies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents and data can be directed to and will be fulfilled by the lead contact, Martin Oft (martinoft@gmail.com). Any sharing of materials or data may be subject to material transfer agreements and data sharing agreements per requirement of the study sponsors.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patient Treatment—Patients were enrolled in a multi-basket trial (NCT02009449). All patients had written consented to the collection of samples and the analysis in accordance with the institutional review board. Patients recorded self-administration of pegilodecakin in a patient diary and drug usage was confirmed and documented in the electronic data capturing system showing overall compliance of 93% in the study. Patients on pegilodecakin alone administered daily self-injection of pegilodecakin at 20 μg/kg, SC, if not noted otherwise. Patients in the pembrolizumab $^+$ pegilodecakin received in addition pembrolizumab at 2 mg/kg IV, Q3W. All samples were collected at the indicated day, prior to dose administration. Normal healthy volunteers (NCT03267732) received a single (day 1) and multiple doses (days 4–9) of 5 μg/kg or 10 μg/kg pegilodecakin SC.

PBMC Derived $CD8^+$ T cell Analysis—Peripheral blood monocytic cells were received from Stanford blood bank.

METHOD DETAILS

PBMC Cell Derived $CD8^+$ T cell Culture and Analysis

$CD8^+$ T cells were isolated from human buffy coats and activated in AIM V media for 3 days on plates coated with 10 μg/mL anti-CD3 and 2 μg/mL anti-CD28, then replated and stimulated for 3 days with 100 ng/mL pegilodecakin. For IL-10 receptor analysis, cells were initially split into three groups: untreated, anti-CD3; anti-CD3/anti-CD28 activation. Cells

were collected and washed before FACS staining. For phosphorylated-STAT3 analysis, cells were stimulated with anti-CD3/anti-CD28, or anti-CD3 alone, or left without stimulation. After three days, cells were collected, washed, and treated with or without 100 ng/mL pegilodecakin for 20 minutes, and immediately stained for pSTAT3 expression.

Serum Cytokine Analysis

Serum cytokine analysis was performed using luminex bead assays (Myriad, San Antonio TX). Serum cytokine for normal health volunteer study was performed by Pacific Biomarkers, Seattle, WA.

$CD8^+$ T cell Analysis in Tumor Samples

Formaldehyde fixed archival, pretreatment biopsies and on treatment biopsies of cancer tissue were analyzed with double immune fluorescence staining for CD8, GranzymeB, phospho-STAT-3 or LAG-3 or immunohistochemistry for T-bet/CD3 and HLA-A The analysis was performed by an independent pathologist. For this, at least 10 representative fields were quantified. Representative images are depicted in Figure 4. The following antibodies were used CD8 (Abcam, ab101500), Phospho-STAT3 (Cell Signaling Technologies, 9138, LAG-3 (Cell Signaling Technologies, 15372), GranzymeB (Leica, NCL-L-GRAN-B), HLA-A (Abcam, ab52922), Tbet (Abcam, ab91109), CD3 (Abcam, ab16669).

Flow Cytometry

PBMC samples at the indicated visits pre- and post-treatment were thawed and stained with a fixable Aqua viability dye (Invitrogen) and a cocktail of antibodies to the following surface markers: CD8-Qdot605 (3B5, Invitrogen), CD4-Qdot655 (S3.5, Invitrogen), PD-1-PE (MIH4, BD Biosciences), LAG-3-FITC (17B4, Enzo), ICOS-PE-Cy7 (ISA-3, eBioscience), TIM-3-APC (344823, R&D Systems). Cells were next fixed and permeabilized with the FoxP3/Ki-67 Fixation/Permeabilization Concentrate and Diluent (eBioscience), and subsequently stained intracellularly with CD3-BV570 (UCHT1, Biolegend), Ki-67-AlexaFluor700 (B56, BD Biosciences), FoxP3-eFluor450 (PCH101, eBioscience), and CTLA-4-PerCP-eFluor710 (14D3, eBioscience). Stained cells were acquired on a BD Biosciences LSRFortessa and analyzed using FlowJo software (FlowJo, LLC). The analysis was performed at the MSKCC immune phenotyping core lab, New York.

T cell Clonal Analysis by TCR Deep Sequence Analysis

TCR deep sequencing was performed at Adaptive biotechnology, Seattle, WA.

Clonal quantification was performed as previously described. DNA was isolated from EDTA blood samples using a DNeasy kit (Qiagen). For further analysis, expanding and contracting clones were defined as T cell clones with more than 10-fold change between the pre-treatment and the on-treatment samples.

QUANTIFICATION AND STATISTICAL ANALYSIS

Significance of experimental results was carried out using paired and unpaired t-test, as appropriate. Correlation analysis between biomarkers and clinical responses were carried out

using linear regression analysis. All statistical analysis was performed using graphpad prism 7.04 software or earlier versions. P values are indicated at each figure.

ADDITIONAL RESOURCES

Patients were enrolled in a phase 1 multibasket clinical trial, registered at www.clinicaltrials.gov (NCT02009449).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Pegilodecakin induces systemic and intratumoral $CD8^+$ T cell activation in patients
- $PD\text{-}1^+$ $Lag3^+$ $CD8^+$ T cells and previously undetected T cell clones are expanded
- IFN-γ, IL-18, GranzymeB, and FasL are elevated across tumor types
- The magnitude of systemic immune activation correlates with tumor response

Significance

Tumor immunity in cancer patients is limited by low prevalence of anti-tumor, immune checkpoint-positive $CD8^+$ T cells and their exhaustion. Conversely, immune therapies are limited by the induction of autoimmunity. Despite its anti-inflammatory nature, PEGylated interleukin-10 (pegilodecakin) enhanced the activity of $CD8^+$ T cells and led to objective tumor response in patients, without induction of autoimmunity. Pegilodecakin systemically induced Th1 cytokines, while moderately reducing Th17 cytokines. Without a change in total lymphocytes, pegilodecakin led to the expansion of previously rare immune checkpoint-positive $CD8^+$ T cells to become a sizable fraction of the T cell repertoire. These data may explain the observed tumor responses in the absence of autoimmunity and introduces a therapeutic mechanism for cancer immunotherapy.

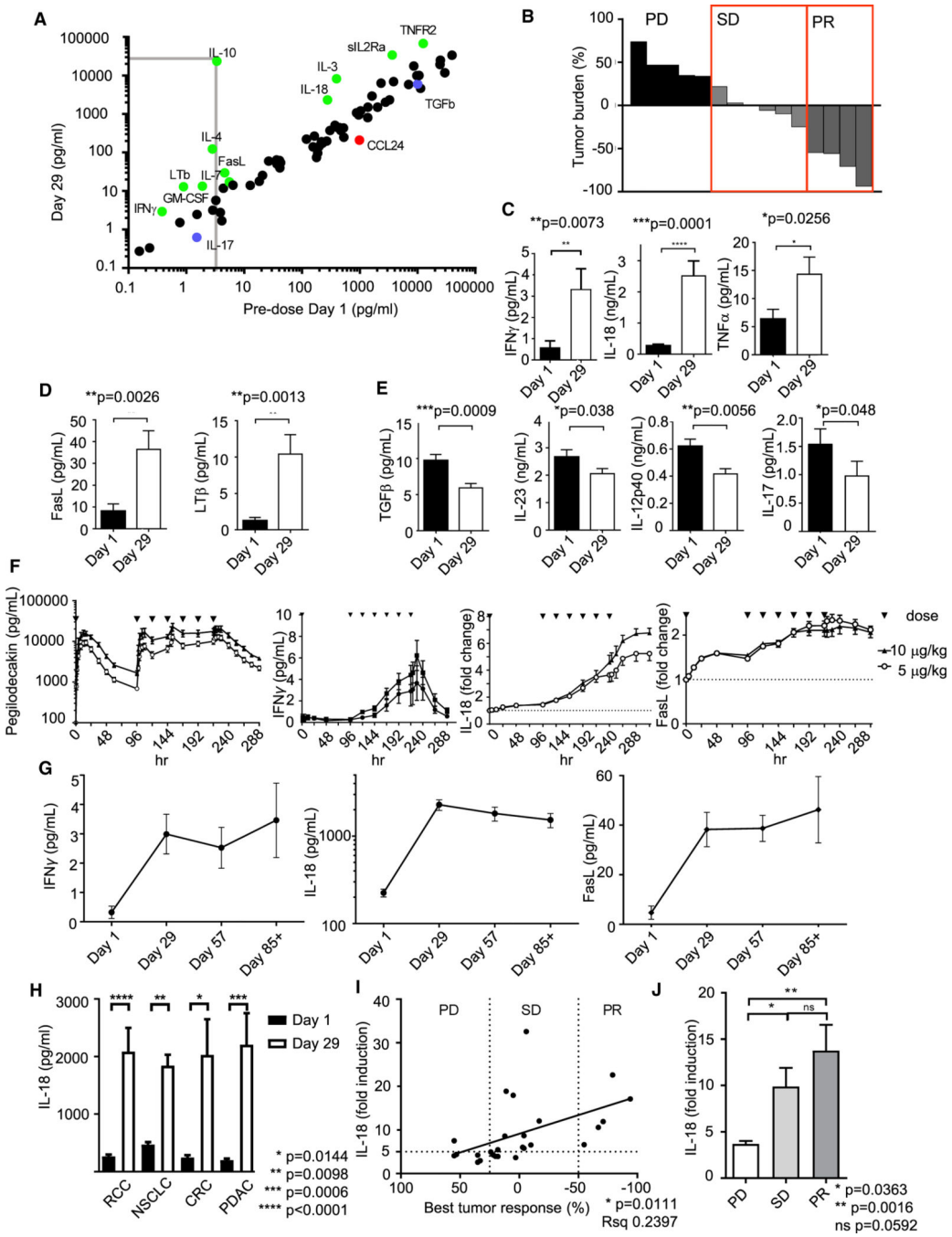

Figure 1. Immune Activation in the Serum on Pegilodecakin Monotherapy Correlates with Objective Response

(A) Comparison of the average concentration of 83 cytokines and proteins in the serum of cancer patients prior to and on pegilodecakin (20 µg/kg for 28 days; n = 30). Green, increased more than 3-fold; black, not changed; red, reduced more than 3-fold; blue, Th17. (B) Waterfall plot for the best tumor response in RCC patients on pegilodecakin monotherapy (n = 15) (PD, progressive disease; SD, stable disease; PR, partial response).

(C–E) Th1 cytokines (IFN-γ, IL-18, and TNF-α) (C), FasL and lymphotoxin b (D), and TGF-β, and Th17 cytokines (IL-23, IL-12p40, and IL-17) (E) in the serum of RCC patients (predose day 1 and day 29, n = 16).

(F) Thirteen-day time course of pegilodecakin in normal healthy volunteers (NHV) (n = 12), after single dose (day 1) and multiple dose (day 5–10) of pegilodecakin at indicated doses; serum concentration of pegilodecakin and serum cytokines (IFN-γ [pg/mL], IL-18, and FasL [fold induction over baseline]).

(G) Three-month time course of daily pegilodecakin (20 μg/kg) in RCC patients (n = 6) (serum cytokines [IL-18, IFN-γ, and FasL]).

(H) IL-18 induction in RCC (n = 16), non-squamous cell lung cancer (NSCLC) (n = 2), CRC (n = 6), and pancreatic cancer (PDAC) (n = 7) patients.

(I) Correlation between best tumor response and IL-18 induction (fold over baseline; Pearson correlation, two-tailed p).

(J) IL-18 (fold induction over baseline) after 28 days of treatment in RCC patients with progressive disease (n = 5); stable disease (n = 6) or partial response (n = 4). Data are represented as means ± SEM, p values represent results of t test, unless indicated. See also Figure S1 and Table S1.

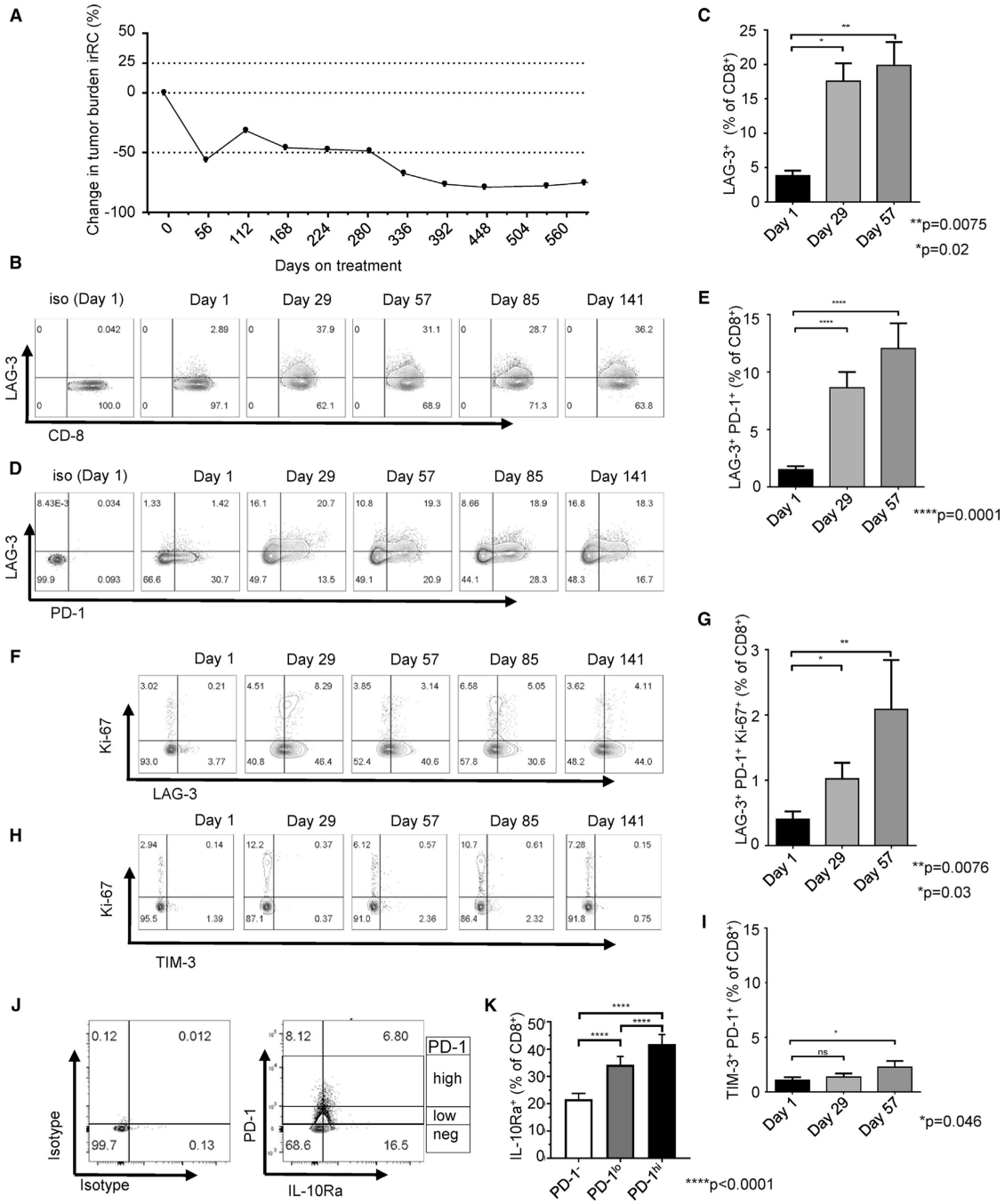

Figure 2. Sustained Expansion of LAG-3$^+$ CD8$^+$ T Cells in the Blood of RCC Patients Treated with Pegilodecakin

(A) Relative change in tumor burden (irRC) of an RCC patient over time on treatment with pegilodecakin (maximal tumor reduction −79%).

(B) LAG-3 on CD8$^+$ T cells in blood of the RCC patient in (A) by fluorescence-activated cell sorting (FACS).

(C) LAG-3$^+$ CD8$^+$ T cells in all patients for pretreatment, after 1 or 2 months of pegilodecakin (n = 22).

(D) LAG-3$^+$ PD-1$^+$ CD8$^+$ T cells in the RCC patient in (A).

(E) LAG-3^+ PD-1^+ $CD8^+$ T cells in all patients (n = 22).
(F) LAG-3^+ PD-1^+ Ki-67^+ $CD8^+$ T cells in patient (A).
(G) LAG-3^+ PD-1^+ Ki-67^+ $CD8^+$ T cells in all patients (n = 22).
(H) Ki-67^+ TIM-3^+ PD-1^+ $CD8^+$ T cells in the patient in (A).
(I) Ki-67^+ TIM-3^+ PD-1^+ $CD8^+$ T cells in all patients (n = 22).
(J and K) IL-10Ra and PD-1 expression on $CD8^+$ T cells from pretreatment PBMCs; FACS analysis of one patient (J) and all patients (K) (n = 22, PD-1 is stratified to negative, low mean fluorescence intensity [MFI] < 1,000 and high MFI > 1,000).
Data are represented as means ± SEM, p values represent results of t test (unless indicated).
See also Figures S2 and S3.

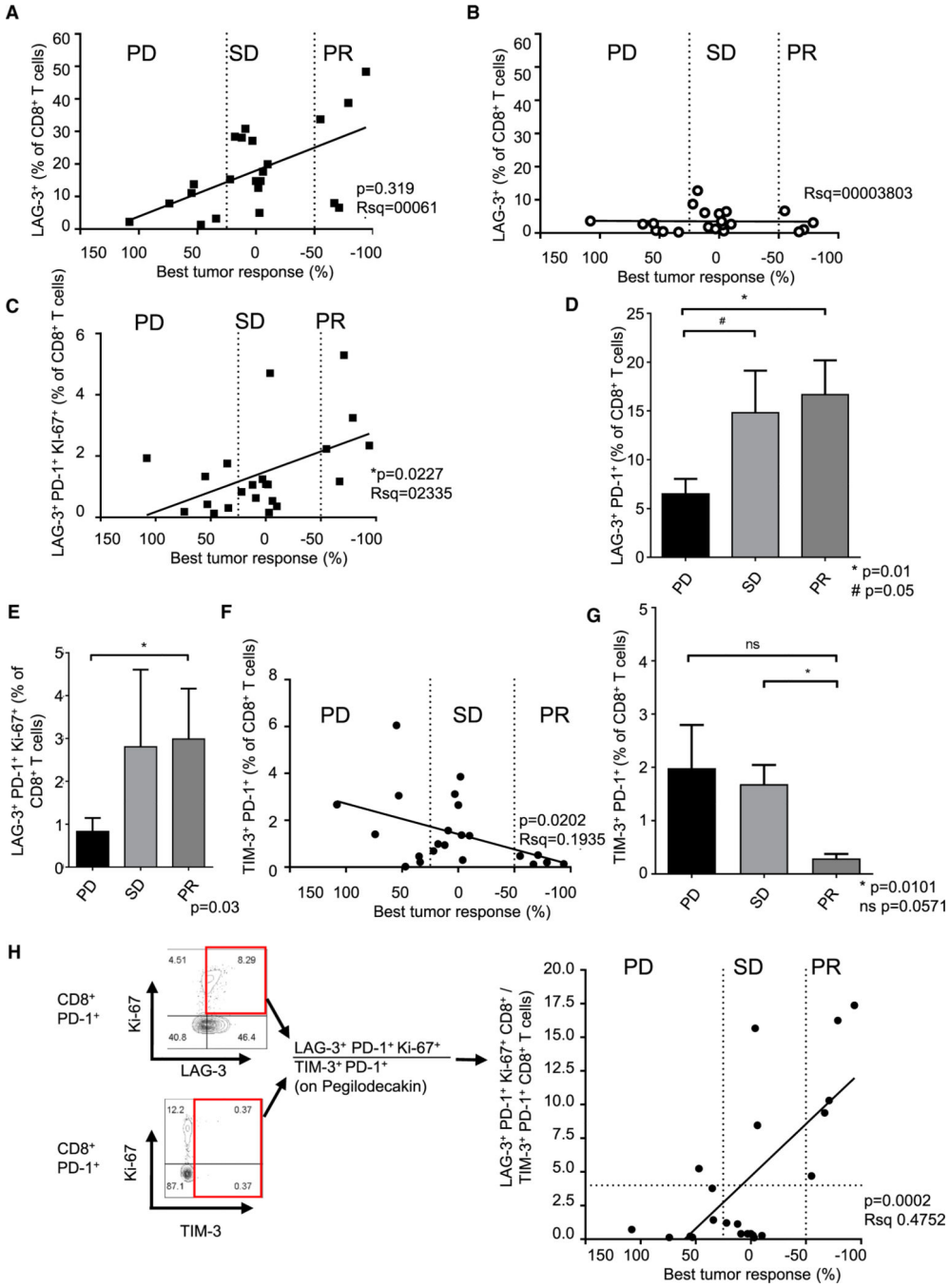

Figure 3. Correlation of CD8$^+$ T Cell Phenotype in the Blood on Pegilodecakin with Tumor Response

(A and B) Correlation of LAG-3$^+$ CD8$^+$ T cells on pegilodecakin (day 57) (A) or before treatment (B) with best tumor response (following irRC criteria, PD ≥ 25% increase; PR ≥ 50% decrease).

(C) Correlation of LAG-3$^+$ PD-1$^+$ Ki67$^+$ CD8$^+$ T cells (day 57) per patient with tumor response (C).

(D and E) Percentage of LAG-3$^+$ PD-1$^+$ CD8$^+$ T cells (day 57) (D) and LAG-3$^+$ PD-1$^+$ Ki-67$^+$ CD8$^+$ T cells (day 57) (E) stratified by tumor response.

(F) Correlation of TIM-3^+ PD-1^+ $CD8^+$ T cells (day 57) with best tumor response.
(G) Percentage of TIM-3^+ PD-1^+ $CD8^+$ T cells (day 57) stratified by tumor response.
(H) Correlation of the best tumor response with the ratio of LAG-3^+ PD-1^+ Ki-67^+ $CD8^+$/ TIM-3^+ PD-1^+ $CD8^+$ T cells (day 57). Data are represented as best tumor response in each individual patient (A–C, F, and H) or means ± SEM (D, E, and G) (n = 22 patients). Spearman correlation was performed for (C, F, and H). A t test was used in (D, E, and G). See Figure S4.

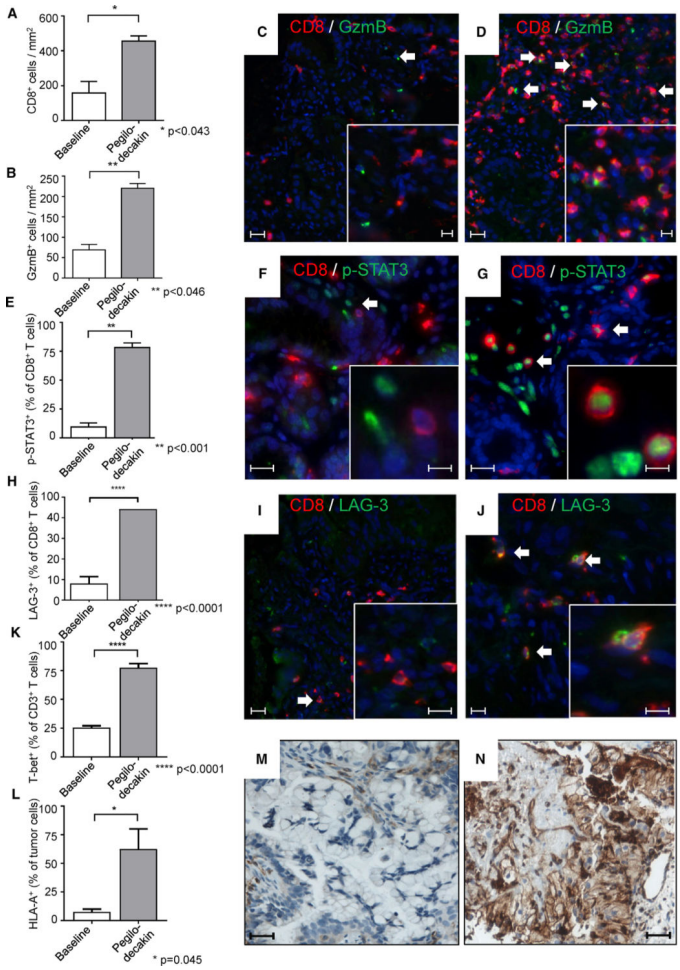

Figure 4. Increased Activation of Intratumoral CD8$^+$ T cells in Patients Treated with Pegilodecakin

(A and B) Quantitation of intratumoral CD8$^+$ T cells (A) and GranzymeB$^+$ (GzmB) CD8$^+$ T cells (B) based on analysis per mm^2 of tumor section at baseline and on treatment with pegilodecakin.

(C and D) Immunofluorescence for CD8 (red) and GranzymeB (GzmB) (green, arrows point to GzmB-positive cells) in tumor sections prior to (C) and on treatment

(D) with pegilodecakin.

(E–G) Quantitation of phospho-STAT3$^+$ CD8$^+$ T cells in relation to all CD8$^+$ T cells (E) and representative tumor section prior to (F) (arrow pointing to CD8$^+$ T cell neighboring P-STAT3$^+$ non-T cells) and on pegilodecakin (G) (arrows to P-STAT3$^+$ CD8$^+$ T cells).

(H–J) Quantitation of LAG-3$^+$ CD8$^+$ T cells in relation to all CD8$^+$ T cells (H) and representative tumor section prior to (I) (arrows to Lag3$^-$ CD8$^+$ T cell) and on pegilodecakin (J) (arrow to Lag3$^+$ CD8$^+$ T cell).

(K) Quantitation of T-bet$^+$ CD8$^+$ T cells in relation to all intratumoral CD8$^+$ T cells.

(L–N) Quantitation of HLA-A$^+$ tumor cells (L) and representative tumor section (lung cancer) prior to (M) and on pegilodecakin (N). At least 10 representative fields were quantified per section.

Data are represented as means ± SEM (n = 4). Scale bar represents 50 μm (C, D, F, G, I, J, M, and N) in lower-magnification and 10 mm (C, D, F, G, I, and J) in high-magnification inserts.

p Values represent the results of t tests.

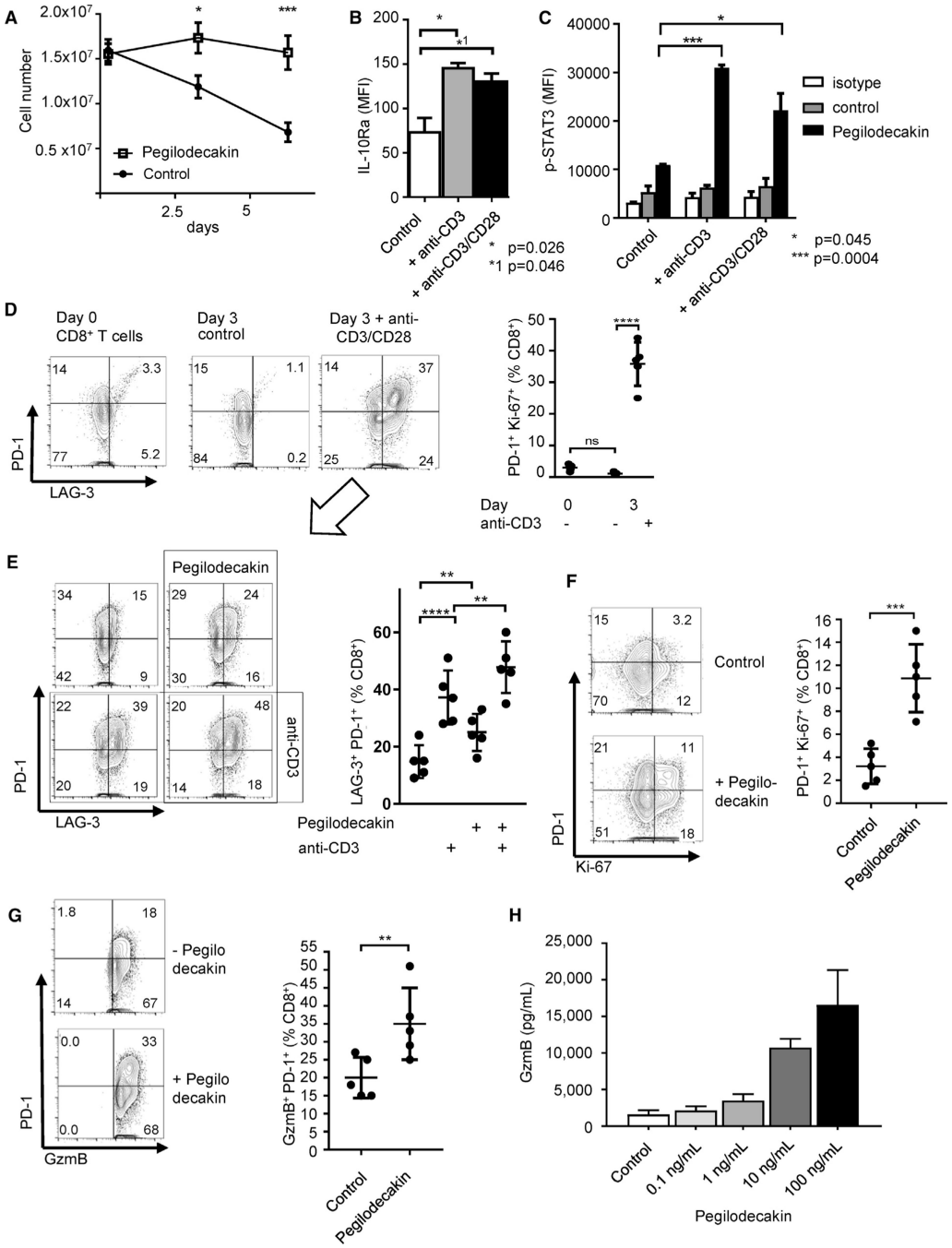

Figure 5. Pegilodecakin Sustains Activation and Proliferation of Human TCR-Activated CD8$^+$ T Cell *In Vitro*

(A) Viability of PBMC-derived CD8$^+$ T cells after activation with anti-CD3/CD28 antibodies (for 3 days) followed by 6 days of pegilodecakin or control treatment.

(B) IL-10 receptor expression (MFI) in PBMC-derived CD8$^+$ T cells (FACS analysis) after anti-CD3 or anti-CD3/CD28 stimulation.

(C) STAT-3 phosphorylation in human CD8$^+$ T cells after anti-CD3 or anti-CD3/anti-CD28 treatment and after pegilodecakin stimulation.

(D) Percentage of PD-1- and LAG-3-expressing human $CD8^+$ T cells after anti-CD3/CD28 stimulation.
(E) Percentage of PD-1- and LAG-3-expressing $CD8^+$ T cells stimulated for 3 days with anti-CD3/CD28 and then rested for 3 days or treated for 3 days with pegilodecakin in the presence or absence of anti-CD3-antibodies as indicated.
(F) Percentage of PD-1- and Ki-67-expressing $CD8^+$ T cells after anti-CD3/CD28 stimulation for 3 days followed by no treatment (control) or pegilodecakin for 3 days.
(G) Percentage of PD-1- and GranzymeB-expressing $CD8^+$ T cells in the presence or absence of pegilodecakin for 3 days, after anti-CD3/CD28 stimulation for 3 days.
(H) GranzymeB in the supernatant of cells cultured as in (G) with increasing concentration of pegilodecakin.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, if not indicated in the panel. All experiments were performed at least three times. Data are represented as means ± SEM of one representative experiment (n = 5 for each experiment). Results were subjected to pairwise t tests. See also Figure S5.

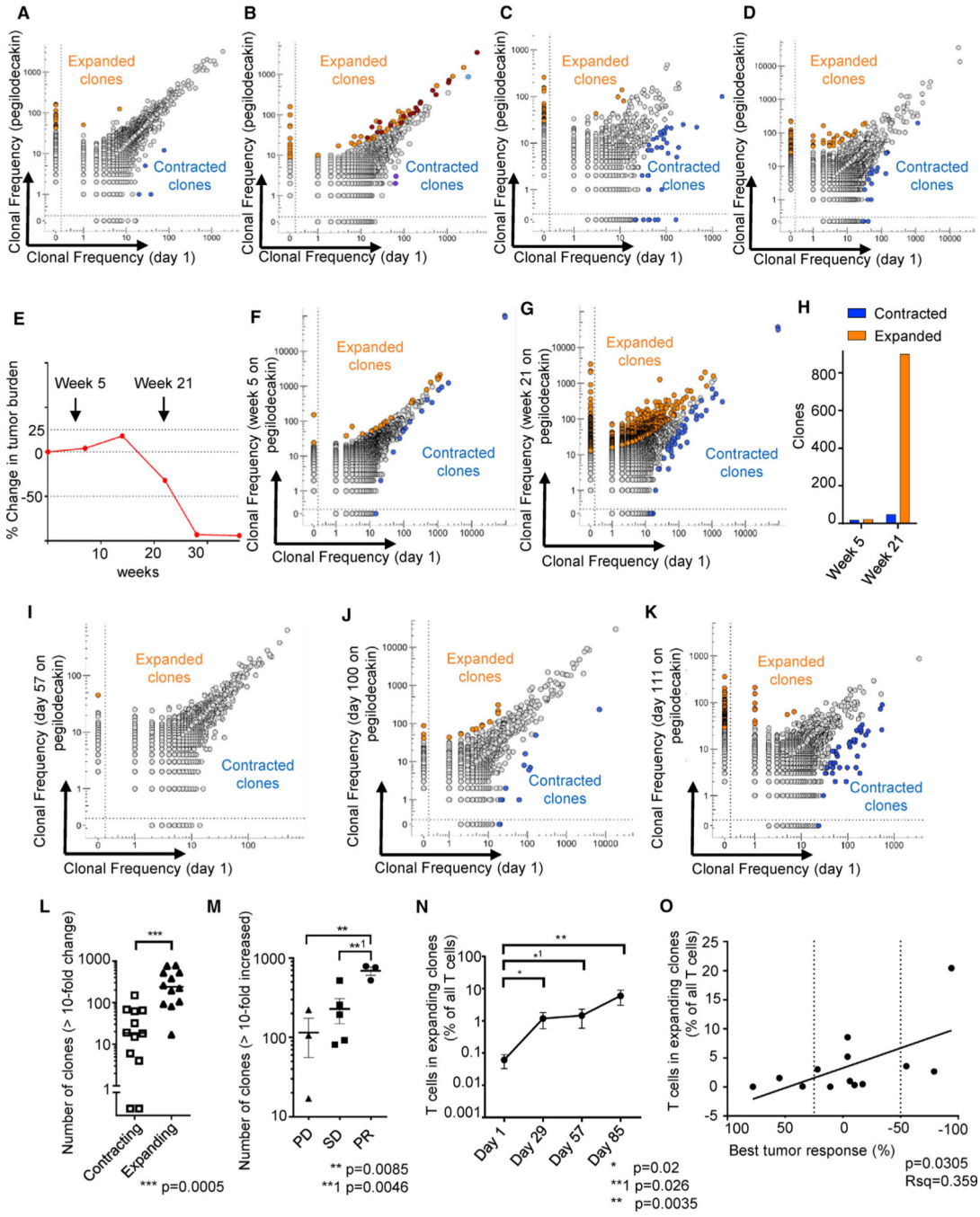

Figure 6. Polyclonal T Cell Expansion of Previously Undetectable T Cells in the Peripheral Blood of Patients

(A–D) Comparison of T cell clonal frequency between pretreatment (x axis) and on treatment (y axis) in the blood of a patient with melanoma (A), colorectal cancer (B), pancreatic cancer (C), and triple-negative breast cancer (D). Significantly expanding clones are colored in yellow or red, contracting clones are colored in blue and purple. Clones not detectable at baseline are shown as x = 0. Clones not detectable on treatment are y = 0. (E) Tumor burden at and after 21 weeks of pegilodecakin monotherapy in an RCC patient with a delayed tumor response. (F–H) The change in T cell clonality in the patient from (E)

at week 5 (F) and at week 21 (G) of treatment. The quantification of expanded and contracted clones at weeks 5 and 21 (H).

(I–K) Comparison of T cell clonal frequency between pretreatment and on treatment (at the indicated day of treatment) in patients with RCC: patient with progressive disease (+35% change [increase] in tumor burden) (I), patient with stable disease (+22%) (J), patient with partial response (−55%) (K).

(L) Comparison of the absolute number of >10× expanding and >10× contracting T cell clones per patient (n = 12).

(M) Total number of expanding T cell clones in the blood of patients based on the objective tumor response of the patient (n = 3 [PD]; n = 6 [SD]; n = 3 [PR]).

(N) Time course of >10× expanding T cell clones on pegilodecakin as a percentage of the total T cell repertoire in the blood per patient (n = 12).

(O) Correlation of the percentage of expanding T cell clones per patient (n = 12) with objective tumor response; “expanding clones” are defined as expanding at least 10-fold during the treatment period, the sum of the frequency of all expanding clones per time point per patient is plotted.

Data in (L–N) are represented as means ± SEM, p values represent results of pairwise t test. Data in (O) represent Spearman correlation (r) and p.

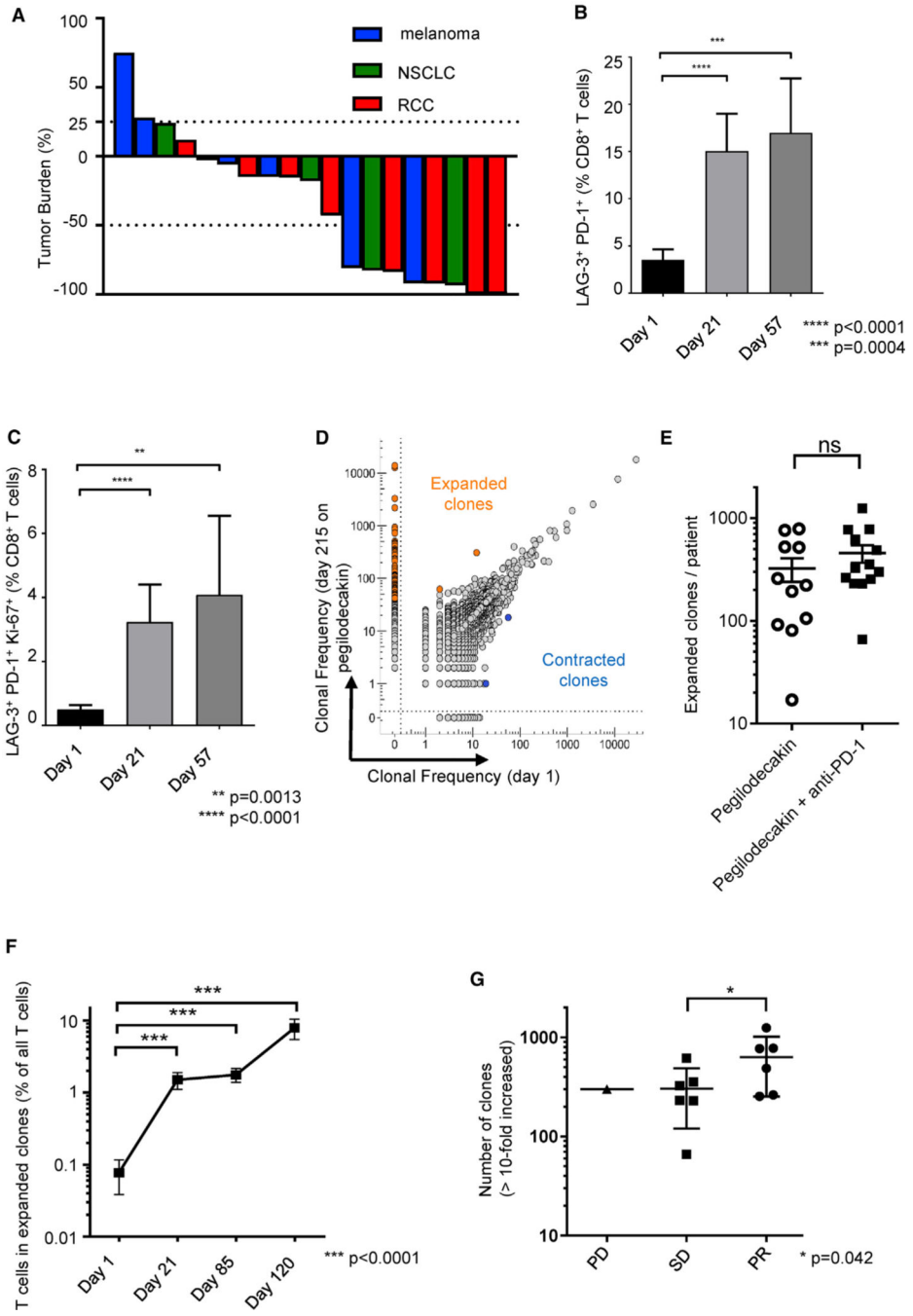

Figure 7. T Cell Responses in the Blood of Patients on Pegilodecakin and Pembrolizumab in Pretreated Cancer Patients

(A) Best response in pretreated cancer patients on pegilodecakin + pembrolizumab (indication as indicated; n = 19 evaluable patients). (B and C) Percentage of peripheral LAG-3$^+$ PD-1$^+$ CD8$^+$ T cells (B) and LAG-3$^+$ PD-1$^+$ Ki-67$^+$ CD8$^+$ T cells (C) from patients on pegilodecakin and pembrolizumab (n = 23) after 21 and 57 days of treatment.

(D) Comparison of T cell clonal frequency between pretreatment (x axis) and on treatment (y axis) in the blood of one RCC patient with a complete response (CR) on combination.

(E) Comparison of >10× expanding clones in patients on pegilodecakin alone (n = 12) or on pegilodecakin and pembrolizumab (n = 13).
(F) >10× expanding T cell clones in patients on pegilodecakin + pembrolizumab (n = 13) as a percentage of all T cell clones in the peripheral blood.
(G) Comparison of the absolute number of expanded T cell clones with best response (n = 1 [PD]; n = 7 [SD]; n = 6 [PR]). All data are represented as means ± SEM, p values represent results of pairwise t test.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CDS	Abcam	ab101500; RRID: AB_10710024
Phospho-STAT3	Cell Signaling Technologies	9138; RRID: AB_331261
LAG-3	Cell Signaling Technologies	15372
GranzymeB	Leica	NCL-L-GRAN-B; RRID: AB_563751
HLA-A	Abcam	ab52922; RRID: AB_881225
Tbet	Abcam	ab91109; RRID: AB_2050371
CD3	Abcam	ab16669; RRID: AB_443425
CDS	Invitrogen	Q10009; RRID: AB_2556437
CD4	Invitrogen	Q10007, RRID: AB_11180600
PD-1	BD Biosciences	560908; RRID: AB_2033990
LAG-3	Enzo	ALX-804-806F-C100
ICOS	eBioscience	25-9948-42; RRID: AB_1518754
TIM-3	R&D Systems	FAB2365A; RRID: AB_1964725
FoxP3	eBioscience	48-4776-42; RRID: AB_1834364
Ki-67	BD Biosciences	561277; RRID: AB_10611571
CD3	Biolegend	300435; RRID: AB_10898117
CTLA4	eBioscience	46-1529-42; RRID: AB_2573718
Phospho-Stat3	Biolegend	13A3-1; RRID: AB_2616951
IL-10Ra	Biolegend	308813; RRID: AB_2565630
IL-10Rb	R&D Systems	FAB774A; RRID: AB_10717538
IL-1b	R&D Systems	DLB50
IL-6	R&D Systems	D6050
Biological Samples		
EDTA blood	Patient derived	NA
Serum	Patient derived	NA
PBMC	Patient derived	NA
Chemicals, Peptides, and Recombinant Proteins		
Human Interleukin 10	R&D Systems	217-IL-010
Critical Commercial Assays		
FasL	Myriad - RBM	Discovery Map 3.0
LTβ	Myriad - RBM	Discovery Map 3.0
TGFβ	Myriad - RBM	Discovery Map 3.0
IL-23	Myriad - RBM	Discovery Map 3.0
IL-12p40	Myriad - RBM	Discovery Map 3.0
IL-17	Myriad - RBM	Discovery Map 3.0
IL-1	Myriad - RBM	Discovery Map 3.0
IFNγ	Myriad - RBM	Discovery Map 3.0

REAGENT or RESOURCE	SOURCE	IDENTIFIER
IL-18	Myriad - RBM	Discovery Map 3.0
IL-4	Myriad - RBM	Discovery Map 3.0
IL-7	Myriad - RBM	Discovery Map 3.0
TNFa	Myriad - RBM	Discovery Map 3.0
GranzymeB	Myriad - RBM	Discovery Map 3.0
FasL	Aushon	http://www.aushon.com/14000083-L-Cira-Multiplex.pdf
IFNg	Aushon	http://www.aushon.com/140–0083-L-Cira-Multiplex.pdf
IL-18	MSD	F213X
GranzymeB	Aushon	http://www.aushon.com/140–0083-L-Cira-Multiplex.pdf
Immunoseq	Adaptive Biotechnologies	https://www.adaptivebiotech.com/immunoseq
Luminex assays	Myriad RBM	Discovery Map 3.0
Experimental Models: Organisms/Strains		
C57 B/6	Jackson Lab	000664
Software and Algorithms		
Prism 7.4	Graphpad	www.graphpad.com
Flow Jo 8.7	FlowJo	www.flowjo.com