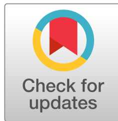

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CD57 identifies T cells with functional senescence before terminal differentiation and relative telomere shortening in patients with activated PI3 kinase delta syndrome

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ABSTRACT

Premature T cell immunosenescence with $CD57^{+}CD8^{+}$ T cell accumulation has been linked to immunodeficiency and autoimmunity in primary immunodeficiencies including activated PI3 kinase delta syndrome (APDS). To address whether CD57 marks the typical senescent T cell population seen in adult individuals or identifies a distinct population in APDS, we compared $CD57^{+}CD8^{+}$ T cells from mostly pediatric APDS patients to those of healthy adults with similarly prominent senescent T cells. $CD57^{+}CD8^{+}$ T cells from APDS patients were less differentiated with more $CD27^{+}CD28^{+}$ effector memory cells showing increased PD1 and Eomesodermin expression. In addition, transition of naïve to $CD57^{+}CD8^{+}$ T cells was not associated with the characteristic telomere shortening. Nevertheless, they showed the increased Interferon-gamma secretion, enhanced degranulation and reduced *in vitro* proliferation typical of senescent $CD57^{+}CD8^{+}$ T cells. Thus, hyperactive PI3 kinase signaling favors premature accumulation of a $CD57^{+}CD8^{+}$ T cell population, which shows most functional features of typical senescent T cells, but is different in terms of differentiation and relative telomere shortening. Initial observations indicate that this specific differentiation state may offer the opportunity to revert premature T cell

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immunosenescence and its potential contribution to inflammation and immunodeficiency in APDS.

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INTRODUCTION

Premature immunosenescence, involving T cells in particular, has recently been linked to the development of infection susceptibility and autoimmunity in patients with primary immunodeficiencies such as TPP2 deficiency and activated PI3 kinase delta syndrome (APDS)[1-3]. However, the concept of T cell “immunosenescence” remains poorly defined. The term “replicative senescence” describes a state in which cells, after having proliferated previously, are unable to further divide[4,5]. Originally based on observations in fibroblasts, this process is due to erosion of telomeres and therefore also called “telomere-dependent senescence”. Senescence can also occur when DNA is damaged by telomere-independent mechanisms such as reactive oxygen species or ionizing radiation, chromatin perturbation or genomic instability due to oncogene activation, called “stress induced senescence”[4,6,7]. T cell immunosenescence has mainly been associated with telomere-dependent replicative senescence of cells with a characteristic phenotypic and functional profile linked to expression of the marker CD57[8].

The terminally sulfated carbohydrate epitope CD57 is expressed on $CD8^+$ T cells that have shortened telomeres and low amounts of T cell receptor excision circles (TRECs)[8]. In adult individuals, its expression is typically coupled to a phenotype of late to terminal differentiation and upregulation of inhibitory receptors. $CD57^+CD8^+$ T cells show low IL-2 production but potent cytotoxicity and pronounced IFN-γ secretion[8,9]. $CD57^+CD8^+$ T cells are low in children, but accumulate with age and in individuals with persistent viral infections, particularly CMV and HIV[10,11]. CD57 expression may thus reflect extensive cell divisions in response to persisting antigen. The potent effector functions of $CD57^+CD8^+$ T cells have

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been linked to inflammatory phenotypes associated with age and persistent viral infection[12,13].

The observed premature accumulation of CD57$^+$CD8$^+$ T cells in APDS patients could thus be linked to their immunodeficiency and inflammatory manifestations. APDS patients carry heterozygous gain-of-function mutations in *PIK3CD* (APDS1) or loss-of-function mutations in *PIK3R1* (APDS2) leading to enhanced PI3K and downstream Akt/mTOR signaling[2,14,15]. While Akt/mTOR signaling promotes activation and proliferation, sustained hyperactivity drives differentiation into terminal effector T cells[16,17]. Indeed, patient CD8$^+$ T cells show enhanced effector differentiation with accumulation of CD57$^+$CD8$^+$ T cells which poorly proliferate[2]. Data addressing effector functions are more controversial. While enhanced IFN-γ production was observed after PMA/ionomycin or CD3 crosslinking of T cell blasts in one study[2], another study reported that secretion of all cytokines measured including IFN-γ and IL-2 was reduced to nearly absent in anti-CD3/28 activated patient lymphocytes[1]. Increased cytokine levels including IFN-γ were also found in the serum of APDS patients[18]. CD8$^+$ T cell degranulation was enhanced in one study and associated with normal cytotoxicity in a redirected lysis assay[2]. Shortened telomeres have been observed in lymphocytes of one APDS2 patient[15]. An increased expression of inhibitory receptors such as PD1 and KLRG1 was noted by several groups[2,18,19], raising the question whether senescence or exhaustion best characterizes the status of these cells[19].

We wanted to contribute to this discussion by focusing on the analysis on the expanded population of senescent CD57$^+$CD8$^+$ T cells. We addressed the question to which extent the premature senescent state marked by CD57 expression in young APDS patients reflects the typical senescent state of adult healthy donors. Understanding similarities and differences may offer opportunities for intervention. We show that CD57$^+$CD8$^+$ T cells in young APDS patients and adult controls share strong IFN-γ secretion and degranulation combined with *in vitro* proliferation impairment as functional features of senescence. However, these features

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occur at an earlier differentiation state and are not stringently associated with a terminal differentiation marker profile and with telomere shortening in the transition from a naïve to a CD57$^+$ stage. This may explain the described effects of P3Kdelta inhibition or rapamycin on the APDS CD57$^+$ T cell population that we could confirm in this study.

RESULTS

Early accumulation of CD57$^+$CD8$^+$ T-cells in APDS patients

We analyzed CD57$^+$CD8$^+$ T cells of 6 pediatric and 1 adult APDS patient presenting with recurrent sinopulmonary infections and lymphoproliferation aged 6 to 41 (median 15) years (Supplementary table 1). At the time of investigation, none of the patients was under immunosuppressive therapy including treatment with rapamycin. All patients had percentages of CD57$^+$CD8$^+$ T cells far above the age-related normal range (median 61%; mean: 58%; range 37-70%). As a control cohort we selected 10 out of 30 healthy adult donors who had similar proportions of CD57$^+$CD8$^+$ T cells (median 59%; mean: 60%; range 40-85%) (Figure 1a). The median age of controls was 47 (range: 33-63) years. As a second control group of similar age as the patients, we selected 15 of 30 healthy individuals < 25 years (median age 20 (range: 2-25) years) with >2% CD57$^+$CD8$^+$ T cells (median 16%; range: 2-32%) for some of the analyses (Figure 1a).

Since persistent viral infections influence the frequency of CD57$^+$CD8$^+$ T-cells [10,11], we screened patients and adult controls for CMV and EBV specific antibodies and performed PCR in patients receiving immunoglobulins and/or lacking specific antibodies. Two patients were seropositive for EBV and CMV and one patient was positive for EBV and CMV by PCR (marked in all figures as “virus+”). The other 4 patients were sero- and/or PCR negative for both viruses including the patient displaying the highest percentage of CD57$^+$CD8$^+$ T cells (Supplementary table 1, P4). These results suggest that these persistent viral infections

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were not the main driver for the abundance of $CD57^+CD8^+$ T cells in our APDS patients. Among the 10 older controls, 9 were seropositive for CMV and EBV and one was EBV^+/CMV^-.

$CD57^+CD8^+$ T cells from APDS patients are less advanced in their differentiation than $CD57^+CD8^+$ T cells from older controls

Several previous studies have shown advanced differentiation of bulk and virus-specific APDS $CD8^+$ T cells[1,2,14,15,18,20,21]. We wanted to focus this analysis on senescent $CD57^+CD8^+$ T cells, which also show advanced differentiation in healthy adults. Unexpectedly, $CD57^+CD8^+$ T cells from young APDS patients contained a lower proportion of TEMRA cells and a higher percentage of $CD45RA^-CCR7^-$ EM cells compared to adult controls (Figure 1b), although these differences did not reach statistical significance. Consistently, the proportion of $CD57^+CD8^+$ cells which had downregulated CD27 and CD28, a typical feature of TEMRA, was lower in patients (Figure 1c). Typical $CD57^+CD8^+$ T cells are characterized by high expression of the inhibitory receptors 2B4 and KLRG1, but down-regulate expression of PD1. While there was no difference in the expression of 2B4 and KLRG1 (Figure 1d,e), PD1 expression was higher among $CD57^+CD8^+$ T cells of APDS patients (Figure 1g), consistent with their less advanced differentiation. Moreover, $CD57^+CD8^+$ T cells of APDS patients showed comparably high expression of the fractalkine receptor CX3CR1 which has been associated with effector functions[22] (Supplementary figure 1). In $CD57^+CD8^+$ TEMRA cells, the transcription factor Eomes is typically downregulated, while Tbet is highly expressed[23,24]. Consistent with their less advanced differentiation, $CD57^+CD8^+$ T cells of APDS patients showed higher expression of Eomes and lower expression of Tbet compared to controls (Figure 2a,b). Differentation marker and transcription factor expression were similar in EBV/CMV positive versus negative patients. To differentiate between age-related and disease-related differences in the $CD57^+CD8^+$ T cell phenotype, we also studied 15 age-matched individuals with a much lower percentage of $CD57^+CD8^+$ T cells. This analysis

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revealed previously unappreciated age differences in the CD57$^+$CD8$^+$ T cell phenotype with a predominance of EM differentiation and preserved CD27 and CD28 expression in this younger age group (Supplementary figure 2a,b).

We then determined the extent of clonal expansions among CD57$^+$CD8$^+$ T cells by analyzing the T cell receptor Vβ repertoire of the 24 most common TCR Vß chains. In adult controls, we found 1-6 expanded populations, while the remaining populations with Vβ receptors, that individually represented less than 5% of the total CD8$^+$ T cell pool added up to a mean of 37,25% (range: 9,73%-65,99%) of all CD8$^+$ T cells. Clonal expansions among CD57$^+$CD8$^+$ T cells of the 4 analyzed APDS patients were less pronounced. Here, the populations individually representing less than 5% added up to a mean of 55,61 (range: 41,94% - 69,78%) (Supplementary figure 3a,b). These reduced clonal expansions are compatible with the less advanced differentiation state in senescent CD8$^+$ T cells from APDS patients.

CD57$^+$CD8$^+$ T cells of APDS patients show poor proliferative capacity *in vitro*

Senescence is characterized by impaired proliferation i*n vitro*[4,5] and impaired proliferation has been demonstrated in APDS bulk CD8$^+$ T cells[2,14]. We first determined Ki67 expression as an indicator of proliferation *in vivo*. With one exception Ki67 expression was similar in patient and adult control CD57$^+$CD8$^+$ T cells (Figure 3a). We then performed CFSE/VPD dilution assays upon PHA or anti-CD3/28 bead stimulation. We excluded modulation of CD57 during the stimulation culture by sorting CD57$^+$ and CD57$^-$CD8$^+$ T cells and confirming similar CD57 expression before and after the 5-day culture period (Supplementary figure 4). Upon stimulation, the fraction of cells undergoing more than two division cycles was lower among CD57$^+$CD8$^+$ than among CD57$^-$CD8$^+$cells in both APDS patients and in older controls (Figure 3 b,c). However, the proliferative capacity of both populations was significantly lower in APDS patients. To test whether PI3Kdelta or mTOR inhibition could improve proliferation, we stimulated PBMC in the absence or presence of different

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concentrations of a PI3Kdelta inhibitor or the mTOR inhibitor rapamycin. The inhibitors did not improve proliferation *in vitro* (Supplementary figure 5).

Transition of naive to $CD57^{+}CD8^{+}$ T cells in APDS patients is not associated with the same telomere shortening as in controls

CD57 is considered a marker of replicative senescence associated with telomere shortening[8] and short telomeres in bulk lymphocytes have been demonstrated in a single APDS patient[15]. We analyzed telomere lengths in different T cell populations of the same individual by using Q-FISH analysis of sorted $CD57^{+}$ and $CD57^{-}CD45RA^{+}$ naïve $CD8^{+}$ T cells (Figure 4 a,b). We found that telomeres in $CD57^{+}CD8^{+}$ T cells of adult control donors were shortened by -2.40 kilo base (kb) pairs on average when compared to their naïve $CD8^{+}$ T cell counterparts. Interestingly, in APDS patients, average telomere shortening in $CD57^{+}CD8^{+}$ T cells relative to naïve $CD8^{+}$ T cells was much less pronounced (-0.41 kb)(Figure 4c). To test whether this difference in telomere shortening was disease-specific or age-related, we analyzed telomere shortening in our second control group. In these young controls, telomeres in $CD57^{+}CD8^{+}$ T cells were shortened by a mean of -1.24 kilo base pairs (Supplementary figure 6 a,b,c). The data show that telomere shortening in CD57 expressing T cells in healthy donors is a function of age. However, since telomere loss in APDS T cells was even lower than in young controls, it appears that under conditions of activated PI3K signaling, the transition of naïve to highly differentiated $CD57^{+}$ CD8+ T cells is not linked to the same degree of telomere shortening as in healthy donors. Of note, absolute telomere lengths of naïve $CD8^{+}$ T cells and of $CD57^{+}$ CD8+ T cells from our mostly pediatric APDS patients were in the range of adult controls and thus shorter than those of controls of similar age (compare Figure 4b and Supplementary figure 6b). This may indicate that these cells already underwent increased cell divisions in an apparently naïve state.

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CD57^{+}CD8^{+} T cells of APDS patients exhibit potent effector functions

Data concerning cytokine production and degranulation of bulk CD8^{+} T cells in APDS are conflicting[1,2]. Here, we again focused our analysis on the CD57^{+}CD8^{+} T cell population to understand whether their functional state resembles that of typical senescent T cells. IFN-γ production after PMA/ionomycin stimulation was as strong in CD57^{+}CD8^{+} T cells of APDS patients as in controls (Figure 5a,b). There was also no difference in the fraction of cells producing IFN-γ and IL-2, neither in patients with nor without persistent viral infection (Figure 5c). We also analyzed IFN-γ secretion in 3 patients after short-term stimulation with PMA/Ionomycin or with CytoStim, a more physiological TCR activation reagent, using a short-term flow cytometry based cytokine secretion assay, which circumvents differences in proliferation and survival of APDS and control T cells during longer cultures[1] (Figure 5d). Again, the responses were similar in patients and controls. Moreover, both patient and control CD57^{+}CD8^{+} T cells showed strong degranulation upon stimulation with anti-CD3 labeled P815 target cells (Figure 5e,f) and most of the cells expressed perforin (Figure 5 g,h). Hence, these results functionally characterize CD57^{+}CD8^{+} T cells in APDS patients as potent effector cells with little differences to typical senescent cells.

CD57^{-}CD8^{+} T cells of APDS patients are biased towards effector differentiation

To better relate these observations to previous findings in bulk CD8^{+} T cells, we also compared the phenotype and function of CD57^{-}CD8^{+} T cells between our mostly pediatric APDS patients and adult controls. As expected from the described loss of naive T cells[2,14,15,20,21], we found enhanced EM differentiation with increased KLRG1, 2B4 and PD1 expression among CD57^{-}CD8^{+} T cells (Figure 6b). Eomes expression was significantly increased (Figure 6c). APDS CD57^{-}CD8^{+} T cells were compromised in their proliferative capacity *in vitro* while their increased Ki67 expression pointed to increased activation/proliferation *in vivo* (Figure 6d,e). Moreover, the fractions of CD57^{-}CD8^{+} T cells secreting IFN-γ (Figure 6f) and producing both IL-2 and IFN-γ (Figure 6g) were similar.

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Degranulation was significantly increased in patient cells (Figure 6h). Combining the observations in $CD57^+$ and $CD57^-$ cells, the total $CD8^+$ T cell pool in patients with hyperactive Akt/mTOR signaling had much less phenotypic and functional diversity than in healthy controls and converged towards an effector phenotype.

Rapamycin treatment reduces the fraction of $CD57^+CD8^+$ T cells and attenuates IFN-γ secretion.

It was shown previously that treatment with rapamycin[2] or with the PI3Kd inhibitor leniolisib[18] led to a moderate reduction in the percentage of $CD57^+CD8^+$ T cells and recovery of naïve and central memory cells. We could confirm this finding in two further patients treated with rapamycin and observed that termination of treatment was associated with secondary increase (Figure 7a). We also found that IFN-γ secretion upon short-term stimulation was significantly reduced both in CD57+ and in $CD57^-CD8^+$ T cell populations (Fig. 7b,c), demonstrating that *in vivo* rapamycin therapy attenuated cytokine secretion in patient $CD8^+$ T cells.

DISCUSSION

A number of recent studies have addressed the altered T cell differentiation in activated PI3Kdelta syndrome[2,14,15,18-21]. The resulting picture is complex and likely to be the consequence of increased PI3Kdelta signaling both in resting cells as well as in the context of antigen stimulation, in particular chronic antigen stimulation by persistent viruses. Features of both immunosenescence and exhaustion have been described[2,18,19]. In this study, we have chosen to further analyze the features of senescent $CD8^+$ T cells in APDS by comparing them to typical senescent cells of adult individuals and have used the currently best established marker CD57 to define our population of interest. The main finding of our study was that despite similar functional features, differentiation and transcription profile of

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CD57$^+$CD8$^+$ T cells in younger APDS patients was different from “bona fide” senescent cells in healthy adult individuals. Moreover, many of the features characterizing senescent cells including shortened telomeres were already present among CD57$^-$CD8$^+$ T cells and the transition from a naïve to the CD57$^+$ state involved less telomere shortening in APDS patients than in healthy controls.

When comparing markers of CD8 T cell differentiation, CD57$^+$CD8$^+$ T cells from APDS patients were less advanced than typical senescent cells of older controls, but more advanced than the small population of CD57$^+$CD8$^+$ T cells in younger individuals. This was particularly obvious when looking at costimulatory molecules and inhibitory receptors. Typical senescent T cells showed reduced expression of CD27 and CD28 and moderate expression of PD1, whereas the inhibitory receptors KLRG1 and 2B4 were highly expressed[8,9]. In contrast, a larger fraction of APDS CD57$^+$CD8$^+$ T cells had effector memory rather than TEMRA phenotype, retained CD27 and CD28 expression and were triple positive for KLRG1, 2B4 and PD1. Expression of multiple inhibitory receptors is also typical for exhausted cells, in which this is associated with deficiencies in cytokine production and cytotoxicity[25-27]. However, inhibitory receptors are also highly expressed during the early/intermediate stage of effector differentiation without associated functional deficiencies[9,27]. The pattern of marker expression does not therefore allow discrimination between exhaustion and senescence without additional consideration of functional features.

Similar to senescent T cells from adult individuals, CD57$^+$CD8$^+$ T cells from APDS patients showed reduced proliferative capacity *in vitro* when compared to the CD57$^-$CD8$^+$ T cell population. In addition, as shown previously, the overall proliferative response in both populations was lower than in control T cells[2,14]. It is not clear how this relates to the lymphoproliferative phenotype of the patients[2,20,21,28]. We observed augmented Ki67 expression mostly in CD57$^-$CD8$^+$ T cells, consistent with significant mitotic activity *in vivo*.

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The impaired proliferative activity may therefore be due to the particular *in vitro* conditions and not reflect the true proliferative potential *in vivo*. Another characteristic functional feature of senescent cells is their enhanced effector function. The great majority of $CD57^+CD8^+$ T cells in our APDS patients showed enhanced IFN-γ production and secretion as well as enhanced perforin expression and degranulation similar to $CD57^+CD8^+$ T cells from adult controls. Again, such increased effector activity could already be observed in $CD57^-CD8^+$ T cells of these patients. These results are in most aspects consistent with previous observations in bulk T cells. However, while Angulo et al. observed reduced cytokine production in 48h patient PBMC cultures[1], we noted normal IFN-γ secretion after short-term TCR mediated stimulation. This may be due to different proliferation/survival of patient cells in longer cultures. Of note, our results are consistent with the observation of increased IFN-γ levels in patient serum[18]. While impaired *in vitro* proliferation is consistent with current concepts of both $CD8^+$ T cell senescence or exhaustion[27] (albeit by different mechanisms), these two functional states differ in cytokine production and cytotoxicity. Specifically, reduced IL-2 and in later stages IFN-γ production as well as impaired degranulation and cytotoxicity characterize exhausted cells, while replicative senescence, frequently enhanced by CMV infection, is associated with elevated cytokine secretion by $CD57^+CD8^+$ T cells[27,29-31]. Hence, the $CD57^+CD8^+$ population of APDS patients showed functional features consistent with senescence rather than exhaustion. Moreover, similar functional features were already observed in the $CD57^-CD8^+$ T cell compartment.

One of the key signatures attributed to senescent cells is the shortening of telomeres. $CD57^+$ and $CD57^-$ populations from APDS patients and adult controls showed similar differences in proliferative capacity *in vitro*, but telomere shortening relative to naïve T cells was much less pronounced in $CD57^+CD8^+$ T cells of APDS patients. Shortened telomeres have been described previously in total lymphocytes of a single 32 year old patient with APDS2[15] and also in our study, naïve $CD8^+$ T cells from young APDS patients had shortened absolute

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telomere lengths compared to age matched controls (compare Figure 4b and Supplementary figure 6b). However, there was significantly less telomere loss during the transition from the naïve state to the CD57^{+} differentiation state. Telomerase activation by hyperactive Akt/mTOR signaling may have contributed to limit additional telomere shortening [32,33].

Taken together, the nature of the aberrant differentiation of the T cell compartment does not easily align with established concepts. Although we found that the CD57^{+}CD8^{+} T cell population in APDS patients was functionally similar to typical senescent T cells of adult individuals, the transition from the naïve to a CD57^{+} late differentiated state was not accompanied the same extent of advanced differentiation and extensive telomere erosion. Nevertheless, our functional findings indicate, that bulk populations of CD57^{+} and also of CD57^{-}CD8^{+} T cells in APDS are better characterized by concepts of senescence than exhaustion. However, our analysis does not preclude that defined populations of virus-specific T cells in persistently infected patients meet all criteria for exhaustion including impaired antigen-specific effector functions[19].

Which mechanism drives the advanced differentiation in patient T cells? Four APDS patients in our study were CMV negative, including the patient with the highest fraction of CD57^{+} cells, suggesting that in these patients, the underlying gain-of-function mutation rather than persistent viral infections are the key driver of the accumulation of CD57^{+}CD8^{+} T cells. The hyperactive AKT/mTOR pathway in APDS patients leads to a metabolic switch towards aerobic glycolysis[2]. Chang *et al.* showed that aerobic glycolysis is essential for the development of effector functions, particularly for IFN-γ production[34]. However, a sustained shift towards glycolysis prevents long-term memory formation, which requires a switch back to energy generation via oxidative phosphorylation. These metabolic considerations may help explain the preferential effector status of CD8^{+} T cells in APDS, irrespective of their

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CD57 expression. The link between Akt/mTOR activation and an effector phenotype including enhanced cytokine release is compatible with the clinical phenotype of APDS patients that frequently includes inflammatory $CD8^+$ T cell infiltrates.

Why is it important to discriminate between senescence and exhaustion? APDS is characterized by lymphoproliferative and inflammatory manifestations, but also in some patients by the failure to control persistent viruses. The features of premature senescence may be of key relevance for the former, while features of exhaustion may additionally favor the latter. Importantly, signaling pathways leading to senescence or exhaustion and therapeutic concepts for these two functional states probably differ[27]. Thus, PD1 inhibition may help to enhance antiviral effector functions, but may also further stimulate the enhanced effector state of T cells with other specificities favouring lymphoproliferation and inflammation. For long-term treatment of these manifestations, it appears a more rational approach to treat and prevent T cell senescence. The effects of the specific PI3K inhibitor leniolisib[18] and of rapamycin documented by Lucas et al.[2] and in this study regarding the reduction of $CD57^+CD8^+$ T cells, the reduction of the differentiation bias towards effector memory cells as well as the reduction of excessive IFN-γ production are encouraging initial observations in this respect.

METHODS

Patient and control cohorts

We recruited 5 patients with germline mutations in *PIK3CD* and 2 patients with germline mutations in *PIK3R1* for this study. Their clinical characteristics, basic immunological parameters and mutations are documented in the ESID APDS registry[28] and are summarized in Supplementary table 1. As a control group we screened 30 healthy adult donors for the percentage of $CD57^+$ cells among $CD8^+$ T cells. Of these we selected 10 donors with a fraction of $CD57^+CD8^+$ T cells in the range of the patient values for inclusion

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into the extended analysis. Since all of these controls were above 30 years of age, we screened additional 25 healthy individuals between 2 and 25 years of age and selected 15 of them with $CD8^{+}CD57^{+}$ T cells above 2%. Because of the low number of senescent cells that could be sorted from these individuals, analysis was restricted to differentiation markers and telomere lengths in this second, age-matched control group. All subjects or their legal guardians in this study gave informed consent. The study was approved by the ethics committee of the University of Freiburg (protocol number 40/09).

Flow cytometry

Antibodies used for flow cytometry including cell sorting are listed in Supplementary table 2. For Fixation and Permeabilization, Versalyse and Fixative Solution were used (BD Bioscience). Data acquisition was performed with a Gallios or Navios Flow cytometer (Beckman Coulter, Brea, USA). Data were analysed using FlowJo7.2.5 software (Tree Star, Ashland, USA).

Proliferation assays

For the analysis of T cell proliferation, 3-5x10^{5} PBMCs cells were labeled with violet proliferation dye (VPD) (BD Biosciences) or with Carboxyfluorescein succinimidyl ester (CFSE) for 15 min at 37°C. Cells were seeded into a 96 well-flat bottom-plate and left untreated or stimulated with CD3/CD28 Beads (Dynabeads Thermo Scientific), plate bound anti-CD3 antibody 500 ng mL^{-1} (OKT3, eBioscience) plus soluble anti-CD28 antibody 1µg mL^{-1} (CD28.2, BD Biosciences) or PHA at a final concentration of 2.5 µg mL^{-1}. Cells were harvested after 5 days, stained for CD8 and CD57 and analyzed by flow cytometry. To test the effect of defined inhibitors on T cell proliferation, the mTOR inhibitor Rapamycine or the PI3K δ inhibitor IC87114 (both Selleckchem) were added to the cultures at indicated concentrations.

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Telomere length analysis

Sorted $CD57^{-}CD45RA^{+}$ “naïve” $CD8^{+}$T cells and $CD57^{+}$ "senescent" $CD8^{+}$ T cells were fixed in a 3:1 solution of ice-cold methanol:acetic acid. Cell suspensions were dropped onto microscope slides and dried at room temperature (RT) for 2 hours. Q-FISH staining was carried out as described previously[35-38]. Briefly, cells were stained for telomeres with a Cy3-conjugated (C3TA2) 3 PNA telomeric probe (Panagene, Korea). After DNA denaturation and hybridization of 3h at RT, slides were washed, rehydrated and counterstained with DAPI for subsequent mounting using Vectashield medium (Vector Laboratories, USA). All experiments were carried out single blinded and confocal microscopy images captured under identical experimental conditions within one experimental were run to reduce inter-experimental variability. At least 40 interphase nuclei were captured. Telomere fluorescence was acquired using the LSM 710 confocal laser scanning microscope running ZEN 2012 software (Zeiss, Germany). A 63x 1.4 NA oil immersion objective was used for image acquisition. DAPI (laser 405) nm and Cy3 (laser 561 nm) signals were acquired simultaneously into separate channels with constant laser intensity of the DPSS-561 laser. Multitracking mode was used for image acquisition. Three section stacks were acquired with a step size of 1 μm and maximal projections of z-stacks were generated. Definiens Developer software (XD 64, Cell Porta, Definiens GmbH, Germany) was used for quantitative image analysis. Quantified intensity of all detected telomere spots was exported and used for further analysis. Peripheral blood lymphocytes of a control donor and a patient with dyskeratosis congenita with known telomere length (measured in parallel by flowFISH) were used as control cells to transform arbitrary units into absolute telomere length in kilobases (kb).

T cell cytokine ~~productions~~

For the analysis of cytokine production, $5x10^{5}$ PBMC’s were left untreated or stimulated with PMA and ionomycin for 4 hours at 37°C in the presence of Golgi-Plug (BD Biosciences).

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Cells were surface stained for CD3, CD8, and CD57, fixed and permeabilized and intracellularly stained for IL-2 and IFN-γ. To test IFN-γ secretion of T cells, $1x10^6$ PBMC's were rested overnight at 37°C and subsequently left untreated or stimulated with PMA/ ionomycin or CytoStim (Miltenyi) for 1 h. CytoStim causes activation of T cells by binding the T cell receptor (TCR) and crosslinking it to a major histocompatibility complex (MHC) molecule of an antigen-presenting cell. IFN-γ secreting cells were detected using the IFN-γ Secretion Assay (Miltenyi) according to the manufacturer's instructions and quantified by flow cytometry.

Degranulation assay

The assay was performed as previously described[39]: $1\text{-}2x10^6$ PBMCs were rested overnight. $2x10^6$ P815 target cells were pre-incubated with purified anti-CD3 antibodies (clone Okt III) for 15 minutes at RT and washed. A 96 well-V-Bottom plate was prepared with medium, P815 or anti-CD3 coated P815 cells. 1 µl of CD107 PE antibody and $2x10^5$ PBMCs were added to each well. After 3 hours of incubation at 37°C, cells were harvested into FACS tubes and stained for CD57, CD3, CD107 and CD8. After 20 minutes, 500 µl of Optilyse C was added, cells were washed and analysed by flow cytometry.

Statistical analysis

Analyses were performed using PRISM-software (Graphpad software, San Diego, USA). Populations were compared using the Mann-Whitney *U*-test. The paired *t*-test was used for analysis of the telomere data. A p-value < 0.05 was considered statistically significant.

Authorship contributions:

PCD, SA, UW, SF, MSFV and FB performed experiments and/or analyzed data. MRL and KS performed genetic investigations. IF carried out basic immunological investigations. CK, TRL, NC, JR, PS-P, CS, PO and ON provided clinical, laboratory and genetic information.

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SE and AR-E supervised the project. PCD, AR-E and SE wrote the manuscript. All authors commented on/revised the manuscript.

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CONFLICTS OF INTEREST

None of the authors declare conflicts of interest.

Disclosure of Conflicts of Interest: The authors do not report any conflicts of interest.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. CD57$^+$CD8$^+$ T-cells of APDS patients are less advanced in their differentiation than the corresponding cells from adult controls

(a) Percentages of CD57$^+$CD8$^+$ T-cells in APDS patients (n=7, black squares: EBV/CMV+, black triangles: EBV/CMV-) and young (<25 years, n=15) and older (>25 years, n=10) control donors. The normal range is depicted by the grey area with the upper and lower lines showing the 95th and 5th percentile, respectively. **(b)** Representative plots showing the gating of CD57$^+$CD8$^+$ T-cells (left panel). Proportions of Naïve (CCR7$^+$/CD45RA$^+$), Central Memory (CM) (CCR7$^+$/CD45RA$^-$), Effector memory (EM) (CCR7$^-$/CD45RA$^+$) and Terminally differentiated T-cells re-expressing CD45RA (TEMRA) (CCR7$^-$/CD45RA$^+$) among CD57$^+$CD8$^+$ T-cells of patients (Squares; EBV/CMV-, triangles: EBVCMV+) and controls. Right panel: Illustration of the subdivision of T-cells into Naïve, CM, EM and TEMRA. **(c)** Comparative analysis of the proportion of CD27$^-$/CD28$^-$ cells among CD57$^+$CD8$^+$ T-cells in patients and controls. **(d-f)** Comparative analysis of the proportion of 2B4 **(d)**, KLRG1 **(e)** and PD1 **(f)** expressing cells among CD57$^+$CD8$^+$ T-cells in patients and controls. Bars represent median values. T cell phenotyping was performed once per patient or control.

Figure 2. CD57$^+$CD8$^+$ T-cells of APDS patients showed higher Eomes expression than those of adult controls

(a) Representative plots showing Eomes and Tbet expression in CD8$^+$ T-cells. Percentages of Eomes^{high} and Eomes^{low} and Tbet^{high} and Tbet^{low} T-cells among CD57$^+$CD8$^+$ T-cells were determined. **(b)** Comparison of proportions of Eomes^{low} and Tbet^{high} among CD57$^+$CD8$^+$ T-cells of patients (n=7, squares: EBV/CMV-, triangles: EBV/CMV+) versus controls (n=10). Staining were performed once per patient or control.

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Figure 3. Proliferative behaviour of $CD57^+CD8^+$ T-cells of APDS patients

(a) Comparison of the percentages of $Ki67^+$ T-cells among $CD57^+CD8^+$ T-cells of patients (n=7) versus controls (n=10). **(b)** PBMCs of patients and controls were left untreated or stimulated with PHA for five days. Cells were stained for CD8 and CD57 and then dilution of VPD was assessed in $CD57^+CD8^+$ and $CD57^-CD8^+$ cells (dark grey), compared to the baseline VPD expression in unstimulated $CD57^+CD8^+$ and $CD57^-CD8^+$ cells (light grey), **(c)** Proliferative response of $CD57^+CD8^+$ T-cells in patients (n=4, squares: EBV/CMV-, triangles: EBV/CMV+) and controls (n=10) quantified by the percentage of cells that had undergone more than 2 division cycles. Experiments were performed once or twice per patient or control.

Figure 4. Relative telomere shortening is less pronounced in $CD57^+CD8^+$ T cells of APDS patients

(a) Telomeres of sorted $CD57^-CD45RA^+CD8^+$ T cells (grey dots) and of sorted $CD57^+CD8^+$ T cells (black dots) were analysed by qFish in 7 APDS patients and 10 healthy control donors. Telomere length is expressed in kilobases (kb). Black lines represent mean telomere lengths. **(b)** Mean telomere lengths of $CD57^-CD45RA^+$ versus $CD57^+CD8^+$ T cells are compared within the patient and control cohort. **(c)** Telomere shortening is expressed as the difference of the mean telomere length of $CD57^-CD45RA^+$ T cells (set to zero) minus the mean telomere length of $CD57^+CD8^+$ T cells for each patient and control (delta telomere length= delta TL). Bars represent means of telomere shortening for patients (n=7, squares: EBV/CMV-, triangles: EBV/CMV+) versus controls. Experiments were performed once per patient or control.

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Figure 5. $CD57^+CD8^+$ T cells of APDS patients possess potent effector functions

(a) Patient (n=7) and control (n=10) PBMCs were stimulated with PMA/Ionomycin and intracellularly stained for the expression of Interleukin 2 (IL-2) and Interferon-gamma (IFN-γ). **(b)** Summarized results showing the proportions of IL-2 or IFN-γ producing $CD57^+CD8^+$ T cells, respectively. **(c)** Frequency of IL-2/IFN-γ double producing $CD57^+CD8^+$ T cells related to total $CD57^+CD8^+$ and to $CD57^+CD8^+IFN\text{-}\gamma^+$ T cells in APDS patients versus control donors **(d)** Patient (n=3) and control (n=5) PBMCs were stimulated with PMA/Ionomycin or CytoStim and IFN-γ secretion was determined using the IFN-γ secretion assay. Percentages of $IFN\text{-}\gamma^+$ T cells are shown. **(e)** $CD57^+CD8^+$ T cells of patients or controls were left untreated or stimulated with P815 target cells pre-incubated with anti-CD3 antibodies and stained for the expression of the degranulation marker CD107. **(f)** Summarized degranulation results for patient and control $CD57^+CD8^+$ T cells. ΔCD107 was calculated by subtracting the CD107 baseline expression from the CD107 expression after stimulation. **(g)** Representative plots showing perforin expression of $CD57^+CD8^+$ T cells. **(h)** Comparative analysis of perforin expression in $CD57^+CD8^+$ T cells from patients and controls. IFN-γ secretion assays were performed twice in 1 patient, all other experiments were performed once.

Figure 6. $CD57^-CD8^+$ T cells from APDS patients show an advanced effector differentiation

(a) $CD57^-CD8^+$ T cells were analyzed for the expression of CD45RA and CCR7. According to the expression pattern of these markers, $CD57^-CD8^+$ T cells were subdivided into Naïve, Central memory, Effector memory and terminally differentiated T cells re-expressing CD45RA (TEMRA). **(b,c)** Comparative analysis of the percentages of $CD57^-CD8^+$ T cells expressing the inhibitory markers KLRG1, 2B4, PD1 and the transcription factor Eomes. **(d)** Summarized results of the proliferative response of $CD57^-CD8^+$ T cells in APDS patients versus controls *in vitro*. **(e)** Comparative analysis of *in vivo* proliferation assessed by intracellularly staining for Ki67. **(f)** Patient (n=3) and control (n=5) PBMCs were stimulated

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with PMA/Ionomycin or CytoStim and IFN-γ secretion of $CD57^{-}CD8^{+}$ T cells was determined using the IFN-γ secretion Assay. Percentages of IFN-γ^{+} T cells are shown. **(g)** Frequency of IL-2/IFN-γ double producing $CD57^{-}CD8^{+}$ T cells related to total $CD57^{-}CD8^{+}$ and to $CD57^{-}CD8^{+}$IFN-γ^{+} T cells in APDS patients versus control donors. **(h)** Comparative analysis displaying the cytotoxic potential of $CD57^{-}CD8^{+}$ in patients and controls. $CD57^{-}CD8^{+}$ T cells were left untreated or stimulated with P815 target cells pre-incubated with anti-CD3 antibodies and stained for the expression of the degranulation marker CD107. Delta CD107 was calculated by subtracting the CD107 baseline expression from the CD107 expression after stimulation. IFN-γ secretion assays were performed twice in 1 patient, all other experiments were performed once.

Figure 7: Rapamycin reduces the fraction of $CD57^{+}CD8^{+}$ T cells and attenuates IFN-γ secretion.

(a) Percentages of $CD57^{+}CD8^{+}$ T cells of 2 APDS patients before and under Rapamycin. In patient 4 the percentage of $CD57^{+}CD8^{+}$ T cells is also shown 12 months after Rapamycin was stopped. **(b)** Cumulative IFN-γ production in response to PMA/ionomycin (PMA/iono) was analysed by intracellular IFN-γ staining, IFN-γ secretion was investigated in response to PMA/ionomycin or CytoStim using the IFN-γ secretion Assay. The percentages of IFN-γ producing or secreting $CD57^{+}CD8^{+}$ (CD57+) and $CD57^{-}CD8^{+}$ (CD57-) T cells are depicted in the lower right quadrant. Representative plots are shown for one APDS patient both untreated and after 18 months of Rapamycin treatment. **(c)** Summary plots for IFN-γ production and secretion for patients P4 and P1 (both virus negative). IFN-γ secretion assays were performed twice in 1 patient, all other experiments were performed once.

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Figure 1

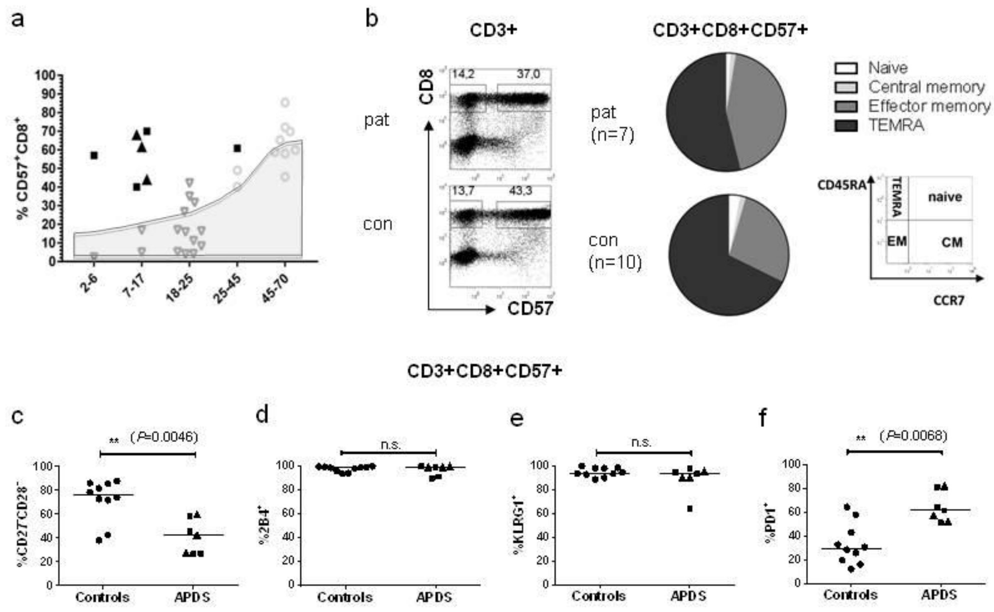

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Figure 2

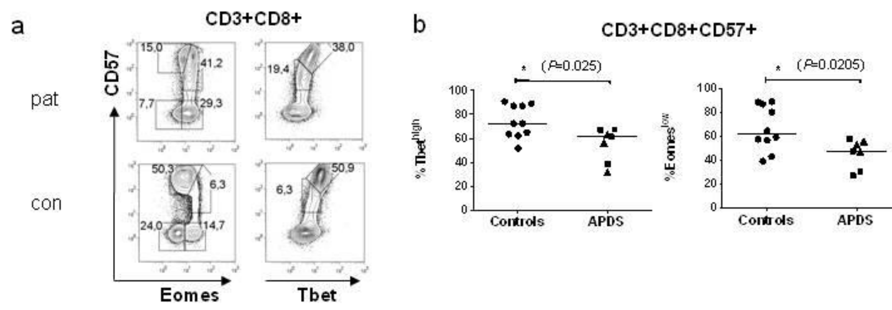

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Figure 3

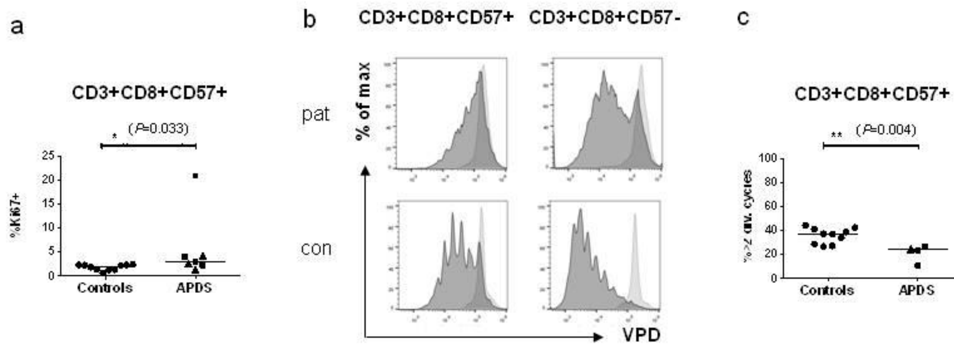

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Figure 4

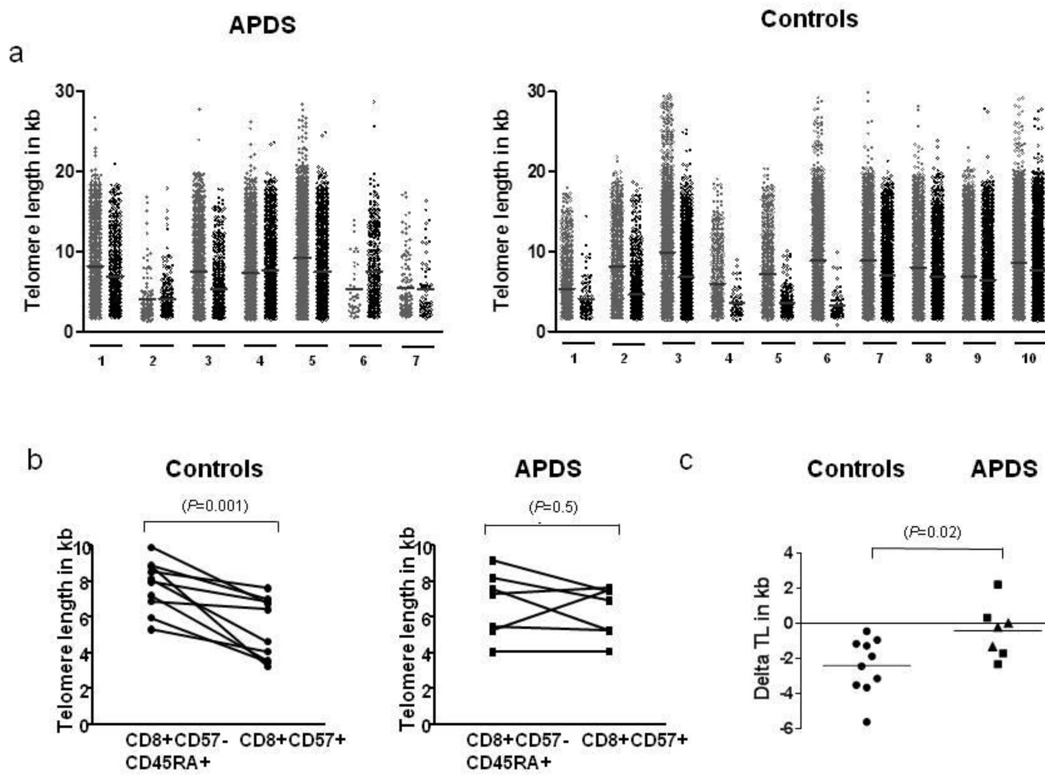

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Figure 5

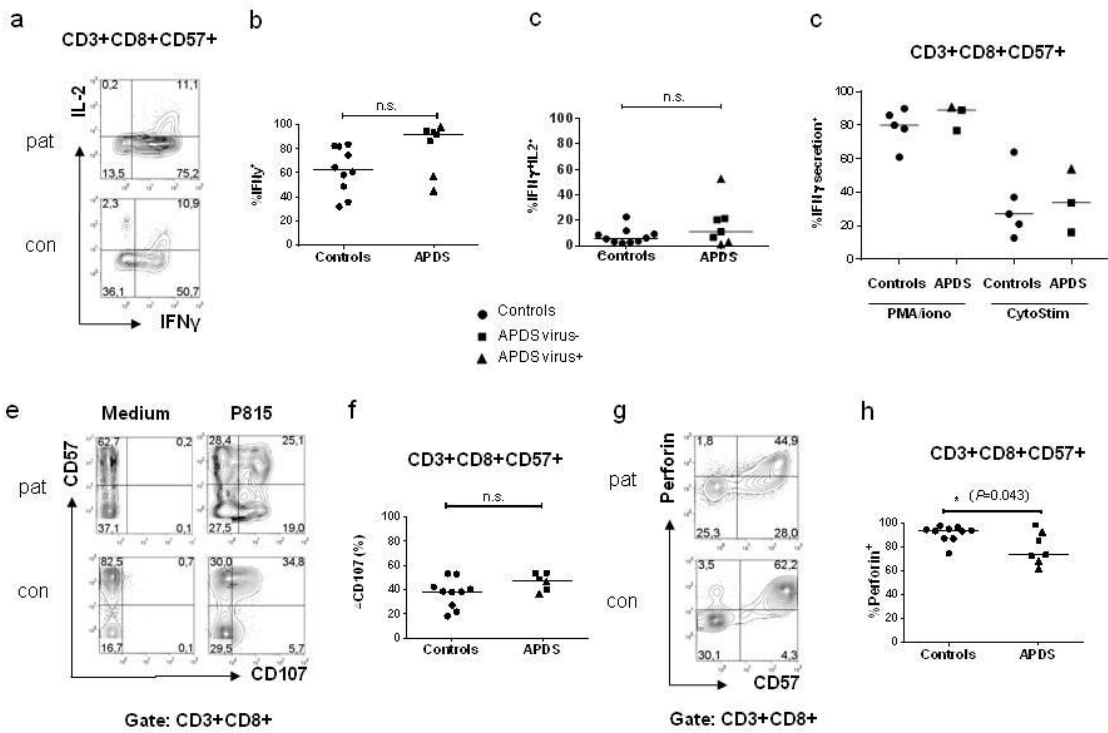

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Figure 6

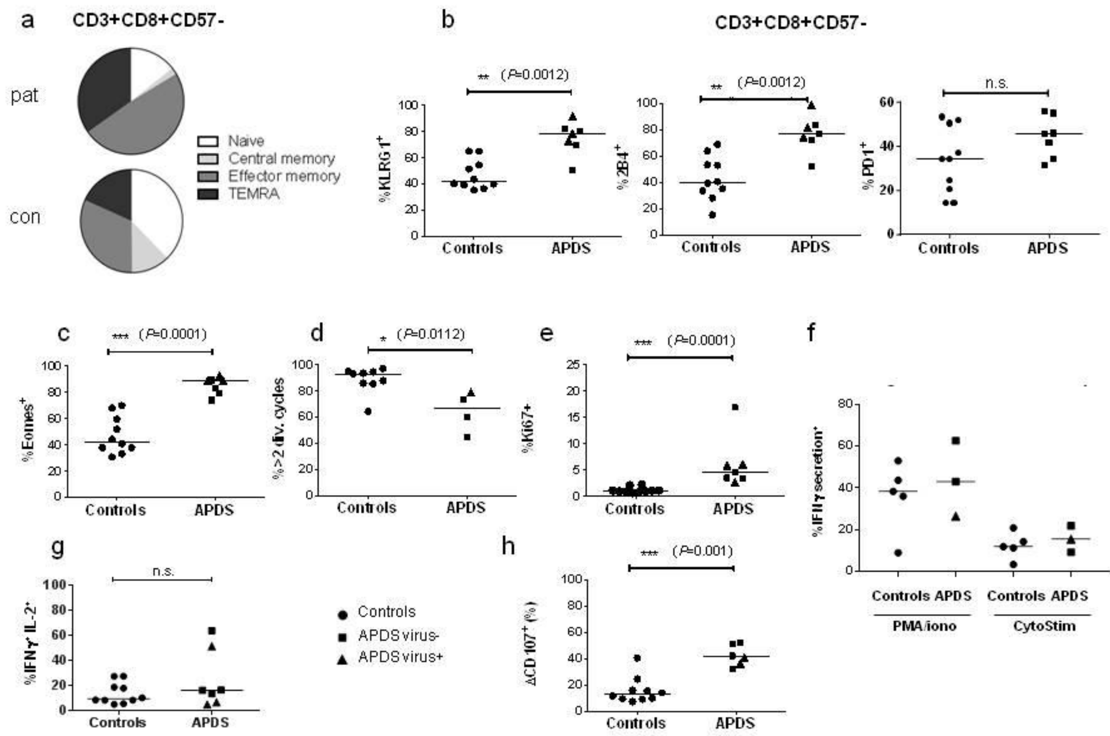

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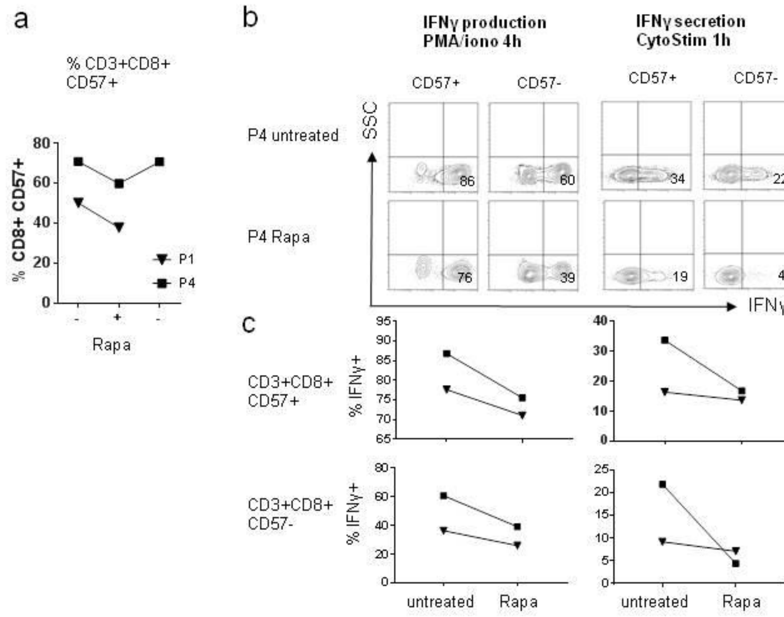
Figure 7



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