

# Dictating Phenotype, Function, and Fate of Human T Cells with Co-Stimulatory Antibodies Presented by Filamentous Immune Cell Mimics

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T cells require a co-stimulatory signal in addition to T-cell receptor (TCR) stimulation to achieve full activation. While most studies focus on the co-stimulatory receptor CD28, little is known about the role of the other co-stimulatory receptors in T-cell signaling. A deeper understanding of how co-stimulatory receptor signaling cooperates with TCR signaling could improve the ability to control T-cell function and benefit the design of T-cell based immunotherapies. Artificial antigen presenting cells (aAPCs) enable tight control over the signals given to T cells. In this study, filamentous polyisocyanopeptide (PIC) polymers (immunofilaments) are used as nanosized aAPCs to study the role of the engagement of six distinct co-stimulatory molecules on human T-cell phenotype, function, and fate in the context of TCR signaling. The immunofilaments highlight important roles for CD28 and CD2 signaling in T-cell priming, proliferation, cytokine production, and multifunctionality. Taken together, this work provides insight into the role of combined TCR and co-stimulation on T-cell phenotype, function, and fate using immunofilaments. Notably, the findings on the roles of co-stimulatory molecule function can be used for the rational design of future cancer immunotherapies.

## 1. Introduction

T cells are important players of the anti-cancer immune response, as they can recognize and specifically kill tumor cells. Several successful immunotherapeutic strategies focus on increasing the number of active tumor antigen-specific T cells in patients. For example, immune checkpoint blockade works

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by blocking co-inhibitory receptors, which can unleash T cells within the tumor.<sup>[1]</sup> In other strategies such as adoptive cell transfer, patients are treated with autologous T cells after ex vivo stimulation and/or genetic modification (e.g., with Chimeric antigen receptors (CARs) or high-affinity T-cell receptors (TCRs)).<sup>[2]</sup> Therefore, understanding, controlling, and balancing T-cell activation is essential due to their vital role in anti-tumor immunity.

Three different signals are important for proper T-cell activation, which are naturally provided by antigen presenting cells (APCs) such as dendritic cells (DCs).<sup>[3,4]</sup> The first signal ensures antigen-specific stimulation of the TCR, which is triggered by specific peptide-MHC complexes on APCs. The second signal comprises co-stimulation, inducing full activation, proliferation and differentiation of T cells and thereby preventing T-cell anergy or T-cell senescence.<sup>[5,6]</sup> Cytokines encompass the third signal, further

shaping the immune response.<sup>[7]</sup> The impact of several cytokines (signal 3) on T-cell differentiation, phenotype, and function has been extensively studied, but research into the importance of signal 2 in shaping T-cell responses has been limited. So far, most studies on signal 2 have focused on triggering the co-stimulatory receptor CD28 on T cells. CD28 is especially important for the priming of naïve T cells, mainly serving as a TCR signal amplifier, inducing IL-2 production and enhancing T-cell survival and proliferation.<sup>[8,9]</sup> However, T cells express a variety of other co-stimulatory molecules that have been studied to a lesser extent for their role in T-cell activation and function in the context of TCR signaling.<sup>[8]</sup> For example, monoclonal antibodies (mAbs) directed against OX-40 and 4-1BB have been tested as immunotherapies and their signaling domains are used in CAR-T cell constructs, where they enhance CAR-T cell persistence, survival, and counteract T-cell exhaustion.<sup>[10–12]</sup> Still, the direct impact of combined TCR and OX-40 or 4-1BB stimulation on T cells remains unclear. Understanding how triggering of different co-stimulatory receptors on T cells cooperate with TCR signaling could improve our ability to control T-cell function, allowing for the development of more potent T cell-based immunotherapies.

To study the impact of co-stimulation on T-cell function and fate in the context of TCR signaling, it is crucial to precisely control the combined presentation of the two signals. Artificial APCs (aAPCs), which mimic DCs in their ability to activate T cells, allow for stringent control over the signals delivered and do not require laborious cell culture practices.<sup>[13]</sup> The majority of synthetic aAPCs developed so far trigger the CD28 co-stimulatory receptor to induce robust T-cell expansion by presenting  $\alpha$ CD28 agonistic antibodies as co-stimulatory signal. A few synthetic aAPCs have been designed to interact with OX-40 or 4-1BB on T cells however, the effect of different co-stimulatory signals on T-cell function and fate in context of signal 1 has not been directly compared.<sup>[14–16]</sup>

Over the past decade, immunofilaments with a polyisocyanopeptide (PIC) polymer backbone have been developed. These 400 nm long aAPCs induce potent T-cell activation via synergistic co-presentation of stimulatory signals.<sup>[17–20]</sup> The PIC-based aAPCs allow multivalent presentation of T-cell activating cues, including  $\alpha$ CD3,  $\alpha$ CD28, IL-2, and IFN $\alpha$  because of their semiflexible character.<sup>[18,20]</sup> However, PICs with other co-stimulatory molecules have not been reported so far. By using this synthetic system with a semiflexible backbone, we can study T-cell responses in a controlled manner. For instance, co-presentation of  $\alpha$ CD3 and  $\alpha$ CD28 on the same immunofilament significantly enhanced T-cell activation compared to PICs presenting either  $\alpha$ CD3 or  $\alpha$ CD28 alone.<sup>[18]</sup> Moreover, the nanosized scale of the immunofilaments not only allows for ex vivo T-cell stimulation but could also support in vivo applications.

In this study, we used PIC-based aAPCs to directly compare the impact of various co-stimulatory antibodies on the phenotype and function of naïve human CD4<sup>+</sup> and CD8<sup>+</sup> T cells. We studied the effect of co-stimulatory immunoglobulin super family receptors CD28, CD2, and SLAM (CD150) and of tumor necrosis factor receptor super family members CD27, OX-40 (CD134), and 4-1BB (CD137). Agonistic mAbs for the co-stimulatory receptors were co-presented with  $\alpha$ CD3 mAbs on PIC-based immunofilaments for polyclonal stimulation of primary human T cells. We profiled the impact of co-stimulation on T-cell activation, differentiation, function, and exhaustion in the context of TCR signaling. This study provides insight into the roles of the different co-stimulatory receptors in T-cell activation and demonstrates the impact of co-stimulation on the T-cell phenotype, function, and fate.

## 2. Results

### 2.1. Potent T-Cell Activation Through Stimulation of Constitutively Expressed Co-Stimulatory Receptors

In order to compare the effect of the different co-stimuli, we prepared immunofilaments bearing  $\alpha$ CD3 (as signal 1) for polyclonal T-cell stimulation and one of the six agonistic mAbs for the co-stimulatory receptors of interest. The mAbs were conjugated to azide-functionalized PIC polymers ( $\approx$ 400 nm in length) using bio-orthogonal click reactions as reported before.<sup>[20,21]</sup> This conjugation resulted in aAPCs that either present  $\alpha$ CD3 alone (**P**) or a combination of  $\alpha$ CD3 with one of the agonistic mAbs against a co-stimulatory receptor (**P- $\alpha$ CD28**, **P- $\alpha$ CD27**, **P- $\alpha$ CD2**, **P- $\alpha$ OX-**

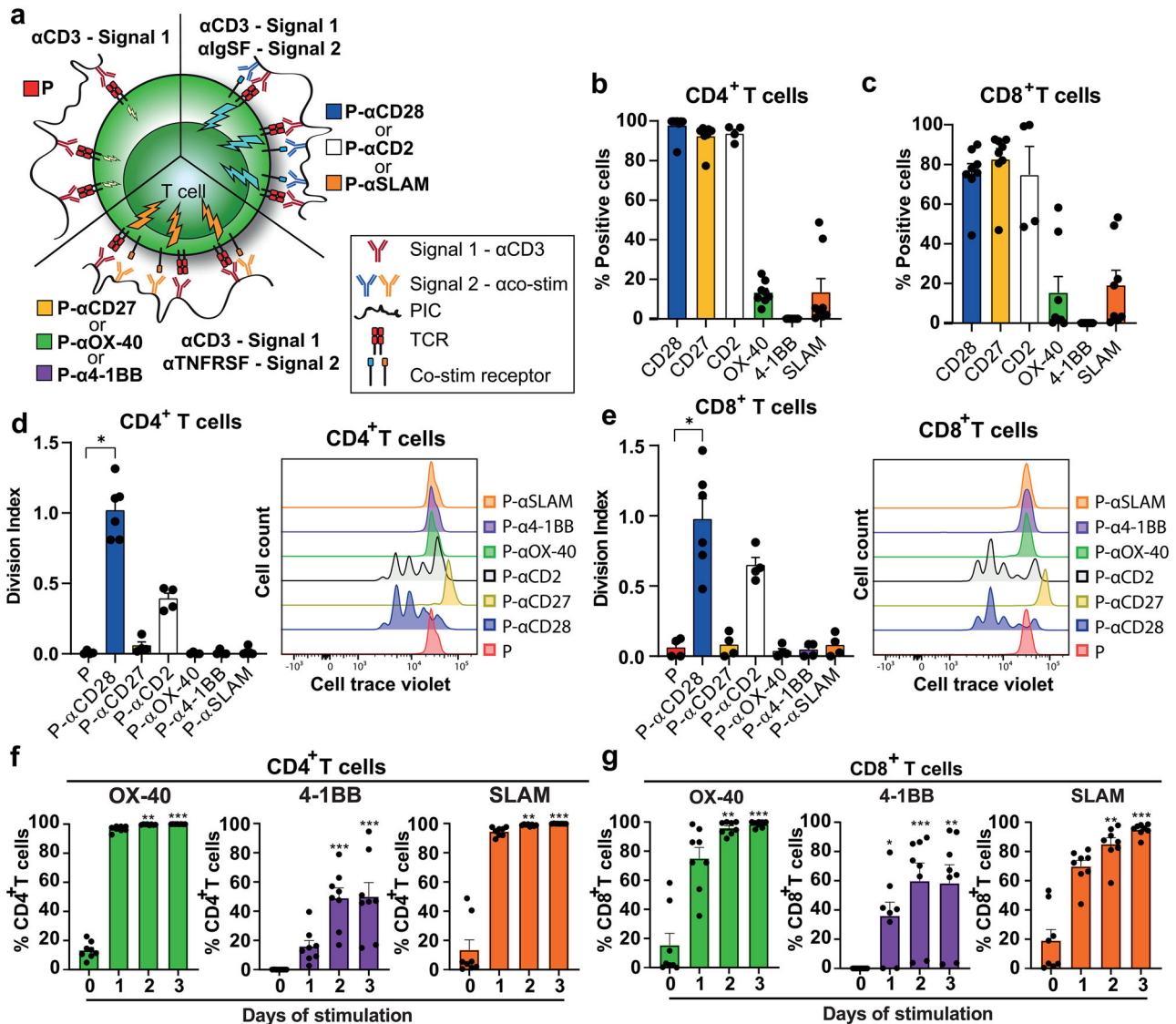
**40**, **P- $\alpha$ 4-1BB**, and **P- $\alpha$ SLAM**) (Figure 1a, Figure S1a, Supporting Information, for  $\alpha$ CD3: $\alpha$ co-stim ratio).

Next, co-stimulatory receptor expression on resting blood-derived primary human total CD3<sup>+</sup> T cells (co-culture of CD4<sup>+</sup> and CD8<sup>+</sup> T cells) from healthy donors was measured to establish baseline expression levels. The receptors CD28, CD27 and CD2 were constitutively expressed on the majority of CD4<sup>+</sup> T cells (Figure 1b) and CD8<sup>+</sup> T cells (Figure 1c). Accordingly, both **P- $\alpha$ CD28** or **P- $\alpha$ CD2** stimulation of resting total CD3<sup>+</sup> T cells resulted in proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> (Figure 1d,e) T cells. Surprisingly, **P- $\alpha$ CD27** was not able to induce potent T-cell stimulation, even though baseline expression of CD27 was observed. By contrast, the T cells showed only low expression of the other co-stimulatory receptors and no T-cell activation with only 4.8% and 13.4% of CD4<sup>+</sup> T cells expressing OX-40 and SLAM, respectively, and 15.3% and 19.0% of CD8<sup>+</sup> T cells expressing OX-40 and SLAM, respectively. 4-1BB expression was limited to less than 1% of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 1b,c). The control polymer **P** and the PIC backbone alone (**P-blank**) did not induce T-cell proliferation (Figure 1d,e; Figure S2a, Supporting Information).

To increase expression of OX-40, SLAM, and 4-1BB, resting total CD3<sup>+</sup> T cells (co-culture of CD4<sup>+</sup> and CD8<sup>+</sup> T cells) were primed with commercially available  $\alpha$ CD3/ $\alpha$ CD28 Dynabeads for 1–3 days. After 2 days of stimulation, expression of OX-40, 4-1BB and SLAM was increased to 99.7%, 48.9%, and 98.9% on CD4<sup>+</sup> T cells, respectively (Figure 1f, Figure S2a, Supporting Information). Similarly, after 2 days of stimulation 95.9%, 59.7%, and 85.0% of CD8<sup>+</sup> T cells expressed OX-40, 4-1BB, and SLAM (Figure 1g, Figure S2b, Supporting Information). The expression of CD27 was also significantly upregulated following 2 days of priming, which may promote potent CD27-mediated co-stimulation (Figure S2a,b, Supporting Information). In accordance with resting total CD3<sup>+</sup> T cells, upregulation of OX-40, 4-1BB, and SLAM was also observed on single culture of naïve CD4<sup>+</sup> and naïve CD8<sup>+</sup> T cells following stimulation for 2 days with  $\alpha$ CD3/ $\alpha$ CD28 Dynabeads (Figure S2c–e, Supporting Information). Stimulation of T cells with  $\alpha$ CD3 alone did not consistently increase the expression of the co-stimulatory receptors (Figure S2f,g, Supporting Information). Stimulation of total CD3<sup>+</sup> T cells with **P- $\alpha$ CD28** resulted in similar upregulation of OX-40, 4-1BB, and SLAM compared to the commercially available Dynabeads (Figure S3a,b, Supporting Information). Together, these results show that priming of total CD3<sup>+</sup> T cells for 2 days with  $\alpha$ CD3 and  $\alpha$ CD28 can drive the expression of the co-stimulatory receptors OX-40, 4-1BB, and SLAM, and increase the expression of CD27.

### 2.2. Immunofilament-Mediated Co-Stimulation Enhances T-Cell Activation

Having established that OX-40, 4-1BB, and SLAM are upregulated after 2 days of priming with  $\alpha$ CD3/ $\alpha$ CD28, we investigated the effect of co-stimulation on cytokine production, proliferation, and differentiation of T cells. To determine differences in T-cell differentiation and to demonstrate the effect of co-stimulation on naïve CD4<sup>+</sup> and naïve CD8<sup>+</sup> T cells, both cell types were primed separately for 2 days with Dynabeads. An advantage of

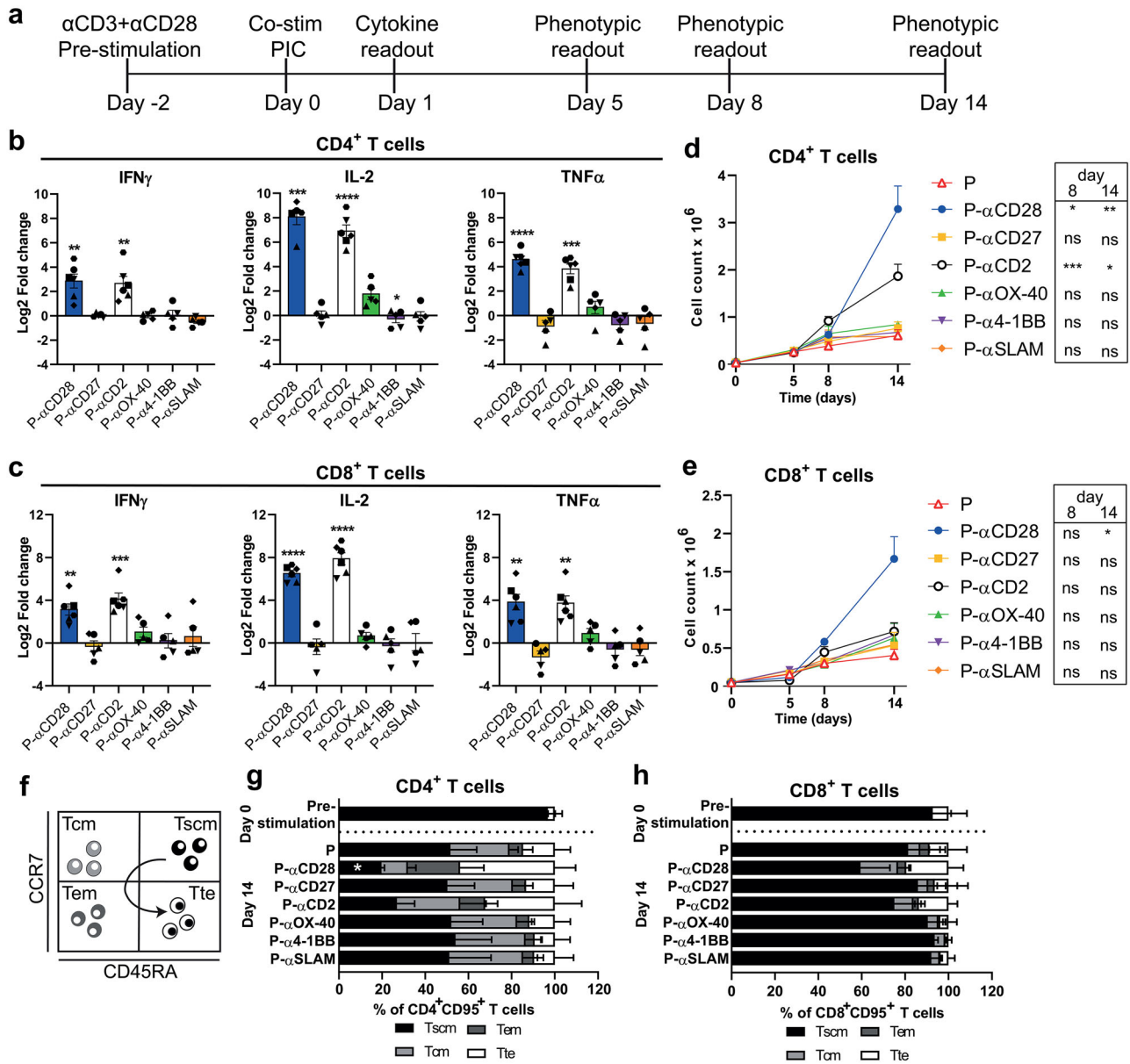


**Figure 1.** Potent T-cell activation through stimulation with constitutively expressed co-stimulatory receptors. a) Artificial antigen presenting cells (aAPCs) were prepared presenting  $\alpha$ CD3 mAb alone (P) or in combination with either  $\alpha$ IgSF mAb (P- $\alpha$ CD28, P- $\alpha$ CD2, and P- $\alpha$ SLAM) or  $\alpha$ TNFRSF mAb (P- $\alpha$ CD27, P- $\alpha$ OX-40, and P- $\alpha$ 4-1BB). Expression levels of the co-stimulatory receptors CD28, CD27, CD2, OX-40 (CD134), 4-1BB (CD137), and SLAM (CD150) on freshly isolated human b) CD4<sup>+</sup> and c) CD8<sup>+</sup> T cells, determined by flow cytometry analysis. Proliferation of d) CD4<sup>+</sup> and e) CD8<sup>+</sup> T cells 72 h after the addition of the different immunofilaments. Proliferation was determined by flow cytometry analysis, measuring Cell Trace Violet dilution. Histograms depict representative donor for CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Expression levels of OX-40, 4-1BB, and SLAM on f) CD4<sup>+</sup> and g) CD8<sup>+</sup> T cells following stimulation for 0, 1, 2, or 3 days with  $\alpha$ CD3/ $\alpha$ CD28 coated magnetic Dynabeads. Expression levels were determined by flow cytometry analysis. Data is represented as means  $\pm$  SEM, each data point represents the mean of a duplicate measurement of cells from a single healthy donor. b,c) Data of three independent experiments. d,e) Data of four independent experiments, differences were evaluated by Kruskal–Wallace test with Dunn’s correction comparing aAPCs to P. f,g) Data of four independent experiments, differences were evaluated by Friedman test with Dunn’s correction comparing days of stimulation to day 0. Stars indicate significance levels \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .

these beads is that they can be magnetically removed from the cells ensuring only stimulation by the specific immunofilaments for the continuing culture time (Figure S1a, Supporting Information, for  $\alpha$ CD3: $\alpha$ co-stim ratio). Subsequently, cells were analyzed for their phenotype on days 1, 5, 8, and 14 (Figure 2a).

At day 1 after stimulation with the respective co-stimulatory immunofilaments, a trend toward increased cytokine production was observed compared to CD3 signaling alone (P) (Figure 2b–d). In particular stimulation of CD4<sup>+</sup> T cells with P- $\alpha$ CD28 signif-

icantly increased interferon gamma (IFN $\gamma$ ), interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF- $\alpha$ ) secretion, showing a log<sub>2</sub> fold increased secretion of 2.9, 8.1, and 4.6, respectively (Figure 2b). Similarly, CD27 proved to be a strong co-stimulator of CD4<sup>+</sup> T cells resulting in a significant log<sub>2</sub> fold increase in IFN $\gamma$ , IL-2, and TNF- $\alpha$  secretion of 1.2, 6.9, and 3.8. Additionally, P- $\alpha$ OX-40 induced a log<sub>2</sub> fold increase of 1.8 for IL-2 production. Secretion of interleukin-4 (IL-4) could not be detected for any of the immunofilaments, interleukin-17 (IL-17) secretion was



**Figure 2.** Triggering of co-stimulatory receptors shapes T-cell activation in addition to T-cell receptor (TCR) signaling. a) Experimental timeline, 2-day priming to ensure OX-40, 4-1BB, and SLAM upregulation followed by stimulation with co-stimulatory immunofilaments. Functional and phenotypic readout were conducted on days 1, 5, 8, and 14. Normalized production of IFN<sub>γ</sub>, IL-2 and TNF- $\alpha$  24 h after co-stimulatory immunofilament stimulation, data normalized to P, for b) CD4<sup>+</sup> and c) CD8<sup>+</sup> T cells. Cell count of d) CD4<sup>+</sup> and e) CD8<sup>+</sup> T cells following 5, 8, and 14 days of stimulation with co-stimulatory immunofilaments. f) Schematic overview of T-cell memory differentiation following the linear T-cell differentiation model, where cells differentiate from Tscm (CD95<sup>+</sup>CCR7<sup>+</sup>CD45RA<sup>+</sup>) > Tcm (CD95<sup>+</sup>CCR7<sup>+</sup>CD45RA<sup>-</sup>) > Tem (CD95<sup>+</sup>CCR7<sup>-</sup>CD45RA<sup>-</sup>) > Tte (CD95<sup>+</sup>CCR7<sup>-</sup>CD45RA<sup>+</sup>). Memory phenotype of g) CD4<sup>+</sup> and h) CD8<sup>+</sup> T cells on day 0 after pre-stimulation and on day 14 after co-stimulatory immunofilament stimulation. Data is represented as mean  $\pm$  SEM, each data point represent the mean of a duplicate of a healthy donor. b–e) Data of three independent experiments g,h) data of two independent experiments. b,c) Log<sub>2</sub> normalized data was analyzed with a T-test comparing immunofilaments to the hypothetical value 0 (P). d,e) Log<sub>2</sub> normalized data (normalized to cell numbers on day 0) was evaluated with a mixed-effects analysis with a Dunnett correction comparing immunofilaments to P on days 5, 8, and 14. g,h) Logit transformed data was evaluated by a mixed-effects analysis with a Dunnett correction comparing, for each memory phenotype individually, immunofilament to P. Stars indicate significance levels \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ .

mainly observed for CD4<sup>+</sup> T cells stimulated with P- $\alpha$ CD28 (Figure S4a, Supporting Information). Moreover, no difference could be detected in the production of the immunosuppressive cytokine interleukin-10 (IL-10), all immunofilaments induced similar levels compared to P (Figure S4b, Supporting Infor-

mation). Similar cytokine production patterns were observed for CD8<sup>+</sup> T cells, where P- $\alpha$ CD28 and P- $\alpha$ CD2 stimulation resulted in significantly higher IFN<sub>γ</sub>, IL-2, and TNF- $\alpha$  secretion, with a log<sub>2</sub> fold change of 3.2 and 4.1 for IFN<sub>γ</sub>, 6.5 and 7.9 for IL-2, and 3.8 and 3.7 for TNF- $\alpha$ , respectively (Figure 2c). When



co-culturing the total CD3<sup>+</sup> T-cell population (CD4<sup>+</sup> and CD8<sup>+</sup> T cells) increased IFN $\gamma$ , TNF- $\alpha$ , and IL-2 secretion can also be observed (Figure S4c, Supporting Information). The increased cytokine secretion indicates that the co-stimulatory antibodies on the immunofilaments, in particular  $\alpha$ CD28 and  $\alpha$ CD2, efficiently enhanced T-cell activation.

In addition to cytokine secretion, T-cell proliferation was increased after co-stimulation with several immunofilaments. While no differences were observed at day 5, naïve CD4<sup>+</sup> T-cells stimulated with **P- $\alpha$ CD28** and **P- $\alpha$ CD2** did induce increased CD4<sup>+</sup> T-cell numbers on days 8 and 14, resulting in  $3.28 \times 10^6$  cells and  $1.8 \times 10^6$  CD4<sup>+</sup> T cells by day 14, respectively (Figure 2d). Naïve CD8<sup>+</sup> T-cell numbers were increased most strongly by stimulation with **P- $\alpha$ CD2** and **P- $\alpha$ CD28** on day 8 but only resulted in significantly higher cell numbers by day 14 for **P- $\alpha$ CD28**, with  $1.6 \times 10^6$  CD8<sup>+</sup> T cells (Figure 2e). This finding indicates that highest cell numbers are obtained when CD28 or CD2 is used for co-stimulation. Interestingly, naïve CD4<sup>+</sup> T cells showed two times higher cell numbers compared to naïve CD8<sup>+</sup> T cells when separately cultured, irrespective of the type of co-stimulation. When co-culturing the total naïve CD3<sup>+</sup> T-cell population a similar increase in CD8<sup>+</sup> T-cell numbers was observed for **P- $\alpha$ CD28** stimulation, CD4<sup>+</sup> T-cell numbers were half in the co-culture compared to single culture (Figure S4d, Supporting Information).

After having established that the co-stimulatory immunofilaments can enhance T-cell activation, we studied the impact of co-stimulation on memory differentiation of T cells. Conform to the linear T-cell differentiation model, we determined the presence of five distinct memory T-cell populations: naïve T cells, stem cell memory T cells (Tscm), central memory T cells (Tcm) effector memory T cells (Tem), and terminal effector T cells (Tte) (Figure 2g).<sup>[22]</sup> Both the naïve CD4<sup>+</sup> and the naïve CD8<sup>+</sup> T-cell populations consisted of low numbers of CD95<sup>+</sup> memory cells before priming, below 20% (Figure S4e, Supporting Information). Priming resulted in expression of memory marker CD95 on approximately all the CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure S4e, Supporting Information). More than 95% of the CD95<sup>+</sup>CD4<sup>+</sup> and CD95<sup>+</sup>CD8<sup>+</sup> T cells displayed a Tscm phenotype (Figure 2h,i), indicating that differentiation induced by priming was limited. The various co-stimulatory immunofilaments did not induce different memory phenotypes in either naïve CD4<sup>+</sup> or naïve CD8<sup>+</sup> T cells on days 5 and 8. CD4<sup>+</sup> T cells mainly displayed an early memory phenotype (Tscm or Tcm) on days 5 and 8 (Figure S4f, Supporting Information). By day 14, the Tscm frequency was significantly reduced to 20% in **P- $\alpha$ CD28**-stimulated CD4<sup>+</sup> T cells compared to 52% of cells stimulated with **P**. Furthermore, the Tem and Tte populations of CD4<sup>+</sup> T cells treated with **P- $\alpha$ CD28** were increased to 24% and 44%, respectively, compared to cells treated with the control polymer **P** (6% and 15%) (Figure 2g). Stimulation with **P- $\alpha$ CD2** leads to a similar pattern but to a lesser extent. In addition, 5 and 8 days of CD8<sup>+</sup> T-cell stimulation with the immunofilaments primarily resulted in Tscm cells for all the co-stimuli (Figure S4g, Supporting Information). At day 14, 59–94% of the CD8<sup>+</sup> T cells retained the Tscm phenotype, while the Tte population was limited to 1–19% of the cells (Figure 2h). CD8<sup>+</sup> T-cell stimulated with **P- $\alpha$ CD28** or **P- $\alpha$ CD2** displayed the highest levels of a Tte phenotype, 19% and 14% respectively. Likewise, the total naïve CD3<sup>+</sup> T-cell population activated with the different aAPCs

demonstrated similar memory phenotypes for the CD4<sup>+</sup> T cells for all the co-stimuli (Figure S4h, Supporting Information). The CD8<sup>+</sup> T-cell population within the co-culture showed high variability between donors and more cells expressing the Tte phenotype compared to the single culture (Figure S4i, Supporting Information).

Taken together, these experiments demonstrate that co-stimulation increased cytokine production and proliferation, but their impact on memory T-cell differentiation cells was limited.

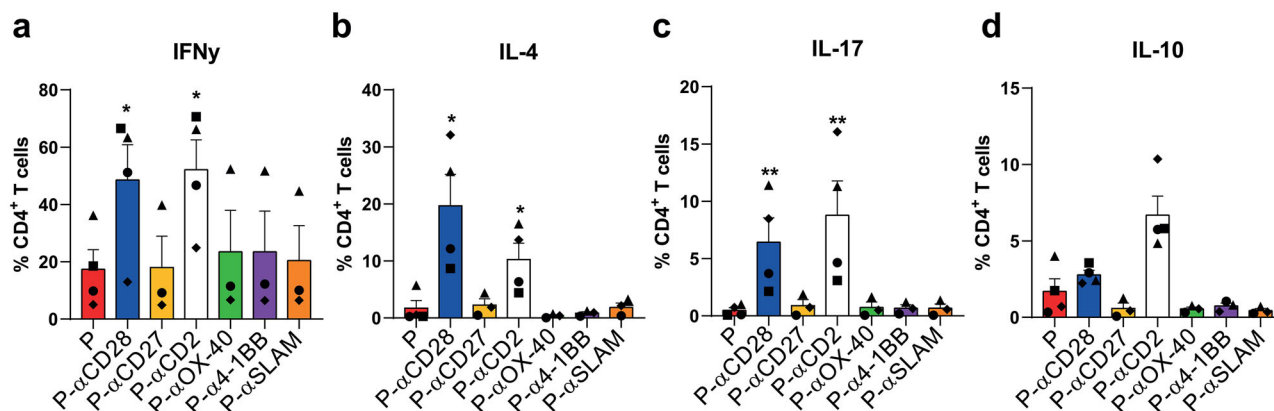
### 2.3. P- $\alpha$ CD28 and P- $\alpha$ CD2 Induce the Expression of Th1 Cytokine IFN $\gamma$ and IL-17 a Th17 Cytokine

Next, we studied the effect of the various immunofilaments on the cytokine production related to the different CD4<sup>+</sup> T helper cell (Th) subsets. The T helper phenotype of CD4<sup>+</sup> T cells can greatly impact their function and the anti-cancer immune response. The Th1 subset is characterized by high IFN $\gamma$  production and is involved in cell-mediated immune responses.<sup>[23]</sup> Th2 cells are primarily involved in humoral immunity and produce IL-4.<sup>[23]</sup> Th17 cells producing IL-17 are mainly involved in immunity against extracellular bacteria and some fungi.<sup>[24]</sup> Both Th1 and Th17 cells have been reported to boost the development of anti-tumor immunity.<sup>[25–27]</sup> As T helper differentiation requires at least 4 days of stimulation, intracellular cytokine production was determined at day 5 of immunofilament co-stimulation.<sup>[28,29]</sup>

At day 5, all immunofilaments induced at least 17% IFN $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells with a significantly bigger population when stimulated with **P- $\alpha$ CD28** or **P- $\alpha$ CD2** (48% and 52%, respectively) (Figure 3a). Limited CD4<sup>+</sup> T cells were IL-4 positive, with only **P- $\alpha$ CD28** and **P- $\alpha$ CD2** inducing significantly higher levels of 20% and 10% (Figure 3b). This finding indicates that the co-stimulatory immunofilaments, and **P- $\alpha$ CD28** and **P- $\alpha$ CD2**, mainly induce the expression of the Th1 cytokine IFN $\gamma$  and induced limited expression of the Th2 cytokine IL-4. Moreover, CD4<sup>+</sup> T cells stimulated with **P- $\alpha$ CD28** or **P- $\alpha$ CD2** showed a clear capacity to induce the expression of IL-17 with 6.4% and 8.8% of the CD4<sup>+</sup> T cells being IL-17<sup>+</sup> T cells, respectively, compared to 0.5% of IL-17<sup>+</sup> cells with **P** (Figure 3c). Besides increased percentages **P- $\alpha$ CD28** and **P- $\alpha$ CD2** also elevated MFI levels of IFN $\gamma$ , IL-4, and IL-17 (Figure S5a–c, Supporting Information). In addition to the expression of IFN $\gamma$ , IL-4, and IL-17, we were also interested in the expression of the immunosuppressive cytokine IL-10. All co-stimulatory immunofilaments induced IL-10 expression in less than 7% of the CD4<sup>+</sup> T cells, with **P- $\alpha$ CD2** stimulation resulting in the highest percentage of IL-10<sup>+</sup>CD4<sup>+</sup> T cells, 6.7% (Figure 3d). Moreover, MFI levels of IL-10 were comparable for all immunofilaments (Figure S5d, Supporting Information). Altogether, these results suggest a different role for CD28 and CD2 compared to the other co-stimulatory molecules in the expression of the T helper cell cytokines.

### 2.4. Co-Stimulation via the CD28 and CD2 Axis Results in Multifunctional but Not Exhausted T Cells

Multifunctionality is another important characteristic of T cells. Multifunctional T cells are defined as T cells that express at least



**Figure 3.** P- $\alpha$ CD28 and P- $\alpha$ CD2 influence Thelper cytokine expression. Intracellular cytokine production, a) IFN $\gamma$ , b) IL-4, c) IL-17, and d) IL-10, following 5.5 h PMA/Ionomycin stimulation in the presence of BFA and monensin. Data is represented as means  $\pm$  SEM, each data point represents the mean of a duplicate of cells from a healthy donor, data of two independent experiments. Logit transformed data was evaluated by a one-way ANOVA with a Dunnett correction comparing immunofilaments to P. Stars indicate significance levels \* $p < 0.05$ , \*\* $p < 0.01$ .

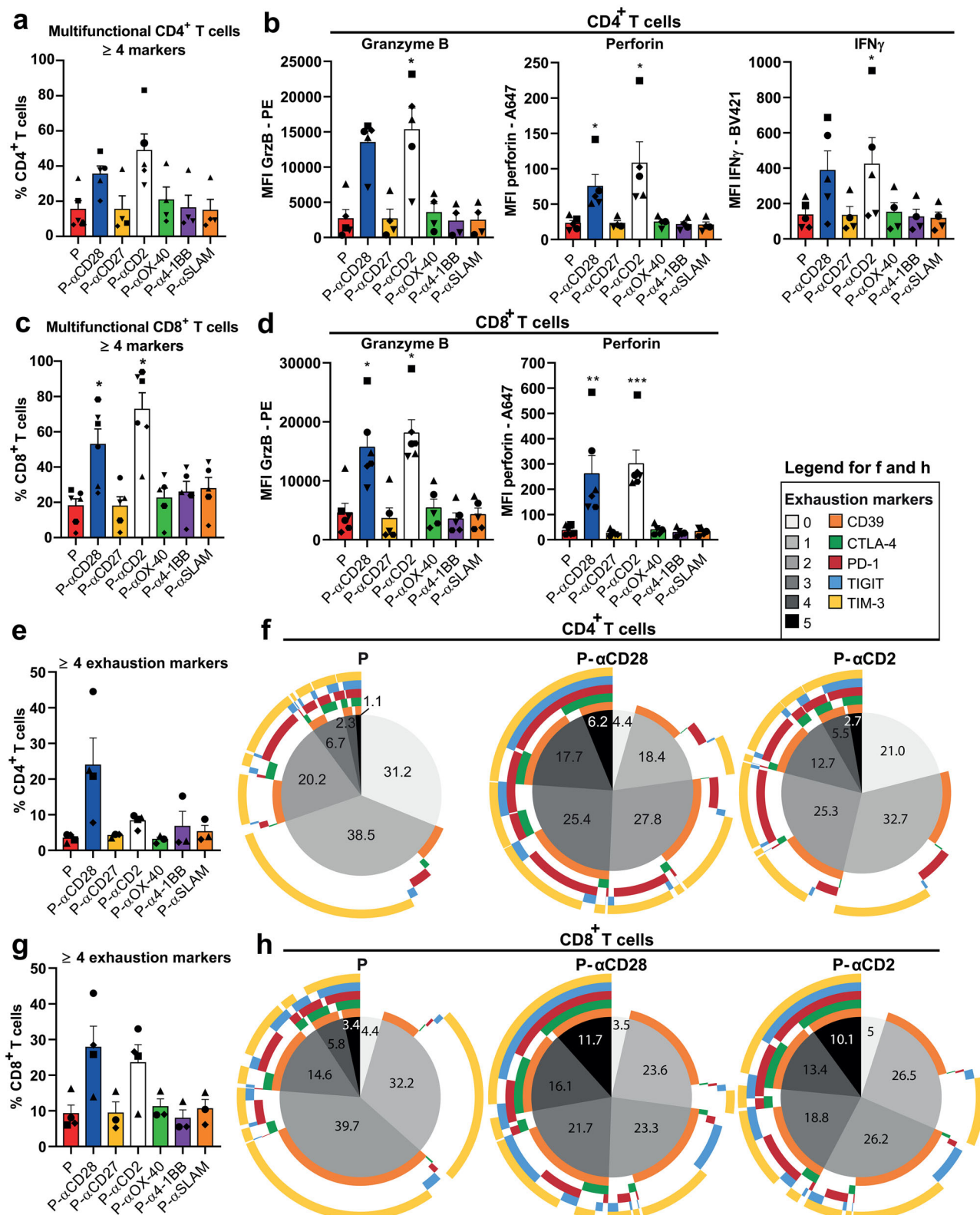
two types of effector markers including cytokines, chemokines, cytotoxic molecules, and degranulation markers.<sup>[30]</sup> Moreover, multifunctional T cells are efficient in killing target cells and can produce high levels of cytokines such as IFN $\gamma$ , allowing signaling to other immune cells.<sup>[30]</sup> Studies both in HIV and in melanoma patients highlighted the correlation of multifunctional T cells with improved clinical outcome.<sup>[31,32]</sup> To understand the effect of co-stimulation on the multifunctional phenotype of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, the expression of the cytokines IL-2, TNF- $\alpha$ , IFN $\gamma$ , and cytotoxic effector molecules perforin and GranzymeB (GrzB) was analyzed.

Most CD4<sup>+</sup> T cells treated with the different co-stimulatory immunofilaments showed a trend toward more cells having a multifunctional phenotype compared to P, as on day 5 all immunofilaments induced the expression of four or more effector markers (Figure 4a, Figure S6a, Supporting Information). P- $\alpha$ CD28 and P- $\alpha$ CD2 induced the highest populations of cells expressing four markers on day 5 compared to P. When looking to the expression levels of the effector markers CD4<sup>+</sup> T cells stimulated with P- $\alpha$ CD2 showed significantly higher MFI values for perforin, GrzB, and IFN $\gamma$  compared to the control polymer (Figure 4b, Figure S6b,c, Supporting Information). Additionally, cells stimulated with P- $\alpha$ CD28 showed elevated levels of perforin, GrzB, and IFN $\gamma$ . Moreover, heatmap analysis indicated a trend toward more CD4<sup>+</sup>T cells with a GrzB<sup>+</sup>IL-2<sup>+</sup>TNF- $\alpha$ <sup>+</sup> phenotype when stimulated with - $\alpha$ CD27, P- $\alpha$ OX-40, P- $\alpha$ 4-1BB, and P- $\alpha$ SLAM (Figure S6d,e, Supporting Information). In comparison, cells stimulated with P- $\alpha$ CD28 and P- $\alpha$ CD2 induced higher levels of CD4<sup>+</sup> T cells expressing all five markers (Figure S6d,e, Supporting Information).

Similar to the CD4<sup>+</sup> T cells, most CD8<sup>+</sup> T cells treated with co-stimulatory immunofilaments displayed a trend toward more cells with a multifunctional phenotype compared to P on day 5 (Figure 4c, Figure S7a, Supporting Information). P- $\alpha$ CD28 and P- $\alpha$ CD2 induced significantly higher populations of CD8<sup>+</sup> T cells expressing at least four effector markers compared to P, 52.8% and 72.7% respectively. Moreover, similar to the CD4<sup>+</sup> T cells P- $\alpha$ CD28 and P- $\alpha$ CD2 stimulation of the CD8<sup>+</sup> T cells resulted in significantly elevated expression levels of GrzB and perforin

(Figure 4d, Figure S7b,c, Supporting Information). Additionally, heatmap analysis of the CD8<sup>+</sup> T cells indicated that the effector phenotype GrzB<sup>+</sup>IL-2<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup> was significantly upregulated with P- $\alpha$ CD27, P- $\alpha$ 4-1BB, and P- $\alpha$ SLAM stimulation (Figure S7d,e, Supporting Information). In contrast, P- $\alpha$ CD28 and P- $\alpha$ CD2 induced the expression of GrzB<sup>+</sup>IFN $\gamma$ <sup>+</sup>Perforin<sup>+</sup>TNF- $\alpha$ <sup>+</sup> and all five markers (Figure S7d,e, Supporting Information).

Next, we investigated the exhaustion phenotype of the T cells. Strong and continuous stimulation of T cells can induce a state of dysfunction, which limits the capacity of T cells to kill target cells and produce cytokines.<sup>[33]</sup> T-cell exhaustion is characterized by sustained expression of multiple inhibitory receptors.<sup>[33]</sup> To determine exhaustion induced by the different co-stimulatory polymers, co-expression of co-inhibitory receptors programmed death 1 (PD-1), CTLA-4, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT), and CD39 was analyzed following 14 days of stimulation. When CD4<sup>+</sup> T cells were activated with the different co-stimulatory immunofilaments, the percentage of cells expressing multiple exhaustion markers on day 14 did not significantly differ from the control polymer (P) (Figure 4e). However, cells stimulated with P- $\alpha$ CD28 did show elevated levels of 23.9% compared to 3.4% for P. Mainly TIM-3, CD39 and PD-1 are expressed on these CD4<sup>+</sup> T cells (Figure 4f, Figure S8a, Supporting Information). Similarly, stimulation of CD8<sup>+</sup> T cells with the co-stimulatory immunofilaments did not induce significantly higher levels of multiple exhaustion markers compared to P following 14 days of culture. CD8<sup>+</sup> T cell stimulated with P- $\alpha$ CD28 and P- $\alpha$ CD2 resulted in the highest expression of multiple exhaustion markers, 27.8% and 23.5%, respectively, compared to 9.2% for P (Figure 4g). Interestingly, both the CD4<sup>+</sup> and CD8<sup>+</sup> T cells mainly expressed the exhaustion markers TIM-3 and CD39, followed by expression of PD-1 and TIGIT with limited expression of CTLA-4 (Figure 4h, Figure S8a,b, Supporting Information). Co-culture of the total naive CD3<sup>+</sup> T-cell population resulted in reduced expression of multiple exhaustion markers on CD4<sup>+</sup> T cells compared to the CD4<sup>+</sup> single culture, especially when stimulated with P- $\alpha$ CD28 or P- $\alpha$ CD2 (Figure S8c, Supporting Information). Compared to the naive CD8<sup>+</sup> single



**Figure 4.** P- $\alpha$ CD28 and P- $\alpha$ CD2 induce multifunctional T-cell phenotypes. a) Percentage of multifunctional CD4<sup>+</sup> T cells (expressing four or more effector functions, Perforin, GrzB, TNF- $\alpha$ , IL-2, IFN $\gamma$ ) following 5 days of immunofilament stimulation. b) Mean fluorescence intensity of the effector markers Granzyme B, Perforin and IFN $\gamma$  of CD4<sup>+</sup> T cells stimulated for 5 days with immunofilaments. c) Percentage of multifunctional CD8<sup>+</sup> T cells (expressing 4 or more effector functions) following 5 days of immunofilament stimulation. d) Mean fluorescence intensity of the effector markers Granzyme B and Perforin of CD8<sup>+</sup> T cells stimulated for 5 days with immunofilaments. e) Percentage of cells expressing four or more exhaustion markers (PD-1, TIGIT, CTLA-4, CD39, TIM-3) following 5 days of immunofilament stimulation. f) Donut charts showing the percentage of CD4<sup>+</sup> T cells expressing 0, 1, 2, 3, 4, or 5 exhaustion markers under different immunofilament conditions. g) Percentage of cells expressing four or more exhaustion markers (PD-1, TIGIT, CTLA-4, CD39, TIM-3) following 5 days of immunofilament stimulation. h) Donut charts showing the percentage of CD8<sup>+</sup> T cells expressing 0, 1, 2, 3, 4, or 5 exhaustion markers under different immunofilament conditions.



culture, co-culture resulted in a reduced expression of multiple exhaustion markers for CD8<sup>+</sup> T cells for all the immunofilaments (Figure S8d, Supporting Information).

Taken together, **P- $\alpha$ CD28** and **P- $\alpha$ CD2** induced highly multifunctional T cells with elevated levels of perforin and GrzB. In addition, triggering co-stimulation via the immunofilaments did not induce an exhausted phenotype in neither CD4<sup>+</sup> nor CD8<sup>+</sup> T cells after a prolonged period of stimulation.

### 3. Conclusion

Co-stimulation is an essential signal required for full-blown T-cell activation. Although T cells express several different co-stimulatory receptors, CD28 in particular has extensively been studied for its role in (artificial) T-cell stimulation. Most information on the roles of co-stimulation on T-cell function and fate have been described using mouse models that are either deficient for the co-stimulatory receptor or their ligand, by using mAbs that block the receptor in vivo, or via in vitro overexpression systems.<sup>[34–36]</sup> These studies often focus on the role of one particular co-stimulatory receptor and do not directly compare the impact of different co-stimulatory cues on T cells. The current study provides a detailed overview of how co-stimulatory signaling affects T cells function and fate and highlights strategies to evoke certain T cell subpopulations.

In this study we opted for TCR signaling and co-stimulation via semiflexible immunofilaments, as they allow for more controlled presentation of co-stimulatory signals when compared to natural APCs and cellular aAPCs. Comparing all six stimuli we noted that CD28 and CD2 in particular play a dominant role in T-cell priming, cytokine production, proliferation, and the induction of multifunctional T cells. Moreover, co-stimulation with **P- $\alpha$ CD28** or **P- $\alpha$ CD2** induced high secreted cytokine levels of IFN $\gamma$ , IL-2, and TNF- $\alpha$  in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Moreover, for both **P- $\alpha$ CD28** and **P- $\alpha$ CD2** there was a trend toward more IL-17 secretion by CD4<sup>+</sup> T cells. Similar to our observations, Leitner et al. showed potent T-cell proliferation and cytokine production for CD2 and CD28 stimulation, using a cellular aAPC system expressing the ligand for CD28 or CD2.<sup>[37]</sup> Cytokine production as a result of CD2 co-stimulation has previously been studied using soluble cross-linked  $\alpha$ CD3 and  $\alpha$ CD2 antibodies. In this context, CD2 stimulation induced high levels of IL-4 and IL-10 but was shown to be inadequate for IL-2 production.<sup>[38]</sup> In contrast, CD2 stimulation using our immunofilaments induced high levels of IL-2 and low levels of IL-10 and IL-4 that were not detectable by ELISA. This indicates that co-stimulatory signaling can be highly dependent on the manner in which the signals are presented to the T cells. Furthermore, stimulation with **P- $\alpha$ CD27** did not result in strong cytokine production, while stimulation with plate-

bound  $\alpha$ CD3 and  $\alpha$ CD27 mAb has previously been shown to result in elevated levels of IFN $\gamma$ , TNF- $\alpha$ , and IL-2.<sup>[39]</sup> Underlining the effect of co-stimulatory signal presentation, moreover, timing of co-stimulatory receptor signaling could play another important role.<sup>[34]</sup>

Although OX-40 and 4-1BB are expressed on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, several studies have highlighted OX-40 signaling to predominantly stimulate CD4<sup>+</sup> T cells while 4-1BB stimulation preferably induces a CD8<sup>+</sup> T-cell response.<sup>[40,41]</sup> Stimulation of CD4<sup>+</sup> T cells with  $\alpha$ CD3 mAb in combination with cross-linking of soluble OX-40L or  $\alpha$ OX-40 mAb can augment proliferation, and production of IL-2 and IL-4.<sup>[40]</sup> Furthermore, CD8<sup>+</sup> T cells show enhanced proliferation, IL-2 and IFN $\gamma$  production when stimulated with  $\alpha$ CD3 mAb in combination with soluble 4-1BBL or  $\alpha$ 4-1BB mAb.<sup>[40]</sup> In this study we did not observe a clear capacity of **P- $\alpha$ OX-40** to preferentially stimulate CD4 T cells, though, we did observe a trend toward an increased IL-2 production. **P- $\alpha$ 4-1BB** stimulation of CD8<sup>+</sup> T cells, moreover, did not result in enhanced CD8<sup>+</sup> T-cell activation, however, we did observe the capacity of **P- $\alpha$ 4-1BB** to induce the multifunctional phenotype GrzB<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL-2<sup>+</sup>TNF- $\alpha$ <sup>+</sup> in CD8<sup>+</sup> T cells. Stimulation with **P- $\alpha$ SLAM** induced limited levels of IFN $\gamma$  and IL-4 after 24 h of stimulation. Limited IFN $\gamma$  production was also seen when SLAM was engaged by its ligand in a cellular aAPC system, although, elevated levels of IL-4 were observed in this system.<sup>[36,42]</sup>

It has been suggested that different co-stimulatory molecules can affect memory formation of CD8<sup>+</sup> T cells.<sup>[8,43]</sup> For example, CD27 signaling was shown to bypass the requirement for CD4<sup>+</sup> T cell help to induce CD8<sup>+</sup> T-cell memory differentiation in vivo studies performed with soluble recombinant CD70 or transgenic DCs expressing CD70.<sup>[44,45]</sup> This effect was not observed in our in vitro immunofilament setting, indicating that other factors besides CD27 co-stimulation might contribute to the in vivo observation. In general, we observed minimal differences in memory phenotypes of both the CD4<sup>+</sup> and CD8<sup>+</sup> T cells after stimulation with the immunofilaments. Only **P- $\alpha$ CD28** or **P- $\alpha$ CD2** depicted a trend toward lower levels of Tscm and higher levels of Tte, especially for the CD4<sup>+</sup> T cells. However, the presence of co-stimulation besides CD28 or CD2 did not significantly alter the memory phenotype when compared to CD3 signaling alone. This may be a result of the priming step with strongly activating Dynabeads.

Immunotherapeutic strategies that target co-stimulatory receptors with mAbs have been limited in the clinic due to the systemic toxicities.<sup>[46]</sup> Nanoparticle-based approaches have been successfully tested to limit this toxicity by passively targeting mAbs to the tumor site.<sup>[46]</sup> Similarly, the size of the PIC-based aAPCs we use allows for in vivo application. We have previously shown that co-presentation of  $\alpha$ CD3 with IL-2 on our

TIM-3, CTLA-4, and CD39) on day 14 for CD4<sup>+</sup> T cells. f) Representative pie chart for CD4<sup>+</sup> T cells stimulated with P, P- $\alpha$ CD28, and P- $\alpha$ CD2, numbers represent percentage of cells expressing 0 to 5 exhaustion markers. Colored arcs represent the five different exhaustion markers and their expression. g) Percentage of cells expressing four or more exhaustion markers on day 14 for CD8<sup>+</sup> T cells. h) Representative pie chart with arcs for CD8<sup>+</sup> T cells stimulated with P, P- $\alpha$ CD28, and P- $\alpha$ CD2. Data is represented as means  $\pm$  SEM, each data point represent the mean of a duplicate of a healthy donor, each symbol represents an individual donor. a–d) Data of three independent experiments. e–h) Data of two independent experiments. a, c, e, g) Logit transformed data was evaluated by a mixed-effects analysis with a Dunnett correction comparing aAPCs to P. b, d) Log transformed data was evaluated by mixed-effects test with Dunn's correction comparing aAPCs to P. f, h) Mean of four donors, numbers indicate percentage, colors represent the different exhaustion marker contribution. Stars indicate significance levels \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .



immunofilaments can target IL-2 to T cells and limit the binding of IL-2 on endothelial cells and possibly limit the toxicity.<sup>[20]</sup> Moreover, the PIC-based aAPC system can be modified to target T cells more specifically by presenting natural ligands instead of mAbs and/or could present multiple co-stimulatory molecules to further shape T-cell phenotypes. Besides changing the mAbs for the natural ligand, changing the mAb isotype to IgG2 might also increase T-cell activating capabilities, as was seen for OX-40 and 4-1BB mAbs.<sup>[47]</sup> In addition, a combination of polymers bearing different co-stimulatory cues could be used to further shape the desired T-cell response. In the current study, **P- $\alpha$ CD28** and **P- $\alpha$ CD2** showed the most potent CD4<sup>+</sup> and CD8<sup>+</sup> T-cell activation. Both of these co-stimulatory receptors signal through specific pathways.<sup>[8,38,48]</sup> Moreover, a recent study by Demetriou et al. has shown that CD2 interaction with its ligand CD58 induces reorganization of several co-stimulatory receptor-ligand pairs, including CD28, to the periphery of the supramolecular activation complex (SMAC).<sup>[49]</sup> Furthermore, distal CD28 signaling has been shown to induce enhanced IL-2 production in naïve CD4<sup>+</sup> T cells.<sup>[50]</sup> Co-presentation of both these mAbs could therefore allow for synergistic T-cell activation by targeting not only these distinct pathways but also induce a more potent SMAC organization. Moreover, **P- $\alpha$ OX-40** has shown a trend toward an increased CD4<sup>+</sup> specific IL-2 production. Therefore, combining an OX-40 mAb with a CD2 and/or CD28 mAb on the polymers could potentially induce a more potent CD4<sup>+</sup> T-cell response. Even though we did not see specific effects of **P- $\alpha$ 4-1BB** on the CD8<sup>+</sup> T cells it could still be worth testing a combination of a 4-1BB mAb with a CD2 and/or CD28 mAb. Additionally, testing other combinations and ratios of co-stimulatory molecules could provide interesting insights into T-cell function and phenotype. In summary, in this study we provided insight into the role of combined TCR and co-stimulation on T-cell phenotype, function, and fate using immunofilaments. Notably, our findings on the roles of co-stimulatory molecule function can be used for the rational design of future cancer immunotherapies.

## 4. Experimental Section

**Reagents and Abs:** T-cell phenotyping panels are listed in Table S1 (Supporting Information). For flow cytometry analysis  $\alpha$ CD27 PE (302 808),  $\alpha$ CD28 PE-Cy7 (302 926),  $\alpha$ CD2 (300 217),  $\alpha$ OX-40 BV421 (350 014),  $\alpha$ SLAM FITC (306 306),  $\alpha$ CD4 PerCP (300 528),  $\alpha$ CD8 BV510 (344 732),  $\alpha$ CD95 BV421 (305 624),  $\alpha$ CD45RA BV510 (304 142),  $\alpha$ Perforin A647 (308 110),  $\alpha$ Granzyme B PE (372 208),  $\alpha$ IL-2 PerCP-Cy5.5 (500 322),  $\alpha$ CD39 BV421 (328 214),  $\alpha$ TIM-3 APC (345 012),  $\alpha$ CD8 PerCP (344 708),  $\alpha$ CD4 PerCP (300 528),  $\alpha$ IL-17 A647 (512 309), and  $\alpha$ IL-10 PE-Cy7 (501 420) were purchased from BioLegend. A4-1BB APC (550 890),  $\alpha$ CD4 APC-Cy7 (557 871),  $\alpha$ IFN $\gamma$  BV421 (562 988),  $\alpha$ TIGIT BB700 (747 846),  $\alpha$ PD-1 BV510 (563 076),  $\alpha$ CLTA-4 PE (555 853), and  $\alpha$ IL-4 PE (559 333) were obtained from BD.  $\alpha$ CD4 PE-Cy7 (25-0047-42) and  $\alpha$ TNF- $\alpha$  PE-Cy7 (25-7349-82) from eBioscience and  $\alpha$ CCR7 A647 (130-099-363) from Miltenyi. For preparation of the aAPCs the following mouse anti-human mAbs were used; CD3 (clone OKT3, BE0001-2 BioXCell), CD28 (clone 9.3, BE0248 InVivoMab), CD27 (clone 1A4LDG, Beckman Coulter), CD2 (clone RPA-2.10, BioLegend), CD134/OX-40 (clone L106, 340 739 BD), CD137/4-1BB (clone 4B4-1, 309 802 BioLegend), CD150/SLAM (clone A12(7D4), 306 310 BioLegend).

All antibodies used for the aAPCs were functionalized with DBCO-PEG4-NHS (click chemistry tools) and Atto488-NHS ( $\alpha$ CD3, atto-tec) or Alexa Fluor 350-NHS (all co-stimuli, ThermoFisher Scientific) using a sim-

ilar protocol as published before.<sup>[20]</sup> Typically, this reaction yielded antibodies with an average degree of labeling of 1–3 DBCO and 1–3 dyes per antibody.

**Preparation of the Immunofilaments:** Water-soluble PICs of  $\approx$ 400 nm were synthesized as described previously.<sup>[17,20]</sup> Briefly, PICs were polymerized from isocyanopeptide monomers either having a methoxy or an azide end group. The ratio between the methoxy and the azide monomers was 30:1. Part of the azides was converted to biotin using a similar protocol as previously published, which allows purification of the polymer bioconjugates over monoavidin beads.<sup>[20,21]</sup>

The final PIC aAPCs were prepared by addition of 0.2 equivalents of  $\alpha$ CD3 and 0.4 equivalents of co-stimulatory antibody relative to azide groups to a 1 mg mL<sup>-1</sup> solution of PIC in PBS. The reaction was incubated overnight on a tube mixer at 4 °C. Next, the polymer-antibody reaction mixtures were purified over monoavidin beads as published before,<sup>[21]</sup> resulting in the final pure PIC aAPCs used in this study. The polymer concentration was analyzed using circular dichroism and the antibody concentration was determined using the Atto488 and Alexa Fluor 350 fluorescent labels. These values were used to calculate the antibody densities in Figure S1A (Supporting Information).<sup>[20]</sup>

**T-Cell Purification:** Total CD3<sup>+</sup> T-cell population, naïve CD4<sup>+</sup>, naïve CD8<sup>+</sup>, and the total naïve CD3<sup>+</sup> T-cell population were isolated from Buffy coats of healthy donors using ficoll density gradient centrifugation (Lymphoprep, Eliteck group). This study was carried out in accordance with the recommendations of institutional guidelines. All subjects gave written informed consent in accordance with the declaration of Helsinki. T cells were isolated from PBLs from healthy donors using the respective isolation kits according to manufacturer's protocol (Miltenyi Biotec).

**Cell Culture:** T cells were cultured for up to 16 days in X-vivo media supplemented with 4% human serum supplemented with IL-2 (30 U mL<sup>-1</sup>). T cells were pre-stimulated using Dynabeads at 1 bead per cell for 1, 2, or 3 days. Following pre-stimulation, Dynabeads were magnetically separated from T cells after which the cells were rested for 2 h, washed two times and counted. PIC-based aAPCs were added to the T cells at a final concentration of 0.5  $\mu$ g mL<sup>-1</sup>  $\alpha$ CD3. Cell culture media was refreshed every 2 days and cell density was adjusted every 3–4 days.

Supernatants were collected at 24 h following co-stimulatory aAPC stimulation and stored at -20 °C. Cytokine production was determined using standard sandwich ELISA kits (Invitrogen) according to manufacturer's protocol. Human IFN $\gamma$ , IL-2, TNF- $\alpha$ , IL-4, IL-17, and IL-10 were quantified.

**T-Cell Stimulation Assays:** For proliferation assays, the total CD3<sup>+</sup> T-cell population was stained with the proliferation dye Cell Trace Violet (2.5  $\mu$ M, Invitrogen) in PBS containing 1% FBS for 10 minutes at 37 °C. To determine intracellular cytokine production, cells were stimulated with PMA (20 ng mL<sup>-1</sup>) and Ionomycin (1  $\mu$ g mL<sup>-1</sup>) for 5.5 h in the presence of brefeldin A and monensin (1:1000, 00-4505-51 eBioscience). The cells were stained for surface markers followed by permeabilization and fixation with the BD cytofix/cytoperm kit following manufacturer's protocol and stained intracellularly. Before cell surface and intracellular staining, T cells were stained with Fixable Viability dye eFluor 780 (BD Pharmingen).

All flow phenotyping was carried out by a single operator and followed the same gating strategy (Figure S1B–D, Supporting Information). Cytometry measurements were performed with the BD FACS Verse and data was analyzed using FlowJo software version 10.0.7. To determine the cell numbers the Miltenyi MACSQuant Analyzer 10 flow cytometer was used.

**Statistical Analysis:** Data is expressed as mean  $\pm$  SEM. Data was analyzed using GraphPad Prism 8.02. For all aAPC activation studies the co-stimulatory aAPCs were compared to the control polymer P ( $\alpha$ CD3 only polymer). Data was considered paired for the treatment and time, all aAPC conditions were tested with the same donors over time. Proliferation data was analyzed using a Kruskal–Wallis test with a Dunn's correction. The raw cytokine data was analyzed using the Friedman test with a Dunn's correction, and is depicted as Log<sub>2</sub> normalized to P. Cell count data was Log<sub>2</sub> normalized to P. All percentage data was LOGIT transformed. Normalized cell counts and LOGIT transformed percentage data were analyzed by a mixed-effects analysis or a one-way ANOVA both with a Dunnett correction. Co-stimulatory expression data was analyzed using a Friedman test

with a Dunn's correction comparing each day to the expression level on day 0. A  $p$  value  $\leq 0.05$  was considered to be statistically significant. NS = non-significant, \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ .

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Keywords

artificial APC, co-stimulation, differentiation, human T cells, phenotype

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