Short Communication

A critical regulatory role for the cytoplasmic domain of CD28 in ligand binding in naive T cells

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T cell activation is a main component of adaptive immunity, which involves two types of signals transmitted by key immune receptors upon engagement with their respective ligands present on the antigen-presenting cells (APCs) \textsuperscript{[1]}. The first signal is induced via the T cell receptor (TCR) upon binding to antigenic peptide-major histocompatibility complex (pMHC); the second signal is induced via the costimulatory receptors, the prototype of which is CD28, which can bind to either B7-1 (also known as CD80) or B7-2 (also known as CD86) \textsuperscript{[2,3]}. CD28 is the only B7 receptor constitutively expressed on naive T cells. Co-stimulation through CD28 is critically required for these cells to achieve clonal expansion, cytokine production and for protection against apoptosis and anergy \textsuperscript{[2,3]}. However, despite many years of study, the relationship between the TCR and CD28-mediated pathways remains to be elucidated. It is considered that CD28 contributes both quantitatively and qualitatively to the signaling pathways driving T cell activation \textsuperscript{[2]}. On the other hand, several studies suggested that the TCR engagement facilitates CD28–B7 interactions \textsuperscript{[4,5]} and consequently boosts the co-stimulatory signal initiation. It is noteworthy, however, that many of the previous studies have investigated the CD28-mediated signal transductions in previously stimulated T cell blasts or established T cell lines, but very rarely in naive T cells. Here, we investigated the mechanism of CD28-mediated signaling pathway activation focusing on the regulation of CD28 binding activity to B7 ligands in mouse naive CD4\textsuperscript{+} T cells.

We first determined the basal CD28–B7 binding avidity, which was found to be very low, consistent with previous studies \textsuperscript{[4]}. We then found that the concurrent TCR stimulation strongly increased the strength of CD28–B7 binding in a manner dependent on tyrosine phosphorylation by SRC kinases. Furthermore, using a mouse model in which CD28 lacked the cytoplasmic tail, we showed that the intracellular domain of CD28 contributed to its binding with B7 and had a mandatory role for binding-avidity enhancement upon TCR stimulation. Altogether, our data establish a critical regulatory role of the cytoplasmic domain of CD28 for its ligand binding and provide new insights on the crosstalk between the TCR- and CD28-mediated signaling pathways in naive T cell activation. All applicable institutional and/or national guidelines for the care and use of animals were followed.

We used naive CD4\textsuperscript{+} T cells from either wild type (WT) or genetically modified mice as a source of T cells, and COS-7 fibroblasts stably transfected with mouse MHC class II (MHCII) I-A\textsuperscript{K} (designated as COS-A\textsuperscript{K}) alone or together with mouse B7-1 (COS-A\textsuperscript{K}/B7\textsuperscript{-1}) as the antigen-presenting cells (APCs). To analyze receptor-ligand interactions following T cell and APC contact, we utilized a centrifugation-based cell-adhesion assay that we adopted from a previously established cell-adhesion test \textsuperscript{[7]} which enables characterizing 2D receptor-ligand binding events \textsuperscript{[8]} (Fig. 1a, and see Supplementary materials online). In the assay, T cells in suspension were first brought onto the monolayer of COS APCs by a mild centrifugation before being centrifuged again in the opposite direction to remove unbound T cells from the COS APCs. The fraction of the remaining T cells bound to the COS APCs was then determined by flow cytometry. The specific cell adhesion frequency ($P_a$) mediated by a given receptor and its ligand was then calculated based on the
total binding frequency ($P_2$) and the non-specific binding frequency ($P_0$) [8] (see Supplementary materials online). We first validated our assay by demonstrating its ability to detect the 2D binding between the TCR and pMHC upon contact between naive CD4$^+$ T cells from 3A9 TCR-transgenic mice (on CBA/J × C3H/HeN background) and COS-Aβ cells loaded with antigen peptides, and this in a dose-dependent manner (Fig. S1a online). We have used the CD4$^+$ T cells from 3A9 TCR-transgenic mice as these cells bear a specific TCR recognizing hen egg lysozyme-derived peptides presented by I-A$. We then went to examine the 2D binding of CD28 with B7-1 in naive CD4$^+$ T cells. For this analysis, we made the use of naive CD4$^+$ T cells purified from C57BL6/J WT and CD28 knockout (CD28KO; C57BL6/J (CD28$^{-/-}$)) mice, respectively. Because T cell development occurs normally in CD28KO mice [9], the only difference between the two types of CD4$^+$ T cells was the presence or absence of CD28 in the entire cell population, respectively. The background binding level was determined using the latter cell type, and the CD28-mediated specific cell adhesion frequency $P_2$ between naive CD4$^+$ T cells and COS-Aβ/B7-1 was then calculated. The initial experiments using a previously described COS-Aβ/B7-1 cell line that displayed a B7-1 density of ~150 molecules/μm$^2$ on their surface [6] did not reveal any CD28-mediated cellular adhesion. This was likely due to the very weak basal 2D interactions between CD28 and B7-1, as previously reported by Bromley and colleagues [4] by analyzing mouse naive CD4$^+$ T cell adhesion onto planar phospholipid bilayers that contained GPI-anchored B7-1. We therefore generated a new COS-Aβ/B7-1 cell line that displayed a higher density of B7-1 (~150 molecules/μm$^2$) on their surface and the expected costimulatory activity (Fig. S1b online). Specific cellular adhesion mediated by CD28–B7–1 2D binding then was efficiently detected (Fig. 1b). As the SRC kinase family member Lck has a key role in the initiation of CD28 signaling [2] whether the constitutive CD28–B7–1 binding was sensitive to PP2, a potent inhibitor of SRC kinases, was next examined. The PP2 treatment, which effectively blocked the activity of SRC kinases in mouse naive CD4$^+$ T cells under our experimental conditions (Fig. S1c online), decreased the CD28-mediated T cell adhesion to COS-Aβ/B7-1 cells (Fig. 1b), indicating that SRC kinase-mediated intracellular tyrosine phosphorylation events promoted 2D binding between CD28 and B7-1. Taken together, CD28 in mouse naive CD4$^+$ T cells displayed a constitutive 2D binding of low avidity with B7-1, which was regulated by the protein tyrosine phosphorylation by SRC kinases.

Recent studies conducted in re-stimulated T cell blasts [10] suggested that TCR engagement may initiate intracellular signaling that induces configurational change on the ligand-binding interface and/or synaptogenic recruitment of CD28, thus favoring CD28-dependent interaction with B7 molecules. We were therefore interested to examine whether TCR triggering regulates CD28–B7 binding in naive CD4$^+$ T cells. To that end, the TCR was stimulated with soluble anti-CD3 monoclonal antibodies (mAbs), which resulted in a significant increase in CD28-specific adhesion frequency $P_2$ between CD4$^+$ T cells and COS-Aβ/B7-1 cells (Fig. 1c). Moreover, we found that this increase was completely abolished when the naive T cells were treated with PP2, indicating that it was taking place via a mechanism dependent on SRC kinase-mediated tyrosine phosphorylation inside T cells (Fig. 1c). We next examined whether the cytoplasmic domain of CD28 (CD28CD) could be involved in the regulation of CD28 binding to its ligands. The adhesion of naive CD4$^+$ T cells from the CD28-tailless mutant knock-in (CD28TL KI; C57BL6/J (CD28$^{TLL}$)) mice with COS-Aβ/B7-1 cells was analyzed. These T cells, which mature normally in vivo as those from WT and CD28KO mice, all express a mutant form of CD28 that lacks the full cytoplasmic domain (amino acids 163–198) but displays normal surface expression and binding to soluble recombinant B7-1–Fc [11]. Such tailless CD28 (CD28TL) was found to exhibit a constitutive 2D binding to B7-1, which was nevertheless significantly weaker than that of wild-type CD28 receptor (CD28WT) (Fig. 1d). Moreover, in contrast to CD28WT, treatment of CD28TL KI T cells with PP2 did not modify their adhesion with COS-Aβ/B7-1 cells. In fact, the B7-1 binding of the CD28TL remained quantitatively the same in the presence of PP2. Our data thus indicated that CD28CD positively contributes to basal 2D interactions between CD28 and B7 ligands, a contribution that required activity of SRC family kinases in T cells. The 2D binding of CD28TL to B7-1 in conjunction with TCR stimulation by anti-CD3 mAbs was then examined, and the binding remained unchanged from basal level (Fig. 1d), in contrasts to that observed with CD28WT. This was despite that CD28TL KI T cells expressed the same amount of TCR on the surface as the wild-type counterparts (Fig. S1d online). Altogether, these data indicated that TCR engagement promotes 2D CD28–B7–1 binding in a manner that critically requires CD28CD, and the SRC family kinases, most likely Lck, regulate not only the basal but also the TCR-augmented CD28–B7–1 interactions. The low and TCR-unregulated ligand-binding activity of CD28TL could account for the residual costimulatory function that is observed in the T cells from CD28TL KI mice [11].

CD28 is a major costimulatory receptor constitutively expressed on naive T cells and is essential for the activation of naive T cells by antigen recognition. However, much knowledge on the molecular mechanism of CD28 signaling in naive T cells has largely been inferred from experiments using T cell lines or previously activated T cell blasts, often in combination with over-expression of CD28-GFP. Nevertheless, it is not clear to what extent the information obtained from these studies can be applied to the activation of naive T cells, as the early signaling steps induced upon T cell antigen recognition between naive T cells and stimulation-experienced T cells exhibit both qualitative and quantitative differences. Here, we conducted experiments directly in naive T cells with a normal expression of CD28. Future studies will need to determine the molecular basis underlying the TCR-regulated CD28–B7 2D binding mechanism in naive T cells. It is noteworthy, that in T cell blasts,
TCR signaling was observed capable of inducing a reorientation of the cytoplasmic domain of the CD28 homodimer [10] modifying its extracellular binding valence to B7 [5] or global membrane changes at the T cell and APC junction augmenting probability for CD28–B7 binding events [12]. Combining observations from both the present work on naive T and various previously published studies on other T cell types, we would like to propose a “CD28 unlocking” model for the enhanced 2D binding activity of CD28 with B7 ligands following TCR signaling in naive T cells (Fig. S2 online). Our data presented in this study thus provide new insights on the crosstalk between the TCR- and CD28-mediated signaling pathways in naive T cell activation. These findings contribute to our understanding of the mechanism of the T cell-mediated immune response, and are of interest for new therapeutic and vaccine strategies against various types of diseases, such as cancer and viral infections.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

Xiao-Jun Guo, Cheng-Rui Qian, Fan Xia designed the study, performed the experiments and analyzed the results. Anne-Marie Sarthe, Anthony Formisano, Sébastien Jaeger, Jacques A. Nunès assisted in the realization and interpretation of the experiments and provided several reagents. Xiao-Jun Guo and Hai-Tao He supervised and directed the research. Xiao-Jun Guo, Cheng-Rui Qian, Fan Xia and Hai-Tao He wrote the manuscript. All authors discussed the results and commented on the manuscript.

Appendix A. Supplementary materials

Supplementary materials to this article can be found online at https://doi.org/10.1016/j.scib.2020.08.007.

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