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CD28 confers CD4$^+$ T cells with resistance to cyclosporin A and tacrolimus but to different degrees

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Abstract

Background: Cyclosporin A (CSA) and tacrolimus (TAC) suppress T-cell activation and subsequent proliferation by inhibiting calcineurin. Though they have the same target, CSA and TAC have quite different molecular structures, indicating quantitative and/or qualitative differences in their effects.

Objective: CD28 is a costimulatory molecule that enhances T-cell activation. It has also been shown to attenuate calcineurin inhibitors. In this study, we compared the CD28-mediated resistance of CD4$^+$ T cells to those calcineurin inhibitors and tried to predict CD28's impact on infectious diseases.

Methods: CD4$^+$ T-cell proliferation was induced with anti-CD3 mAb in the presence or absence of anti-CD28 mAb *in vitro*. CSA or TAC was added at various concentrations, and the half-maximal inhibitory concentration on CD4$^+$ T-cell proliferation was determined. Effects of lipopolysaccharide (LPS) on dendritic cells (DCs) and CD4$^+$ T-cell proliferation were also evaluated *in vitro*.

Results: Anti-CD28 mAb conferred CD4$^+$ T cells with resistance to both CSA and TAC, and CD28's effect on the latter was approximately twice that on the former. LPS induced expression of CD28 ligands CD80/86 on DCs. The addition of LPS to culture containing DCs seemed to make CD4$^+$ T cells slightly resistant to TAC but not to CSA. However, its effect on the former was very weak under our experimental conditions.

Conclusion: CD28 attenuated TAC more strongly than CSA. Although LPS did not demonstrate strong enough resistance in our *in vitro* model, TAC might maintain a better antibacterial immune response than CSA in clinical use.

Key words: Cyclosporin A, Tacrolimus, Calcineurin inhibitor, CD28, T-cell activation

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Introduction

CD4$^+$ T cells express a T-cell receptor (TCR) by which they recognize an antigen (Ag). A TCR's specificity to an Ag differs for each CD4$^+$ T cell, allowing an immune response to a wide range of pathogens. On the other hand, CD4$^+$ T cells stay quiescent without binding of the TCR to the Ag; that is, the TCR engagement triggers intracellular signaling to change the state of CD4$^+$ T cells. The molecular basis by which TCR engagement leads to T-cell activation has been well documented.[1,2] First, CD3 molecules associated with the TCR are phosphorylated. These phosphorylated CD3 molecules recruit Zeta-chain-associated protein kinase 70 (ZAP-70), resulting in the assembly of a linker for activation of T cells (LAT) signalosome. The signalosome then initiates multiple downstream signalings, including those of a mitogen-activated protein kinase (MAPK), a nuclear factor-κB (NF-κB), and a Ca2$^+$-calcineurin pathway.

Among these, the calcineurin that responds to $Ca2^+$ influx dephosphorylates the nuclear factor of activated T cells (NFAT) and consequently induces its translocation from the cytoplasm to the nucleus, which is especially important for interleukin-2 (IL-2) production by activated $CD4^+$ T cells. Thus, TCR signaling is essential for T-cell activation, but it alone is insufficient[3] and an additional costimulatory signal is required. CD28 is a T-cell surface receptor, which delivers such a costimulatory signal[4] and binds to CD80 and CD86 on antigen-presenting cells (APCs).[5] Those CD28 ligands are upregulated on APCs such as dendritic cells (DCs) upon microbial infections,[6] thus assuring the T-cell response. Although IL-2-dependent and -independent roles of CD28 in $CD4^+$ T-cell activation have been identified,[6,7] their underlying molecular mechanisms are still controversial.[5]

Cyclosporin A (CSA) and tacrolimus (TAC) are potent immunosuppressants that are used clinically to prevent rejection following organ transplantation. They are derived from metabolites of microbes. First, CSA was isolated from a fungal extract and found its suppressive effects on T-cell activation[8] and IL-2 production.[9] TAC was later isolated from a culture fluid of Streptomyces and found to be even more effective than CSA.[10,11] Though they are both antibiotics, their chemical structures are quite different. Moreover, they require distinct intracellular receptors called immunophilins: cyclophilin for CSA[12] and FK-506 binding protein (FKBP) for TAC.[13] Nevertheless, these complexes inhibit the same intracellular target, calcineurin.[14,15] The crystal structures of the ternary complexes of those immunophilins, immunosuppressants, and calcineurins revealed that both the CSA-cyclophilin and TAC-FKBP complexes bind to the same composite surface consisting of a catalytic subunit and a regulatory subunit of the calcineurin.[16] These investigations also identified several unique positions for each complex,[16] indicating that CSA and TAC might act differently on $CD4^+$ T cells.

CD28 not only enhances $CD4^+$ T-cell activation but also confers $CD4^+$ T cells with resistance to CSA.[17] Despite the structural difference between CSA and TAC, they have never been directly compared for their sensitivity to the effect of CD28. In this study, we focused on the CD28-mediated resistance to calcineurin inhibitors and compared the potency of CD28 between CSA and TAC. The effects of lipopolysaccharide (LPS) on DCs and $CD4^+$ T-cell proliferation were also evaluated.

Methods

T-cell culture and immunosuppressants

Lymph nodes (LNs: cervical, axillary, brachial, inguinal, mesenteric, periaortic, and pancreatic) were isolated from ddY mice aged 8-10 weeks. The mice had been euthanized according to the guidelines of the Animal Care and Use Committee of Kanazawa University. LNs were crushed to make a single cell suspension.[18,19] LN cells were labeled for 15 min at 37°C with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR, USA) to track mitotic divisions of T cells following stimulation. After 3 washes with PBS containing 5% heat-inactivated fetal bovine serum (FBS), CFSE-labeled LN cells were inoculated at 1.5×10^6 cells/ml along with the same density of unlabeled spleen (SPL) cells prepared from the same mice. The cells were co-cultured in RPMI1640 containing 10% FBS supplemented with gentamycin (10 μg/ml) and stimulated with anti-CD3 monoclonal antibody (mAb, 1 μg/ml), which had been obtained from hybridoma 145-2C11,[20] for 48 h. To provide a costimulatory signal, anti-CD28 mAb from hybridoma PV1[21] was added to the culture at a concentration of 2 μg/ml. In some experiments, LPS (Sigma-Aldrich, St Louis, MO, USA) was added at a concentration of 100 μg/ml instead of anti-CD28 mAb. CFSE-labeled LN cells (7×10^6 cells/ml) from ddY mice were also co-cultured with unlabeled SPL cells (3.5×10^6 cells/ml) from BALB/c mice for 6 days. In this mixed lymphocyte reaction (MLR), $CD4^+$ T cells responded to allogeneic stimulation, and subsequent proliferation was detected by CFSE as well. To evaluate the efficacy of immunosuppressants, CSA (Wako Pure Chemical, Osaka, Japan) or TAC (Cayman Chemical, Ann Arbor, MI, USA) was added at various concentrations from the start of culture. The half-maximal inhibitory concentration (IC_{50}) was obtained from the dose-response curve. In some experiments, methotrexate (MTX, ALEXIS Biochemicals, San Diego, CA, USA) was also tested as an immunosuppressant with a mechanism of action different from those of CSA and TAC.

FACS and CFSE analyses

The cells were harvested from the culture and first treated with anti-FcγR II/III mAb from hybridoma 2.4G2[22] to reduce nonspecific antibody binding. The cells were then stained with PE-labeled anti-CD4 mAb (GK1.5, BioLegend San Diego, CA, USA). The cells were finally treated with 7-amino-actinomycin D (7-AAD, ENZO Life Sciences, Farmingdale, NY, USA) or propidium iodide (PI, Sigma-Aldrich) to exclude dead cells from the analysis. The CFSE profile of $CD4^+$ T cells was obtained by flow cytometry using FACSverse (BD Biosciences, San Jose, CA, USA), and the mean division number was determined by calculating the percentage contribution of the initial cohort in each division peak.[18]

In order to determine the upregulation of costimulatory molecules on DCs following LPS stimulation, SPL cells were cultured in the presence of LPS (100 μg/ml) for 18 h. The cells were harvested and treated with anti-FcγR II/III mAb. The cells were then stained with biotin-labeled anti-CD11c mAb (HL3, BD Biosciences) followed by APC-labeled streptavidin (SouthernBiotech, Birmingham, AL, USA). The cells were also stained with either FITC-labeled anti-CD80 mAb (16-10A1, BioLegend), FITC-labeled anti-CD86 mAb (GL-1, BioLegend), or FITC-labeled anti-CD40 mAb (HM40-3, BD Biosciences) to evaluate the expression of each molecule on the $CD11c^+$ DCs. The cells were finally treated with PI (Sigma-Aldrich) and analyzed by FACS.

Statistical analysis

Statistical tests were carried out between CD4⁺ T cells stimulated with or without anti-CD28 mAb using Student's independent t-test. The results were considered significant at $P < 0.05$.

Results

CD28 signaling confers CD4⁺ T cells with resistance to TAC as well as CSA, but to different degrees

CD28 signaling was first shown to make CD4⁺ T cells resistant to a calcineurin inhibitor using CSA.[17] TAC was later developed as an immunosuppressant with the same target: calcineurin.[14,15] In this study, we compared the efficacy of the CD28-mediated resistance to these compounds using CD4⁺ T-cell proliferation induced with anti-CD3 mAb in the presence or absence of anti-CD28 mAb *in vitro* (**Figure 1**). When CSA was added to the culture, CD4⁺ T-cell proliferation induced by anti-CD3 mAb alone was suppressed in a dose-dependent manner and its IC_{50} was calculated to be 65.5 nM (**Figure 1a, Table 1**). Like CSA, TAC also suppressed anti-CD3 mAb-induced CD4⁺ T cell-proliferation, but its effect was much stronger than that of CSA (**Figure 1b**). From the IC_{50} of TAC, which was calculated to be 2.9 nM, the difference reached around 20-fold (**Table 1**). Simultaneous stimulation of CD28 along with CD3 indeed made CD4⁺ T cells resistant to CSA, reproducing previous results; and the IC_{50} increased to 430.6 nM. This effect was also true for TAC, and its IC_{50} increased to 33.3 nM (**Table 1**). Here, CD28 costimulation increased the IC_{50} of TAC more than it did that of CSA (fold resistance: 11.5 vs 6.6). In contrast, CD28 signaling showed no effect on the efficacy of MTX (IC_{50}: 24.6 nM vs 35.2 nM), which suppresses CD4⁺ T-cell proliferation via a different mechanism from those of CSA and TAC.

Table 1. The half-maximal inhibitory concentration (IC_{50}) and its difference compared to that of anti-CD3 mAb alone

		CSA (nM)	TAC (nM)	MTX (nM)
Exp.1	anti-CD3	65.5	2.9	35.2
	anti-CD3 + anti-CD28	430.6	33.3	24.6
	Fold resistance*	6.6	11.5	0.7
Exp.2	anti-CD3	69.0	4.3	
	MLR	119.2	5.7	
	Fold resistance*	1.7	1.3	
Exp.3	anti-CD3	48.2	5.2	
	anti-CD3 + LPS	61.2	11.3	
	Fold resistance*	1.3	2.2	

*Fold resistance was calculated by dividing each IC_{50} by that of anti-CD3 mAb alone.

These results not only show a unique interaction between CD28 signaling and the calcineurin pathway, but also suggest that it affects CSA and TAC in a quantitatively different manner.

CSA and TAC suppress MLR-induced CD4⁺ T-cell proliferation to a similar extent as that induced with anti-CD3 mAb alone

CSA and TAC are used to prevent organ graft rejection in patients following transplantation. At the time of transplantation, or probably soon after, host CD4⁺ T cells initiate a response via recognition of a donor major histocompatibility complex (MHC) polymorphism without events that lead to the induction of CD28 ligands.

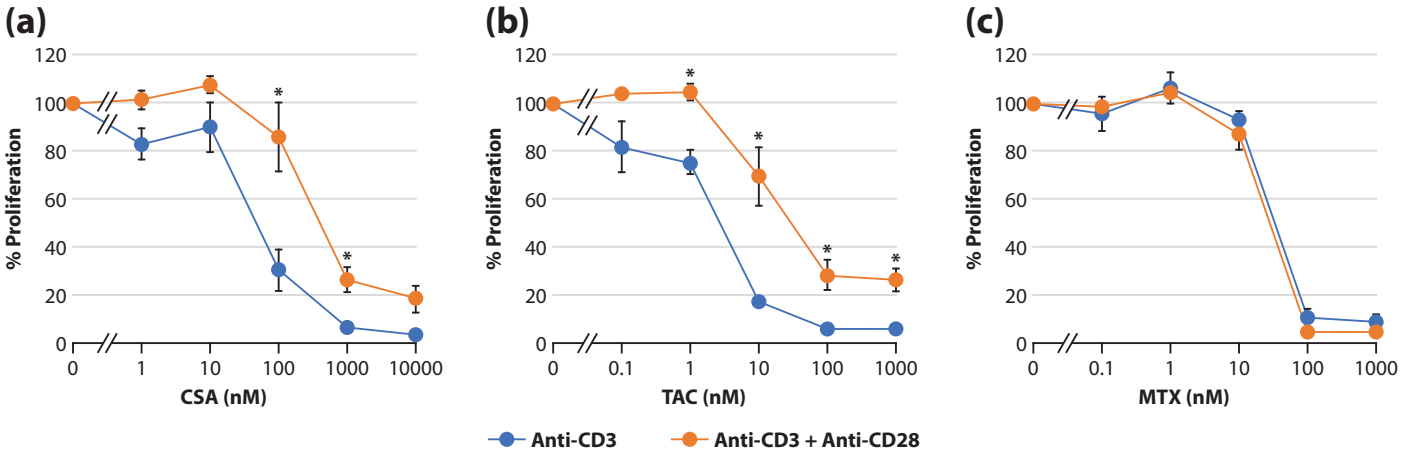

Figure 1. CD28 signaling confers CD4⁺ T cells with resistance to CSA and TAC, but not to MTX.
LN cells were labeled with CFSE and combined with SPL cells. The cells were stimulated with anti-CD3 mAb (1 μg/ml) in the presence (●) or absence (●) of anti-CD28 mAb (2 μg/ml) for 48 h. CSA (a), TAC (b), or MTX (c) was added at various concentrations and the mean division number was determined as described in Methods. Percentage proliferation was calculated by dividing each mean division number by that of the control group (no immunosuppressant) and multiplying by 100. The average ± SEM from three independent experiments is shown. *$P < 0.05$, significantly different from the proliferation without anti-CD28 mAb.

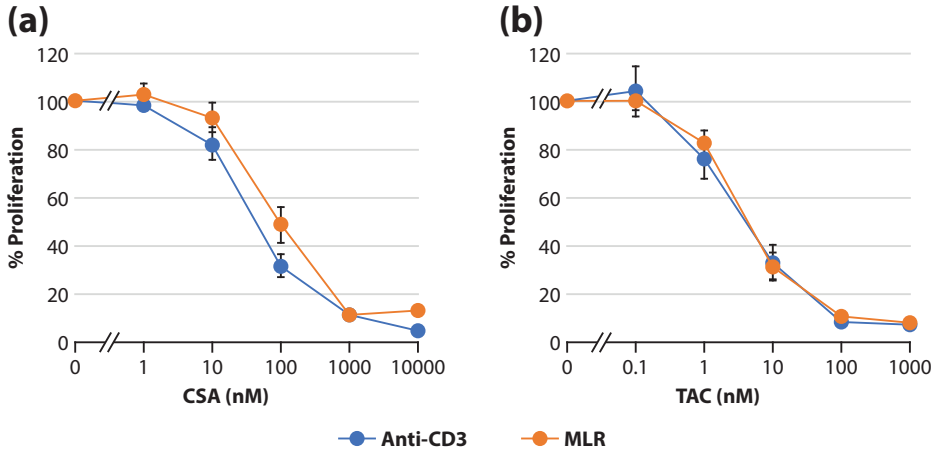

Figure 2. CD4⁺ T cells responding to MLR are as sensitive to CSA and TAC as those stimulated with anti-CD3 mAb alone. LN cells from ddY mice were labeled with CFSE and combined with SPL cells from either the same mice (●) or BALB/c mice (●). CD4⁺ T cells in the former were stimulated with anti-CD3 mAb (1 μg/ml) for 48 h, while nothing was added to the latter, which was cultured for 6 days (MLR). CSA (a) or TAC (b) was added at various concentrations and the mean division number was determined as described in Methods. The percentage proliferation was calculated as in Figure 1, and the average ± SEM from five independent experiments is shown.

In order to evaluate the efficacy of CSA and TAC in transplantation, CD4⁺ T cells responding to MLR were treated with various concentrations of either CSA or TAC and examined for their effects on proliferation (**Figure 2**). CSA suppressed CD4⁺ T-cell proliferation with an IC_{50} of 119.2 nM. On the other hand, the IC_{50} of TAC was 5.7 nM (**Figure 2, Table 1**). These IC_{50} values were roughly equivalent to those obtained by CD4⁺ T cells stimulated with anti-CD3 mAb alone. These results suggest that CD4⁺ T cells responding to MLR are activated under an insufficient intensity of CD28 signaling.

LPS induces various costimulatory molecules including CD28 ligands on DCs

Since CD28 signaling attenuated TAC more than CSA, a T-cell response to bacterial infections, in which CD28 ligands are upregulated via Toll-like receptors (TLRs), might be better preserved to TAC than to CSA. To test this hypothesis, we first examined whether LPS, which is a component of the cell wall of Gram-negative bacteria and shown to stimulate DCs via TLR4,[23] induces CD28 ligands on DCs in our *in vitro* culture (**Figure 3**). After 18 h of the culture in the presence of LPS, both CD80 and CD86 were strongly expressed on CD11c⁺ DCs.

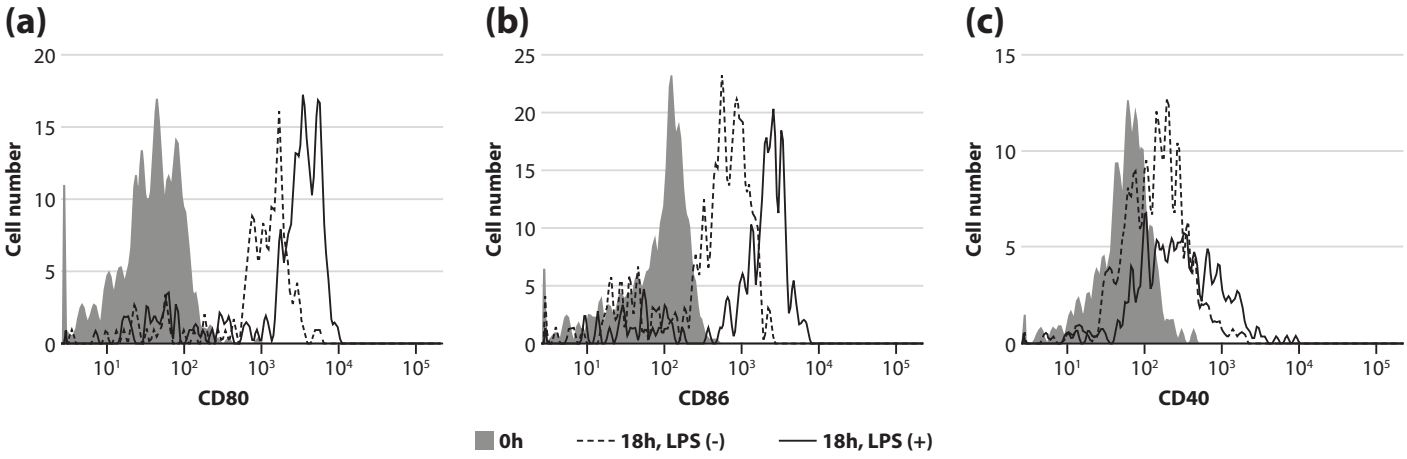

Figure 3. LPS induces expression of CD80, CD86, and CD40 on DCs. SPL cells were cultured in the presence (solid lines) or absence (dotted lines) of LPS (100 μg/ml) for 18 h. The cells were harvested and expression of CD80 (a), CD86 (b), or CD40 (c) was determined on CD11c⁺ DCs by FACS. SPL cells were also analyzed immediately after isolation (gray histograms). The representative data from three independent experiments is shown.

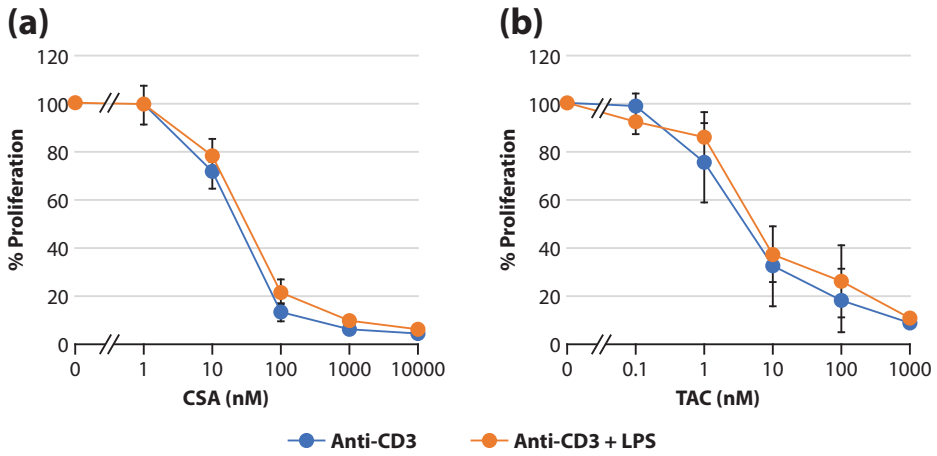

Figure 4. LPS seems to make CD4⁺ T cells slightly resistant to TAC but not to CSA, although the effect on the former was marginal in our culture conditions. LN cells were labeled with CFSE and combined with SPL cells. The cells were stimulated with anti-CD3 mAb (1 μg/ml) in the presence (●) or absence (●) of LPS (100 μg/ml) for 48 h. CSA (a) or TAC (b) was added at various concentrations and the mean division number was determined as described in Methods. The percentage proliferation was calculated as in Figure 1, and the average ± SEM from 3 (a) or 4-7 (b) independent experiments is shown.

However, compared to DCs immediately after isolation, significant expression of CD80 and CD86 was observed on DCs after 18 h of culture in the absence of LPS. These features were also the same with CD40. These results suggest that DCs change their state during culture and induce a substantial amount of CD28 ligands. Nevertheless, LPS clearly stimulates DCs and induces cell surface molecules including CD28 ligands.

LPS makes CD4⁺ T cells slightly resistant to TAC, but its effect is marginal

LPS induced the expression of CD80 and CD86 on DCs in our *in vitro* culture model. We next examined whether those CD28 ligands confer CD4⁺ T cells with resistance to CSA and TAC (**Figure 4, Table 1**). When CD4⁺ T cells were stimulated with anti-CD3 mAb in the presence of SPL cells along with LPS, their proliferation seemed to be slightly resistant to TAC. The IC_{50} (11.3 nM) was 2.2-fold higher than that without LPS (5.2 nM). On the other hand, the IC_{50} of CSA in the presence of LPS was 61.2 nM, which was only 1.3-fold higher than that in the absence of LPS (48.2 nM). Thus, LPS-mediated expression of CD28 ligands might attenuate TAC and to a lesser extent CSA, but to a much smaller extent than that of anti-CD28 mAb.

Discussion

We stimulated CD4⁺ T cells under various conditions and compared the effects of CSA and TAC on their proliferation. CSA and TAC suppressed CD4⁺ T-cell proliferation induced with anti-CD3 mAb in a dose-dependent manner, and their IC_{50} values were determined to be 48.2-69.0 nM for CSA and 2.9-5.2 nM for TAC. These ranges are slightly lower than therapeutic blood concentrations, which are 200-400 μg/ml (167-333 nM) for CSA and 5-15 μg/ml (6-18 nM) for TAC.[24-26] The therapeutic blood concentration would depend on multiple *in vivo* factors, including metabolism, excretion, and distribution. CSA is a cyclic peptide, while TAC is a macrocyclic lactone, and they are metabolized mainly by CYP3A4 and CYP3A5, respectively.[27] Some metabolites retain different immunosuppressive activities. Furthermore, an individual T cell expresses P-glycoprotein, which pumps out drugs from the cell. The metabolites derived from CSA and TAC might have different sensitivities to this efflux transporter. In addition, drug concentrations at the place where CD4⁺ T cells are activated may be slightly different from the blood concentrations. For all these reasons, the IC_{50}s are likely inconsistent with the therapeutic blood concentrations. Nevertheless, these CSA and TAC IC_{50}s suggest that anti-CD3 mAb-induced CD4⁺ T-cell proliferation *in vitro* can be a good tool for evaluating the efficacy of calcineurin inhibitors. When LPS was added to the culture, it did not confer CD4⁺ T cells with resistance clearly to the calcineurin inhibitors. Although LPS induced the expression of CD28 ligands on DCs, CD80 and CD86 were substantially upregulated without LPS in our culture conditions (**Figure 3**). In order to make the experimental conditions the same, SPL cells containing DCs were always co-cultured with CD4⁺ T cells. Therefore, CD4⁺ T cells received some CD28 signals even in the absence of LPS, making its effect difficult to detect. In contrast, anti-CD28 mAb conferred CD4⁺ T cells with resistance clearly to the calcineurin inhibitors. These results not only reveal the effect of CD28 signaling but also suggest that CD4⁺ T cells do not receive optimal CD28 signals from DCs in *in vitro* culture, which allows a limited number of CD28 molecules to engage with the ligand under insufficient numbers of T-DC cell contacts. MLR, which contains allogeneic SPL cells, could also provide CD4⁺ T cells with some CD28 signals, but they are likely insufficient as well.

When anti-CD28 mAb in addition to anti-CD3 mAb was added to the culture, $CD4^+$ T-cell proliferation became resistant to CSA, confirming the previous result.[17] Although slightly different in potency compared to CSA, the anti-CD28 mAb also attenuated TAC. In contrast, MTX was unaffected by the anti-CD28 mAb. These results suggest that CD28 signaling cross-talks somewhere along the $Ca2^+$-calcineurin pathway. With regard to the signal transduction downstream of CD28 whose cytoplasmic tail has no enzymatic activity, several cytoplasmic motifs recruit a variety of molecules in both phosphorylation-dependent and -independent manners, ultimately leading to the activation of transcription factors NFAT, AP-1, and NF-κB.[5,28] Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine kinase that is involved in CD28 signaling[29,30] and that contributes to NFAT activation.[31] NFAT stays in the cytoplasm in resting T cells; it is located in the nucleus following T-cell activation and induces the transcription of target genes. This translocation is induced via the dephosphorylation of NFAT by calcineurin upon T-cell activation. GSK-3β has been shown to rephosphorylate NFAT, inducing its egress from the nucleus and thus terminating transcriptional activity.[31] CD28 signaling inactivates GSK-3β, thereby maintaining NFAT in the nucleus and its transcriptional activity. Thus, CD28 signaling exerts the opposite effect of calcineurin inhibitors, which reduce NFAT entry into the nucleus. Although these mechanisms could explain how CD28 signaling counteracts calcineurin inhibitors, it is difficult to explain why CD28 signaling confers $CD4^+$ T cells with resistance to both CSA and TAC but to different degrees. GSK-3β has been shown to regulate expression of PD-1 on $CD8^+$ T cells.[32] If GSK-3β functions in $CD4^+$ T cells as well, CD28 could downregulate PD-1 and influence subsequent TCR signaling. Thus, CD28 may cross-talk with TCR via PD-1. Besides GSK-3β, VAV and SLP-76, a 76 kDa SH2 domain-containing leukocyte protein, have been found to regulate the function of NFAT downstream of CD28.[33] CD28 assembles a complex of VAV and SLP-76, which could be mediated by growth factor receptor bound protein-2 (Grb-2),[34] and induces NFAT translocation from the cytoplasm to the nucleus. Moreover, IL-2 promoter activation via VAV/SLP-76 is sensitive to CSA. These results suggest that CD28-VAV/SLP-76 signaling intersects with TCR signaling at a point upstream of the calcineurin. On the other hand, CSA and TAC have been shown to inhibit MAPKs.[27] The different sensitivities of $CD4^+$ T cells to CSA and TAC might be attributable to the MAPK pathway, but not to the $Ca2^+$-calcineurin pathway. To resolve this, it would be necessary to elucidate the molecular mechanism underlying the CD28-mediated resistance of $CD4^+$ T cells to CSA and TAC.

Conclusion

In this study, we have found that CD28 confers $CD4^+$ T cells with stronger resistance to TAC than to CSA. Suppression of an immune response to foreign pathogens is a crucial side effect of immunosuppressants. As many of those pathogens induce the expression of CD28 ligands on APCs, TAC might preserve a better antimicrobial immune response than CSA in clinical use.

Disclosure

The authors have no conflicts of interest to disclose.

Author contributions

- H.K., F.Y., and A.T. performed the experiments.
- T. M. and M.I. designed the study.
- M.I. wrote the manuscript.
- All authors contributed significantly to this study.

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