AOP ID and Title:

SNAPSHOT

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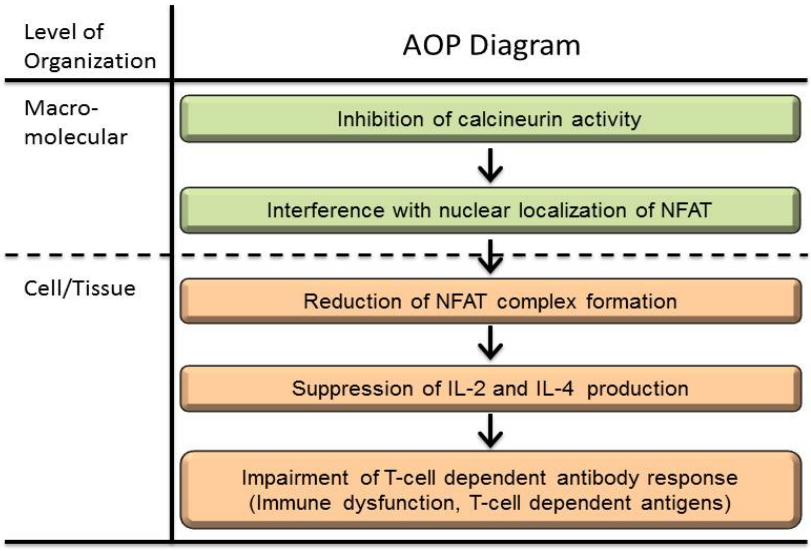
AOP 154: Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response

Short Title: Immunosuppression

Graphical Representation



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Status

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Abstract

Calcineurin (CN) is a type of protein phosphatase that is known to impair immune function when its phosphatase activation is inhibited. The relationship between CN and immune functions is well understood, and immunosuppressants that work by inhibiting CN have been developed.

CN inhibitors (CNIs) suppress many kinds of immune functions leading to increased susceptibility to infections. T-cell dependent antibody response (TDAR) is considered to be the most important endpoint on evaluating immunotoxicity of chemicals; therefore, this AOP describes the linkage between the inhibition of CN activity and impairment of TDAR.

CN activity is inhibited when stressors of CNIs bind to immunophilins, which interferes with the nuclear localization of nuclear factor of activated T cells (NFAT), a substrate of CN. As a result, the formation of functional NFAT complexes that bind at the site of IL-2, IL-4 and other T cell - derived cytokine promoters is reduced, thereby suppressing production of these cytokines. Thus, TDAR is impaired mainly by the suppression of production of IL-2 and IL-4, which affects the proliferation and differentiation of B-cells.

We have identified a number of key events along this pathway and determined the key event relationships, based on which we have created an AOP for inhibition of CN activity leading to impaired TDAR.

Since CN expresses in cells among vast variety of species, this AOP is applicable to many mammal species, including humans and rodents.

Background

Although there are numerous stressors that inhibit CN activity, this AOP is mainly based on an understanding of immunosuppression caused by FK506 and FKBP12 complexes, on which a significant body of scientific literature has been published.

We look forward to future amendments to this AOP with up-to-date information on other stressors, which will more clarify the linkage between inhibition of CN activity and impairment of TDAR.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	980	Inhibition, Calcineurin Activity (https://aopwiki.org/events/980)	Inhibition, Calcineurin Activity
2	KE	979	Interference, nuclear localization of NFAT (https://aopwiki.org/events/979)	Interference, nuclear localization of NFAT
3	KE	981	Reduction, NFAT complex formation (https://aopwiki.org/events/981)	Reduction, NFAT complex formation
4	KE	1202	Suppression, IL-2 and IL-4 production (https://aopwiki.org/events/1202)	Suppression, IL-2 and IL-4 production
5	AO	984	Impairment, T-cell dependent antibody response (https://aopwiki.org/events/984)	Impairment, T-cell dependent antibody response

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Interference, nuclear localization of NFAT (https://aopwiki.org/relationships/1017)	adjacent	Reduction, NFAT complex formation	High	High
Reduction, NFAT complex formation (https://aopwiki.org/relationships/1509)	adjacent	Suppression, IL-2 and IL-4 production	High	High
Suppression, IL-2 and IL-4 production (https://aopwiki.org/relationships/1510)	adjacent	Impairment, T-cell dependent antibody response	High	High
Inhibition, Calcineurin Activity (https://aopwiki.org/relationships/1508)	adjacent	Interference, nuclear localization of NFAT	High	High

Stressors

Name	Evidence
Tacrolimus	
Cyclosporin	

Overall Assessment of the AOP

CN activity is inhibited when stressors bond with immunophilins, which interferes with the nuclear localization of NFAT, a substrate of CN. As a result, the formation of functional NFAT complexes that bind at the site of IL-2 and other cytokine promoters is reduced, thereby suppressing production of these cytokines. Thus TDAR is impaired mainly by the suppression of production of IL-2 and IL-4, which affect the proliferation and differentiation of B-cells. We have identified a number of key events (KEs) along this pathway, and based on these key event relationships (KERs), created an AOP for inhibition of CN activity leading to impaired TDAR.

Since each of the KEs involving MIE and AO is measurable quantitatively and shows clear dose responses with the CNIs, this AOP is useful for understanding of immunosuppression derived from CN activity inhibition. In addition, each of the KERs is based on sufficient scientific evidence and there is no contradiction found between dose responses of the adjacent KEs.

Since CN expresses in cells among vast variety of species, this AOP is applicable to many mammal species, including humans and rodents.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Macaca fascicularis	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Mixed	High

The proposed AOP of inhibited CN activity leading to impaired TDAR is not associated with life stage-, sex-, or age-dependency. The relevant life stages for the AOP are from child to adult, and since tacrolimus (FK506) ointment (Protopic) is approved for pediatric atopic dermatitis, the MOA for immunosuppression appears to be applicable to all of life stages. Since FK506 or CsA -induced outcomes in humans are mimicked by similar responses in a variety of animal models, immunosuppression induced by immunophilin-CNI complexe is considered to be preserved across a variety of mammalian species.

Essentiality of the Key Events

MIE and later events: CnA-knockout (KO) mice

The CN molecule consists of two regions, CnA and CnB, and CnA holds phosphatase activity. In CnA KO mice, T cell proliferation in response to ovalbumin stimulation is lower than for wild-type mice and is not complemented by normal antibody producing cells1. In addition, when stimulated with ovalbumin, CnA KO mice produce less IFN-γ, IL-2, and IL-4 than those in wild-type mice.1 However, primary antibody response in CnA-/- mice is normal in response to TNP-ovalbumin.

Stressor: FKBP12-KO mice

There is no evidence of a relationship between FKBP12 KO and the immune system in the FKBP12 KO mouse model. Steric structure of FKBP12/FK506 complex is the key factor for inhibition of CN phosphatase activity, but not the enzymatic activities of FKBP12.

KE1 and later events: NFAT-KO mice

The following phenotypes are observed in NFAT KO mice2 : moderate hyperproliferation with splenomegaly; moderately enhanced B- and T-cell responses, with bias towards Th2-cell responses; decreased IFN-γ production in response to TCR ligation; reduced proliferative responses by T cells; impaired repopulation of the thymus and lymphoid organs; impaired Th2- cell responses and IL-4 production; grossly impaired T-cell effector functions, profound defects in cytokine production and cytolytic activity; B-cell hyperactivity; impaired development of CD4 and CD8 single-positive cells, increased apoptosis of double -positive thymocytes; mild hyperactivation of peripheral T cells.

Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune-cell phenomenon, and some of these phenomenon are known to be regulated by CN. This indicates that the production of T-cell derived cytokine is regulated by the CN-NFAT system.

Weight of Evidence Summary

Biological Plausibility

T cell functions are regulated by CN-NFAT system and the suppression of CN activity in T cell is well known to induce multiple types of immunosuppression including TDAR.

Experiments with T cells indicates that T cell receptor (TCR) stimulation brings about intracellular increases in concentrations of Ca2+, which triggers CN activity, thereby inducing nuclear localization of its substrate NFAT per dephosphorylation, which forms complexes with activator protein 1 (AP-1) at the promoter sites of the T-cell cytokine genes and induces production of the cytokines.

CN phosphatase activity is well known to be inhibited due to the formation of immunophilin-CNI complexes, such as CsA/cyclophilin complexes or FK506/FK506- binding protein (FKBP) 12 complexes. Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity, but there is no commonality between their two amino acid sequences. The three-dimensional structures of immunophilin complexes, but not these enzyme activities, are essential to the inhibition of CN phosphatase activity.

It is also well known that one of the effects on immune function when FK506 forms complexes with immunophilins and inhibits CN activity is the suppression of IL-2 and other T-cell derived cytokine production. It is further well known that inhibition of CN leads to suppression of TDAR because IL-2 and IL-4 mainly promote the proliferation, class switching, differentiation, and maturation of B-cells.

Furthermore, CN-NFAT also exists in B-cells and it has been reported that CNIs do suppress production of certain cytokines from them; though, at the time of our review of the literature, we did not find any reports of a direct effect of CN inhibition on B-cells such as changes in proliferations, class switching, differentiation, or maturation of B-cells.

Also, although CN-NFAT is known to exist in dendritic cells, natural killer T (NKT) cells, and other types of cells, where it regulates the expression of IL-2 receptors, there are no reports of effects on the production of antibodies due to altered expression of IL-2 receptors in these cells.

KER	KE_{up}-KE_{down}	Plausibility	Rationales supported by literatures
KER1	CN inhibition to interference, NFAT nuclear translocation	Strong	T cell functions are regulated by CN-NFAT system. Activated CN through TCR stimulation dephosphorylates NFAT to promote its nuclear localization. CN in T cells is inhibited by CNI/immunophilin complexes, which does not induce dephosphorylation of NFAT and following its nuclear localization.
KER2	Interference, nuclear localization to reduction, NFAT complex formation	Strong	Activated CN dephosphorylates NFAT to promote its nuclear translocation. Nuclear-located NFAT binds with AP-1 at the promoter regions of the cytokine genes to promote T-cell cytokine production. Inhibition of dephosphorylation of NFAT by CNIs prevents nuclear localization of NFAT and resultant binding with AP-1 at the promoter region of the T cell cytokine genes.
KER3	Reduction, NFAT complex formation to suppression of IL-2 and IL-4 production	Strong	NFAT/AP-1 complexes bind to the promoter regions of the cytokine genes leading to produce these cytokines from T cells. Among these cytokines, IL-2 and IL-4 have a major role in promoting proliferation, maturation and class-switching of B cells, and induction of TDAR. Reduced NFAT complex formation in the nucleus induced by CNIs suppresses production of T-cell derived cytokines including IL-2 and IL-4.

KER4	Suppression of IL-2 and IL-4 production to impaired TDAR	Strong	Inhibition of CN by CNIs is known to suppress production of multiple cytokine species from T cells and other populations of immune cells and in some cases IL-2 receptor expression. Among these cytokines and receptors, suppression of IL-2 and IL-4 production by CNIs are known to affect proliferation, maturation and class switching of B cells leading to impairment of TDAR.. Other cytokines show only minor effects, if any, on TDAR in cases where their production is suppressed through inhibition of CN activity.

Empirical Support

MIE Inhibition, calcineurin activity

Phosphatase activity of CN can be measured quantitatively using a phosphatase assay.

In the phosphatase assay for CN, addition of 1 molar equivalent of FKBP-FK506 complex inhibited over 80% of the CN protein phosphatase activity (mixed with 300 nM calmodulin-CN and 30 µM FK506) (Liu et al. 1992). Dose-response analysis of the effects of FK506 on CN-mediated phosphatase activity in KiSVMC4W cells showed that increased expression of FKBP12 resulted in a greater than ten-fold increase in the sensitivity of the KiSV-MC4W cells containing human FKBP12 cDNA to FK506-mediated inhibition of CN phosphatase activity, as indicted by an IC50 value of ~3 nM (Fruman et al.1995). The phosphatase assay showed that FK506 inhibition of CN activity was concentration-dependent and that IC50 values for CN inhibition were approximately 0.5 nM for FK 506 (Fruman et al.1992).

KE1:Interference, nuclear localization of NFAT

Interference with the translocation of NFAT to the nucleus can be detected quantitatively using a gel mobility shift assay.

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing CNI concentrations up to 1 µM (1000 nM). Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again at maximal concentrations of 1 µM (Maguire et al. 2013).

KE2:Reduction, NFAT complex formation

Activated NFAT that has localized to the nucleus binds cooperatively at the site of the IL-2 promoter with activator protein AP-1, which is a heterodimer comprising a Fos and a Jun protein (Schreiber and Crabtree 1992, Jain et al. 1992 , thereby inducing transcription of IL-2 (Jain et al. 1993). FK506 hinders the formation of the functional NFAT complexes necessary to binding at the site of IL-2 promoters by interfering with nuclear localization of NFAT (Flanagan et al. 1991). Concentration-dependent reduction of in vitro nuclear localization of NFAT by FK506 was evident at the maximum concentration of 1µM (Maguire et al. 2013).

KE3:Suppression, IL-2 and IL-4 production

Quantification of cytokine content can be measured using a Sandwich ELISA kit, and cytokine mRNA levels can be determined using a RiboQuant MutiProbe RPA system (PharMingen, San Diego, CA).

FK506 suppressed production of IL-2, IL-4, and IFN-γ in human T cells stimulated with anti- CD3 mAb in the presence of PMA at 1.2 and 12.5 nM, and inhibited expression of IL-2, IL-4, and IFN-γ mRNA expression in anti-CD3/PMA-activated cells at 10 nM (Dumont et al. 1998). FK506 or CsA suppressed production of IL-2 in mouse and human MLR from 0.1 to 10 nM of FK506 or 10 to 100 nM of CsA (Kino et al. 1987a).

KE4:Impairment, T-cell dependent antibody response

Total IgM and IgG levels as well as antigen-specific antibodies can be determined in vitro and in vivo. The effects on immunoglobulin class switching can also be evaluated in vitro. These all parameters can be determined quantitatively.

In vitro: T cells and B cells isolated from human PBMC were co-cultured with a CNI for nine days in the presence of polyclonal T cell stimulation, after which supernatants were tested for immunoglobulin IgM and IgG levels using a Sandwich ELISA kit. FK506 or CsA reduced the levels of IgM and IgG at the concentrations of 0.3 and 1.0 ng/mL or 50 and 100 ng/mL (Heidt et al, 2009).

SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) were cultured with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. After culturing for four days, IgM produced in the culture supernatants was measured using an ELISA kit. FK506 or CsA reduced the levels of IgM at the concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma et al. 2001b).

In vivo: FK506 reduced serum concentration of anti-KLH antibodies IgM and IgG in rats treated with FK506 at 3 mg/kg for over four weeks and immunized with KLH (Ulrich et al. 2004). FK506 or CsA reduced antigen-specific plaque -forming splenocytes in mice treated with FK506 or CsA for 4 days and immunized with SRBC from 3.2 to 100 mg/kg of FK506 or 32 to 100 mg/kg of CsA (Kino et al. 1987b).

Class switching: T cells derived from human PBMCs were cultured with CNI, and cytokine mRNA levels of IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-13, and other B cell stimulatory cytokines produced in T cells were measured by quantitative PCR. FK506 suppressed production of IL-2, IL-4, and IFN-γ in human T cells stimulated with anti-CD3 mAb in the presence of PMA at 1.2 and 12.5 nM, and inhibited expression of IL-2, IL-4, and IFN-γ mRNA expression in anti- CD3/PMA-activated cells at 10 nM (Dumont et al. 1998).

KER	Empirical support of KERs

MIE=>KE1 Inhibition, calcineurin activity leads to interference, nuclear localization of NFAT	Empirical support of the MIE => KE1 is strong. Rationale Many experimental data support the inhibition of CN activity induced by CNI - immunophilin complexes and following suppression of nuclear localization. CN phosphatase activity is inhibited by CNI of FK506 with IC50 values of 0.5 – 30nM and 80% suppression at 30 µM. Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the maximum concentration of 1µM. Dose responses and temporality deem to be similar between these two key events.
KE1=>KE2 Interference, nuclear localization of NFAT leads to reduction, NFAT complex formation	Empirical support of the KE1 => KE2 is strong. Rationale The relationship between interfered nuclear localization of NFAT and resultant reduced NFAT complex formation is well known by the sufficient experiments. There have been no data on the direct measurement of NFAT/AP-1 complex bound at the promoter sites of cytokine genes in the presence of CNIs; however, the amounts of NFAT/AP-1 complexes and the transcribed mRNAs are expected to be the alternative parameters. Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the maximum concentration of 1µM. NFAT/AP-1 complexes at the site of cytokine promoters is dependent on CNI dosage. Dose responses and temporality deem to be similar between these two key events.
KE2=>KE3 Reduction, NFAT complex formation leads to suppression, IL-2 and IL-4 production	Empirical support of the KE2 => KE3 is strong. Rationale It is well known that inhibition of NFAT complex formation composed of NFAT, AP-1 at the promoter sites reduces the production of T cell cytokines including IL-2 and IL-4, which are mainly involved in T cell -dependent antibody response. NFAT/AP-1 complexes at the site of cytokine promoters is dependent on CNI dosage. In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA in a dose-dependent (10 nM) manner. (Dumont et al. 1998). Dose responses and temporality deem to be similar between these two key events.

KE3=>AO: Suppression, IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response	Empirical support of the KE3 => AO is strong. Rationale CN-NFAT system functions in many cell types throughout the body including T cells, B cells and other immune cells, and inhibition of CN-NFAT by CNIs affects production of cytokines from these immune cells. Among these cytokines, it is well known that reduced production of IL-2 and IL-4 from T cells plays a major role in CNI-induced suppression of TDAR, and our present literature research showed that decreased production of cytokines other than the two showed only minor effect on TDAR. After 9-day culture of B cells and non-pre-activated T cell stimulation with FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL of FK506 or 50 and 100 ng/mL of CsA. After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL of FK506 or 0.1 to 1000 ng/mL of CsA. Rats were treated with FK506 for over four weeks and immunized with KLH, after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day. Mice were treated with FK506 or CsA for 4 days, and immunized with SRBC, after which antigen-specific plaque -forming splenocytes reduced at the dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA. In vitro class switching; in CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA at the concentrations of 10 nM. FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA. In vitro suppressions of T cell -derived cytokines and T cell -dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs, however, time gaps were found between the two events, which showed earlier onset of cytokine production and delayed onset of antibody production.

Quantitative Consideration

In the present AOP, information on the effects of CN inhibition depends mainly on the findings for one of the CNIs, FK506; therefore, the relationships between two KEs in each of the KERs could be evaluated based on the dose responses to FK506.

As described in the Empirical Support, each of the MIE, KEs and AO except for KE2, are able to be measured directly and quantitatively; accordingly, clear dose responses to CNIs could be found in each of the KEs.

As for KE2, there have been little data on the amounts of NFAT complex formation at the cytokine promoter sites influenced by CNIs; however, NFAT/AP-1 complex formation or mRNA levels of related cytokines are measurable and served as the alternative parameters to NFAT complex formation at the promoter sites. The changes in the amounts of NFAT/AP-1 complex or mRNA levels of cytokines also showed clear dose-responses to CNIs.

In vitro dose responses are found to be similar between all KEs from MIE to AO, and temporality is also similar from MIE to KE3; however, in KER4, the temporality showed that delayed onset of AO (in vitro antibody responses) compared with KE3 of cytokine production; therefore, no contradictions in dose response and temporality are found in each of the KERs.

Each of the KEs and KERs of the present AOP is supported by sufficient scientific evidence and each KE shows clear dose response relations without any contradiction in dose response and temporality; therefore, measurement of each of the KEs could serve as an appropriate predictor of the adverse outcome of CN inhibition-induced impairment of TDAR.

Considerations for Potential Applications of the AOP (optional)

CN is expressed in T cells as well as other types of immune cells like B cells and dendritic cells. CNIs suppress many kinds of immune functions leading to increased susceptibility to infections and decreased hyper immune reactions such as rejection and graft versus host disease (GVHD). Among these, TDAR is considered to be the most important endpoint of immunotoxicity, because T cells, B cells and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR and changes in at least one of these immune cell populations influencing TDAR.

Moreover, on evaluation of immunotoxicity of pharmaceuticals, the ICH S8 immunotoxicity testing guideline recommends to evaluate TDAR in cases where target cells of immunotoxicity are not clear based on its pharmacology and findings in standard toxicity studies.

The present AOP could be applied to predict whether a compound might affect TDAR in cases where the compound shows a possibility to act on T cells, meanwhile, it is inappropriate that the present AOP would be used as an alternative method to TDAR measurement in the ICH S8 immunotoxicity testing guideline.

References

- Alessiani, M., Kusne, S., Martin, M., Jain, A., Abu-Elmagd, K., Moser, J., Todo, S., Fung, J. and Starzl, T. (1991). Transplantation proceedings 23 (1 Pt 2): 1501-3.
- Antiga, E., Volpi, W., Torchia, D., Fabbri, P. and Caproni, M. (2011). Clinical and experimental dermatology 36 (3): 235-41.
- Beals, C.R., Clipstone, N.A., Ho, S.N. and Crabtree, G.R. (1997). Genes & development 11 (7): 824-34.
- Bhattacharyya, S., Deb, J., Patra, A.K., Thuy Pham, D.A., Chen, W., Vaeth, M., Berberich-Siebelt, F., Klein-Hessling, S., Lamperti, E.D., Reifenberg, K., Jellusova, J., Schweizer, A., Nitschke, L., Leich, E., Rosenwald, A., Brunner, C., Engelmann, S., Bommhardt, U., Avots, A., Müller, M.R., Kondo, E. and Serfling, E. (2011). The Journal of experimental medicine 208 (4): 823-39.
- Bierer, B.E., Holländer, G., Fruman, D. and Burakoff, S.J. (1993). Current opinion in immunology 5 (5): 763-73.
- Boussiotis, V.A., Nadler, L.M., Strominger, J.L. and Goldfeld, A.E. (1994).Proceedings of the National Academy of Sciences of the United States of America 91 (15): 7007-11.
- Bram, R.J., Hung, D.T., Martin, P.K., Schreiber, S.L. and Crabtree, G.R. (1993). Molecular and cellular biology 13 (8): 4760-9.
- Cameron, A.M., Nucifora, F.C. Jr., Fung, E.T., Livingston, D.J., Aldape, R.A., Ross, C.A. and Snyder, S.H. (1997). The Journal of biological chemistry 272 (44): 27582-8.
- Chung, B.H., Kim, K.W., Yu, J.H., Kim, B.M., Choi, B.S., Park, C.W., Kim, Y.S., Cho, M.L. and Yang, C.W. (2014). Transplant immunology 30 (4): 159-67.
- Cohan, V.L., Undem, B.J., Fox, C.C., Adkinson, N.F. Jr., Lichtenstein, L.M. and Schleimer, R.P. (1989). The American review of respiratory disease 140 (4): 951-4.
- Conboy, I.M., Manoli, D., Mhaiskar, V., and Jones, P.P. (1999). Proceedings of the National Academy of Sciences of the United States of America 96 (11):6324-9.
- Dumont, F.J., Staruch, M.J., Fischer, P., DaSilva, C. and Camacho, R. (1998). Journal of immunology 160 (6): 2579-89.
- Ekberg, H., Tedesco-Silva, H., Demirbas, A., Vítko, S., Nashan, B., Gürkan, A., Margreiter, R., Hugo, C., Grinyó, J.M., Frei, U., Vanrenterghem, Y., Daloze, P. and Halloran, P.F.; ELITE-Symphony Study. (2007). The New England journal of medicine 357 (25): 2562-75.
- Ekberg, H., Bernasconi, C., Tedesco-Silva, H., Vítko, S., Hugo, C., Demirbas, A., Acevedo, R.R., Grinyó, J., Frei, U., Vanrenterghem, Y., Daloze, P. and Halloran, P. (2009). American journal of transplantation 9 (8): 1876-85.
- Flanagan, W.M., Corthésy, B., Bram, R.J. and Crabtree, G.R. (1991). Nature 352 (6338): 803-7.
- Foletta, V.C., Segal, D.H. and Cohen, D.R. (1998). Journal of leukocyte biology 63 (2): 139-52.
- Fruman, D.A., Bierer, B.E., Benes, J.E., Burakoff, S.J., Austen, K.F. and Katz, H.R. (1995). Journal of immunology 154 (4): 1846-51.
- Fung, J., Abu-Elmagd, K., Jain, A., Gordon, R., Tzakis, A., Todo, S., Takaya, S., Alessiani, M., Demetris, A., Bronster, O., Martin, M., Mieles, L., Selby, R., Reyes, J., Doyle, H., Stieber, A., Casavilla, A. and Starzl, T. (1991). Transplantation proceedings 23 (6): 2977-83.
- Glynne, R., Akkaraju, S., Healy, J.I., Rayner, J., Goodnow, C.C. and Mack, D.H. (2000). Nature 403 (6770): 672-6.
- Goldfeld, A.E., Flemington, E.K., Boussiotis, V.A., Theodos, C.M., Titus, R.G., Strominger, J.L. and Speck, S.H. (1992). Proceedings of the National Academy of Sciences of the United States of America 89 (24): 12198-201.
- Goldfeld, A. E., Tsai, E., Kincaid, R., Belshaw, P. J., Schrieber, S. L., Strominger, J. L. and Rao, A. (1994). Journal of experimental medicine. 180(2): 763-768.
- Heidt, S., Roelen, D. L., Eijsink, C., Eikmans, M., van Kooten, C., Claas, F. H. and Mulder, A. (2010). Clinical and experimental immunology. 159(2): 199-207.
- Hiroi, J., Sengoku, T., Morita, K., Kishi, S., Sato, S., Ogawa, T., Tsudzuki, M., Matsuda, H., Wada, A. and Esaki, K. (1998). Japanese journal of pharmacology. 76(2): 175-183.
- Hultsch, T., Albers, M. W., Schreiber, S.L. and Hohman, R. J. (1991). Proceedings of the national academic science of the United States of America. 14: 6229-6233.
- Imai, A., Sahara, H., Tamura, Y., Jimbow, K., Saito, T., Ezoe, K., Yotsuyanagi, T. and Sato, N. (2007). European journal of immunology. 37(7): 1730-1738.
- Jain, J., McCaffrey, P. G., Valge-Archer, V. E. and Rao, A. (1992). Nature. 356(6372): 801-804.

- Jain, J., Miner, Z. and Rao, A. (1993). Journal of immunology. 151(2): 837-848.
- Jennings, C., Kusler, B. and Jones, P. P. (2009). Innate immunity. 15(2): 109-120.
- Kang, Y. J., Kusler, B., Otsuka, M., Hughes, M., Suzuki, N., Suzuki, S., Yeh, W. C., Akira, S., Han, J. and Jones, P. P. (2007). Journal of immunology. 179(7): 4598-4607.
- Kang, C. B., Hong, Y., Dhe-Paganon, S. and Yoon, H. S. (2008). Neurosignals. 16: 318-325.
- Kim, T., Kim, N. and Kang, H. J. (2010). Journal of leukocyte biology. 88:1089-1097.
- Kino, T., Hatanaka, H., Miyata, S., Inamura, N., Nishiyama, M., Yajima, T., Goto, T., Okuhara, M., Kohsaka, M. and Aoki, H. (1987a). Journal of antibiotics. 40(9): 1256-1265.
- Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H. and Imanaka, H. (1987b). Journal of antibiotics. 40(9): 1249-1255.
- Klee, C. B., Draetta, G. F. and Hubbard, M. J. (1988). Advances in enzymology and related areas of molecular biology. 61:149-200.
- Lee, Y. R., Yang, I. H., Lee, Y. H., Im, S. A., Song, S., Li, H., Han, K., Kim, K., Eo, S. K. and Lee, C. K. (2005). Blood. 105(10): 3951-3955.
- Liu, J., Farmer, J. D. Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991). Cell. 66(4): 807-815.
- Liu, J (https://www.ncbi.nlm.nih.gov/pubmed/?term=Liu%20J%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Albers, M. W (https://www.ncbi.nlm.nih.gov/pubmed/?term=Albers%20MW%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Wandless, T. J (https://www.ncbi.nlm.nih.gov/pubmed/?term=Wandless%20TJ%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Luan, S (https://www.ncbi.nlm.nih.gov/pubmed/?term=Luan%20S%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Alberg, D. G (https://www.ncbi.nlm.nih.gov/pubmed/?term=Alberg%20DG%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Belshaw, P. J (https://www.ncbi.nlm.nih.gov/pubmed/?term=Belshaw%20PJ%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Cohen, P (https://www.ncbi.nlm.nih.gov/pubmed/?term=Cohen%20P%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., MacKintosh, C (https://www.ncbi.nlm.nih.gov/pubmed/?term=MacKintosh%20C%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Klee, C. B (https://www.ncbi.nlm.nih.gov/pubmed/?term=Klee%20CB%5BAuthor%5D&cauthor=true&cauthor_uid=1373650). and Schreiber, S.L (https://www.ncbi.nlm.nih.gov/pubmed/?term=Schreiber%20SL%5BAuthor%5D&cauthor=true&cauthor_uid=1373650).. (1992). Biochemistry. (https://www.ncbi.nlm.nih.gov/pubmed/1373650) 31(16):3896-901.
- Liu, J. (1993). Immunology today. 14(6): 290-305.
- Macian, F. (2005). Nature reviews. Immunology. 5(6): 472-84.
- Magari, K., Miyata, S., Ohkubo, Y., Mutoh, S. and Goto, T. (2003). British journal of pharmacology. 139: 927-934.
- Matsuda, S., Koyasu, S. (2000). Tanpakushitsu kakusan koso. 45(11): 1823-1831.
- Meingassner, J.G. and Stütz, A. (1992). Journal of investigative dermatology 98(6): 851-5
- Nalesnik, MA., Todo, S., Murase, N., Gryzan, S., Lee, PH., Makowka, L., and Starzl, TE. (1987). Transplantation Proceedings 19(5 Suppl 6): 89-92.
- Panhans-Gross, A., Novak, N., Kraft, S., and Bieber, T. (2001). Journal of Allergy and Clinical Immunology 107(2): 345-52.
- Pirsch, JD., Miller, J., Deierhoi, MH., Vincenti, F., and Filo, RS. (1997). Transplantation 63(7): 977-83.
- Rao, A., Luo, C., and Hogan, PG. (1997). Annual Review of Immunology 15: 707-47.
- Sakuma, S., Kato, Y., Nishigaki, F., Sasakawa, T., Magari, K., Miyata, S., Ohkubo, Y., and Goto, T. (2000). British Journal of Pharmacology 130(7): 1655-63.
- Sakuma, S., Higashi, Y., Sato, N., Sasakawa, T., Sengoku, T., Ohkubo, Y., Amaya, T., and Goto, T. (2001a). International Immunopharmacology 1(6): 1219-26.
- Sakuma, S., Kato, Y., Nishigaki, F., Magari, K., Miyata, S., Ohkubo, Y., and Goto, T. (2001b). International Immunopharmacology 1(4): 749-57.
- Sasakawa, Y., Sakuma, S., Higashi, Y., Sasakawa, T., Amaya, T., and Goto, T. (2000). European Journal of Pharmacology 403(3): 281-8.
- Sasaki, T., Nakamura, W., Inokuma, S., and Matsubara, E. (2015). Journal of Clinical Rheumatology Feb 3.
- Schreiber, SL., and Crabtree, GR. (1992). Immunology Today 13(4): 136-42.
- Serfling, E., Berberich-Siebelt, F., Chuvpilo, S., Jankevics, E., Klein-Hessling, S., Twardzik, T., and Avots, A., (2000). Biochimica et Biophysica Act 1498 (1): 1-18.
- Siekierka, JJ., Hung, SH., Poe, M., Lin, CS., and Sigal, NH. (1989a). Nature 341(6244): 755-57.
- Siekierka, JJ., Staruch, MJ., Hung, SH., and Sigal, NH. (1989b). Journal of immunology 143(5): 1580-3.
- Siekierka, JJ., Wiederrecht, G., Greulich, H., Boulton, D., Hung, SH., Cryan, J., Hodges, PJ., and Sigal, NH. (1990). Journal of Biological Chemistry 265(34): 21011-5.
- Sonoda, T., Takahara, S., Takahashi, K., Uchida, K., Ohshima, S., Toma, H., Tanabe, K., Yoshimura, N.; Japanese Tacrolimus Study Group. (2003). Transplantation 75(2): 199-204.
- Standaert, RF., Galat, A., Verdine, GL., and Schreiber, SL. (1990). Nature 346(6285): 671-4.

- Tamura, F., Masuhara, A., Sakaida, I., Fukumoto, E., Nakamura, T., and Okita, K. (1998). Journal of Gastroenterology and Hepatology 13(7): 703-8.
- Ulrich, P., Paul, G., Perentes, E., Mahl, A., and Roman D. (2004). Toxicology Letters 149(1-3): 123-31.
- Vacher-Coponat, H., Brunet, C., Moal, V., Loundou, A., Bonnet, E., Lyonnet, L., Ravet, S., Sampol-Manos, E., Sampol, J., Berland, Y., George, FD., and Paul, P. (2006). Transplantation 82(4): 558-66.
- Vandewalle, A., Tourneur, E., Bens, M., Chassin, C., and Werts, C. (2014). Cell Communication and Signaling 12: 8
- Weiwad, M., Edlich, F., Kilka ,S., Erdmann, F., Jarczowski, F., Dorn, M., Moutty, M.C. and Fischer, G. (2006). Biochemistry 45(51): 15776-84.
- Wicker, L.S., Boltz, R.C. Jr., Matt, V., Nichols. E.A., Peterson, L.B. and Sigal, N.H. (1990). European journal of immunology 20(10): 2277-83.
- Yoshimura, N., Matsui, S., Hamashima, T. and Oka, T. (1989). Transplantation 47(2): 356-9.
- Yoshino, T., Nakase, H., Honzawa, Y., Matsumura, K., Yamamoto, S., Takeda, Y., Ueno, S., Uza, N., Masuda, S., Inui, K. and Chiba, T. (2010). Inflammatory bowel disease. 16(12): 2022-33
- Zhang, B.W., Zimmer, G., Chen, J., Ladd, D., Li, E., Alt, F.W., Wiederrecht, G., Cryan, J., O'Neill, E.A., Seidman, C.E., Abbas, A.K. and Seidman, J.G.. (1996). Journal of experimental medicine 183(2): 413-20.
- Zhu, J. and McKeon, F. (1999). Nature. 398(6724): 256-60.
- de Paulis, A., Cirillo, R., Ciccarelli, A., de Crescenzo, G., Oriente, A. and Marone, G. (1991). Journal of immunology 147(12): 4278-85.
- de Paulis, A., Stellato, C., Cirillo, R., Ciccarelli, A., Oriente, A. and Marone, G. (1992). Journal of investigative dermatology 99(6): 723-8.
- van Dieren, J.M., Lambers, M.E.H., Kuipers, E.J., Samsom, J.N., van der Woude, C.J. and Nieuwenhuis, E.E.S. (2010). Digestive diseases and sciences 55(9): 2514-19.
- van Lierop, P.P., de Haar, C., Lindenbergh-Kortleve, D.J., Simons-Oosterhuis, Y., van Rijt, L.S., Lambrecht, B.N., Escher, J.C., Samsom, J.N. and Nieuwenhuis, E.E. (2010). Inflammatory bowel disease 16(3): 442-51.
- Maruho Co.,Ltd. (2014) Drug interview form Protopic ointment 0.1% Revised 16th edition.
- Astellas Pharma Inc. (2014) Drug interview form Prograf capsules 0.5mg, 1mg, 5mg, granules 0.2mg, 1mg. Revised 34th edition
- Astellas Pharma Inc. (2014) Drug interview form Prograf capsules 0.5 mg, 1 mg, 5 mg, granules 0.2 mg, 1 mg. Revised 34th edition
- Fyjii Y., Gogi H., Takamura K., Sakuma A. and Goto T. Kisotorinsyo 31(8): 2693-2700 (in Japanese)
- Sengoku T., Morita K., Sato A., Sakuma S., Ogawa T., Hiroi J., Fujii T and Goto T. (1998) Folia Pharmacol. Jpn. (Nippon Yakurigaku Zasshi) 112, 221-232

Appendix 1

List of MIEs in this AOP

Event: 980: Inhibition, Calcineurin Activity (https://aopwiki.org/events/980)

Short Name: Inhibition, Calcineurin Activity

Key Event Component

Process	Object	Action
binding	FK506-binding protein 15	increased
binding	FKBP12 (Arabidopsis thaliana)	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	MolecularInitiatingEvent

Stressors

Name
Tacrorimus

Name
Cyclosporin

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus rattus	Rattus rattus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10117)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

CN is broadly distributed in T-cells, B-cells, and throughout the body. The structure of CnA and CnB is highly conserved from yeasts to humans. Also highly conserved are the amino acid sequences of the catalytic and regulatory domains of CnA isoforms from different organisms (Kincaid. 1996).

As for immunophilins, of which complexes inhibit the CN activity, FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989a). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, langerhans cells and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all creat have PPIase activity (Wang P et al. 2005).

Therefore, inhibition of CN phosphatase activity through immunophilin-CNI complex deems to be in common among mammalian species.

Key Event Description

Calcineurin (CN) is a heterodimer that comprises a catalytic subunit (CnA), which handles phosphatase activity as well as calmodulin binding, and a Ca-binding regulatory subunit (CnB), which regulates intracellular calcium as well as CnA (Klee et al. 1988, Zhang et al. 1996). CnA, a 59kDa protein, has a serine-threonine phosphatase domain.

Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity (Barik. 2006) and an immunophilin-CN

inhibitor (CNI) complex such as FKBP12- FK506 and cyclophilin-CsA binds directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which inhibits the phosphatase activity of CN without any contribution of PPIase activity (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

How it is Measured or Detected

Phosphatase activity can be measured using a phosphatase assay. CN, calmodulin, FK506, and FKBP are incubated together, and the phosphatase activity is measured at various concentrations of FKBP. Kinetic analysis of FKBP12 concentration-dependent phosphatase activity and calculation of Ki inhibition of CN by the FKBP12-FK506 complex are conducted. (Bram et al. 1993). Phosphatase activity of CN in the presence of cyclosporin A (CsA) and cyclophilin can also be determined in the manner described above.

Immunophilin-CNI complexes directly inhibit phosphatase activity of CN, therefore, as a surrogate measurement of the CN activity, the binding of CsA with cyclophilin can be detected using an ELISA kit. Microtiter plates precoated with BSA and conjugated to cyclosporin are incubated with cyclophilin. Bound cyclophilin is then revealed by incubation with anti-cyclophilin rabbit antiserum followed by incubation with anti-rabbit globulin goat IgG coupled to alkaline phosphatase (Quesniaux et al. 1987).

References

[1] Barik, S. (2006). Immunophilins: for the love of proteins. Cellular and Molecular Life Sciences 63(24): 2889-900.

[2] Bierer, B.E., Holländer, G., Fruman, D. and Burakoff, S.J. (1993). Cyclosporin A and FK506: molecular mechanisms of immunosuppression and probes for transplantation biology. Current opinion in immunology 5 (5): 763-73.

[3] Bram, R.J., Hung, D.T., Martin, P.K., Schreiber, S.L. and Crabtree, G.R. (1993). Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: roles of calcimeurin binding and cellular location. Molecular and cellular biology 13 (8): 4760-9.

[4] Cameron, A.M., Nucifora, F.C. Jr., Fung, E.T., Livingston, D.J., Aldape, R.A., Ross, C.A. and Snyder, S.H. (1997). FKBP12 binds the inositol 1, 4, 5-trisphosphate receptor at leucine-proline (1400-1401) and anchors calcineurin to this FK506-like domain. The Journal of biological chemistry 272 (44): 27582-8.

[5] Hultsch, T., Albers, M. W., Schreiber, S.L. and Hohman, R. J. (1991). Immunophilin ligands demonstrate common features of signal transduction leading to exocytosis or transcription. Proceedings of the national academic science of the United States of America. 14: 6229-6233.

[6] Kang, C. B., Hong, Y., Dhe-Paganon, S. and Yoon, H. S. (2008). FKBP family proteins: immunophilins with versatile biological function. Neurosignals. 16: 318-325.

[7] Kincaid, R..L. (1993). Calmodulin-dependent protein phosphatases from microorganisms to man. A study in structural conservatism and biological diversity. Adv Second Messenger Phosphoprotein Res. 1993;27:1-23.

[8] Klee, C. B., Draetta, G. F. and Hubbard, M. J. (1988). Calcineurin. Advances in enzymology and related areas of molecular biology. 61:149-200.

[9] Liu, J., Farmer, J. D. Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell. 66(4): 807-815.

[10] Liu, J. (1993). FK506 and cyclosporin, molecular probes for studying intracellular signal transduction. Immunology today. 14(6): 290-305.

[11] Panhans-Gross, A., Novak, N., Kraft, S., and Bieber, T. (2001). Human epidermal Langerhans' cells are targets for the immunosuppressive macrolide tacrolimus (FK506). Journal of Allergy and Clinical Immunology 107(2): 345-52.

[12] Rao, A., Luo, C., and Hogan, PG. (1997). Transcription factors of the NFAT family: regulation and function. Annual Review of Immunology 15: 707-47.

[13] Schreiber, SL., and Crabtree, GR. (1992). The mechanism of action of cyclosporin A and FK506. Immunology Today 13(4): 136-42.

[14] Siekierka, JJ., Hung, SH., Poe, M., Lin, CS., and Sigal, NH. (1989a). A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature 341(6244): 755-57.

[15] Siekierka, JJ., Wiederrecht, G., Greulich, H., Boulton, D., Hung, SH., Cryan, J., Hodges, PJ., and Sigal, NH. (1990). The cytosolic-binding protein for the immunosuppressant FK-506 is both a ubiquitous an highly conserved peptidyl-prolyl cis-trans isomerase. Journal of Biological Chemistry 265(34): 21011-5.

[16] Zhang, B.W., Zimmer, G., Chen, J., Ladd, D., Li, E., Alt, F.W., Wiederrecht, G., Cryan, J., O'Neill, E.A., Seidman, C.E., Abbas, A.K. and Seidman, J.G.. (1996). T cell responses in calcineurin A alpha-deficient mice. Journal of experimental medicine 183(2): 413-20.

List of Key Events in the AOP

Event: 979: Interference, nuclear localization of NFAT (https://aopwiki.org/events/979)

Short Name: Interference, nuclear localization of NFAT

Key Event Component

Process	Object	Action
genetic interference	NFAT protein	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus
Cyclosporin

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

Key Event Description

The nuclear factor of activated T cells (NFAT) is a substrate of calcineurin (CN) (Rao et al. 1997). A NFAT has an N-terminal with a plurality of SP motifs rich in serine and proline, which are controlled by means of phosphorylation and dephosphorylation. There is a nuclear localization signal (NLS) held between these SP regions as well as a nuclear export signal (NES) in the N-terminal adjacent to the SP motifs (Beals et al. 1997, Zhu and McKeon 1999, Serfling et al. 2000). SP motifs ordinarily are phosphorylated, which covers the NLS and leaves NES exposed, so NFAT localizes in cytoplasm. When SP motifs are dephosphorylated by activated CN to expose NLS and cover NES, thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999). When T-cell activation takes place, T-cell receptor (TCR)-mediated stimulus increases the intracellular concentration of calcium and activates a regulatory subunit (CnB), which subsequently induces a catalytic subunit (CnA) phosphatase activation, leading to dephosphorylation of NFAT followed by nuclear localization. CN inhibitor -immunophilin complexes inhibit CN phosphatase activation, thereby interfering with NFAT nuclear localization (Bhattacharyya et al.2011).

How it is Measured or Detected

Interference with translocation of NFAT to the nucleus can be detected using a gel mobility shift assay of nuclear or cytoplasmic extracts (Flanagan et al. 1991).

References

[1] Rao, A., Luo, C., and Hogan, PG. (1997). Transcription factors of the NFAT family: regulation and function. Annual Review of Immunology 15: 707-47.

[2] Beals, C.R., Clipstone, N.A., Ho, S.N. and Crabtree, G.R. (1997). Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. Genes & development 11 (7): 824-34.

[3] Zhu, J. and McKeon, F. (1999). NF-AT activation requires suppression of Crm1-dependent export by calcineurin. Nature. 398(6724): 256-60.

[4] Serfling, E., Berberich-Siebelt, F., Chuvpilo, S., Jankevics, E., Klein-Hessling, S., Twardzik, T., and Avots, A., (2000). The role of NF-AT transcription factors in T cell activation and differentiation. Biochimica et Biophysica Act 1498 (1): 1-18.

[5] Matsuda, S., Koyasu, S. (2000). A second target of cyclosporin A and FK506. Tanpakushitsu kakusan koso. 45(11): 1823-1831.

[6] Bhattacharyya, S., Deb, J., Patra, A.K., Thuy Pham, D.A., Chen, W., Vaeth, M., Berberich-Siebelt, F., Klein-Hessling, S., Lamperti, E.D., Reifenberg, K., Jellusova, J., Schweizer, A., Nitschke, L., Leich, E., Rosenwald, A., Brunner, C., Engelmann, S., Bommhardt, U., Avots, A., Müller, M.R., Kondo, E. and Serfling, E. (2011). NFATc1 affects mouse splenic B cell function by controlling the calcineurin-NFAT signaling network. The Journal of experimental medicine 208 (4): 823-39.

[7] Flanagan, W.M., Corthésy, B., Bram, R.J. and Crabtree, G.R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 352 (6338): 803-7.

Event: 981: Reduction, NFAT complex formation (https://aopwiki.org/events/981)

Short Name: Reduction, NFAT complex formation

Key Event Component

Process	Object	Action
cytokine production involved in inflammatory response	NFAT activation molecule 1	decreased
cell activation		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus
Cyclosporin

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
memory T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

CN-NFAT system functions in common among mammalian species including human and rodents. Indeed, FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene might be in common among mammalian T cells including humans and rodents (Flanagan et al. 1991).

Key Event Description

Activated nuclear factor of activated T cells (NFAT) that has localized to the nucleus binds cooperatively at the site of the Interleukin-2 (IL-2) promoter with activator protein-1 (AP-1), which is a heterodimer comprising a Fos and a Jun protein (Schreiber and Crabtree 1992, Jain et al. 1992), thereby inducing transcription of IL-2. (Jain et al. 1993) FK506 hinders the formation of the functional NFAT complexes necessary to binding at the site of IL-2 promoters by interfering with nuclear localization of NFAT (Flanagan et al. 1991).

NFAT is known to bind cooperatively at the promoters of Interleukin-4 (IL-4) and other T-cell cytokines as well as that of IL-2 (Macian et al. 2005).

How it is Measured or Detected

Nevertheless, there are no reports of direct evidence that interference with the binding of NFAT–AP -1 complexes at the site of cytokine promoters is dependent on calcineurin (CN) inhibitor dosage. However, the amount of NFAT/AP-1 complexes is expected to be the alternative parameters. Reduction of NFAT/AP-1 complex formation can be detected using a gel shift assay (Jain et al. 1992) to test nuclear extracts from either stimulated or unstimulated Ar-5 T cells with radio-labelled murine NFAT oligonucleotide.

References

1. Flanagan, W.M., Corthésy, B., Bram, R.J. and Crabtree, G.R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506

and cyclosporin A. Nature 352 (6338): 803-7.
2. Jain, J., McCaffrey, P. G., Valge-Archer, V. E. and Rao, A. (1992). Nuclear factor of activated T cells contains Fos and Jun. Nature. 356(6372): 801-804.
3. Jain, J., Miner, Z. and Rao, A. (1993). Analysis of the preexisting and nuclear forms of nuclear factor of activated T cells. Journal of immunology. 151(2): 837-848.
4. Macian, F. (2005). NFAT proteins: key regulators of T-cell development and function. Nature reviews. Immunology. 5(6): 472-84.
5. Schreiber, SL., and Crabtree, GR. (1992). The mechanism of action of cyclosporin A and FK506. Immunology Today 13(4): 136-42.

Event: 1202: Suppression, IL-2 and IL-4 production (https://aopwiki.org/events/1202)

Short Name: Suppression, IL-2 and IL-4 production

Key Event Component

Process	Object	Action
interleukin-2 production	interleukin-2	decreased
interleukin-4 production	interleukin-4	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus
Cyclosporin

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

CNIs suppress production of IL-2, IL-3, IL-4, IL-5, IFN-γ, GM-CSF and other cytokines, as induced by CD2/CD3 or CD3/CD26 stimulation, in human peripheral blood mononuclear cells (PBMC) (Sakuma et al. 2001a). Also, CNIs (FK506 and CsA) suppress production of IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, TNF-α, IFN-γ, and GM- CSF, as induced by CD3/PMA stimulation, in human PBMC (Dumont et al. 1998).

CNIs (FK506 and CsA) exhibit suppression of IL-2 production induced from mixed lymphocyte reactions in mice and humans (Kino, T et al. 1987a).

These facts indicate that CN-NFAT system-mediated suppression of cytokines is common phenomenon found in mammalian species including human and mice.

Key Event Description

Production of T cell cytokines including Interleukin-2 (IL-2) and interleukin-4 (IL-4) is regulated by NFAT/AP-1 complexes, that is, NFAT that has localized to the nucleus binds cooperatively at the site of the IL-2 and IL-4 promoters with activator protein-1 (AP-1), which is a heterodimer comprising a Fos and a Jun protein (Schreiber and Crabtree 1992, Jain et al. 1992), thereby inducing transcription of IL-2 (Jain et al. 1993). Calcineurin inhibitors (CNIs) such as FK506 and cyclosporin A (CsA), by interfering with NFAT nuclear localization, hinder the formation of the functional NFAT complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991). Transcription of IL-4 is also inhibited by CNIs in the same manner as IL-2 (Dumont et al. 1998).

How it is Measured or Detected

Quantitation of cytokine content was done on appropriately diluted samples, run in duplicate, using Sandwich ELISA kits to test matched Ab pairs with biotin-horseradish peroxidase (HRP)-streptavidin detection and TMB substrate. ELISA plates were scanned in a Molecular Devices UVmax plate reader (Menlo Park, CA), using SOFT max software (Molecular Devices) (Dumont et al. 1998).

Total RNA was extracted using RNeasy mini kit (Qiagen, Chatsworth, CA) and quantitated by absorbance at 260 nm. Cytokine mRNAs were detected using a RiboQuant MultiProbe RPA system (PharMingen, San Diego, CA). Riboprobes were 32P-labeled and hybridized overnight with 10 to 30 mg of the RNA samples. The hybridized RNA was treated with RNase and purified according to the RiboQuant protocol. The samples were then electrophoresed in 6% polyacrylamide-Tris-borate-EDTA-urea gels using the Seqi -Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad, Hercules, CA), or minigels (Novex, San Diego, CA). The gels were dried, exposed and quantitated in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant software (Dumont et al. 1998).

References

1. Dumont, F.J., Staruch, M.J., Fischer, P., DaSilva, C. and Camacho, R. (1998). Inhibition of T cell activation by pharmacologic disruption of the MEK1/ERK MAP kinase or calcineurin signaling pathways results in differential modulation of cytokine production. Journal of immunology 160 (6): 2579-89.
2. Flanagan, W.M., Corthésy, B., Bram, R.J. and Crabtree, G.R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 352 (6338): 803-7.
3. Jain, J., McCaffrey, P. G., Valge-Archer, V. E. and Rao, A. (1992). Nuclear factor of activated T cells contains Fos and Jun. Nature. 356(6372): 801-804.
4. Jain, J., Miner, Z. and Rao, A. (1993). Analysis of the preexisting and nuclear forms of nuclear factor of activated T cells. Journal of immunology. 151(2): 837-848.
5. Kino, T., Hatanaka, H., Miyata, S., Inamura, N., Nishiyama, M., Yajima, T., Goto, T., Okuhara, M., Kohsaka, M. and Aoki, H. (1987a). FK-506, a novel immunosuppressant isolated from a Streptomyces. II. Immunosuppressive effect of FK-506 in vitro. Journal of antibiotics. 40(9): 1256-1265.
6. Sakuma, S., Higashi, Y., Sato, N., Sasakawa, T., Sengoku, T., Ohkubo, Y., Amaya, T., and Goto, T. (2001a). Tacrolimus suppressed the production of cytokines involved in atopic dermatitis by direct stimulation of human PBMC system. (Comparison with steroids). International Immunopharmacology 1(6): 1219-26.
7. Schreiber, SL., and Crabtree, GR. (1992). The mechanism of action of cyclosporin A and FK506. Immunology Today 13(4): 136-42.

List of Adverse Outcomes in this AOP

Event: 984: Impairment, T-cell dependent antibody response (https://aopwiki.org/events/984)

Short Name: Impairment, T-cell dependent antibody response

Key Event Component

Process	Object	Action
Immunosuppression		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	AdverseOutcome

Stressors

Name
Tacrolimus
Cyclosporin

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

CNI induced impairment of TDAR is demonstrated with rodent studies. That is, oral administration of FK506 or CsA to mice for 4 days impaired the response of PFC in splenocytes after intravenous immunization with sheep erythrocytes (Kino et al. 1987). Likewise, oral administration of FK506 to rats over a four-week period reduced production of both anti-KLH-IgG and IgM antibodies after subcutaneous immunization with KLH (Ulrich et al. 2004). As for humans, in vitro experiments showed that treatment with FK506 or CsA of peripheral blood mononuclear cells from blood-bank donors suppressed the production of IgM and IgG antibodies specific to T-cell dependent antigens. (Heidt et al, 2009) Also, in SKW6.4 cells (IL-6-dependent IgM-secreting human B- cell line) cultures, FK506 or cyclosporin suppressed the production of IgM antibodies in the presence of T-cell activation. (Sakuma et al. 2001b) These findings strongly suggest that CNI -induced impairment of TDAR occurs at least in common among humans and rodents.

Key Event Description

Antibody production to T cell -dependent antigens is established through the coordination of B cells, antigen-presenting cells as well as T cell -derived cytokines which stimulates B cells to proliferate and differentiate. T cell -dependent antibody response (TDAR) might be altered if at least one of these cell populations is affected. Calcineurin inhibitors (CNIs) are known to impair T-cell dependent antibody response, but have not been shown to directly affect B cells to reduce antibody production.

FK506 and cyclosporine A (CsA) suppresses the production of IL-2, IL-4 and other classes of cytokines in T cells. IL-2 stimulates B cells to proliferate through surface IL-2 receptors. IL-4 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. Suppressing the production of these B-cell -related cytokines appears to be the main factor in impairment of TDAR by FK506 (Heidt et al, 2009).

How it is Measured or Detected

TDAR could be examined in vivo and in vitro.

In usual in vivo studies, antigen-specific antibodies are evaluated with measuring of serum antibody levels by ELISA or with plaque-forming cell (PFC) assay.

- Rats were repeatedly administered FK506 orally for 4 weeks and immunized with KLH, after which the serum was examined for T cell dependent, antigen-specific IgM and IgG levels using a Sandwich ELISA kit (Ulrich et al. 2004).
- Mice were repeatedly administered CNI including FK506 and CsA orally for 4 days and immunized with SRBC, after which spleen cells were examined using a PFC assay (Kino et al. 1987).

In vitro studies, total IgM and IgG levels in culture supernatant are often measured after polyclonal T-cell activation instead of antigen stimulation in immune cell cultures.

- T cells and B cells isolated from human PBMC were co-cultured with a CNI for nine days in the presence of polyclonal T cell stimulation, after which supernatants were tested for immunoglobulin IgM and IgG levels using a Sandwich ELISA kit. FK506 or CsA reduced the levels of IgM and IgG at the concentrations of 0.3 and 1.0 ng/mL or 50 and 100 ng/mL (Heidt et al, 2009).
- SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) were cultured with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. After culturing for four days, IgM produced in the culture supernatants was measured using an ELISA kit. FK506 or CsA reduced the levels of IgM at the concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma et al. 2001b).
- In order to examine class switching, T cells derived from human PBMCs were cultured with CNI, and cytokine mRNA levels of IFN-gamma, IL-2, IL-4, IL-5, IL-10, IL-13 and other B cell stimulatory cytokines produced in T cells were measured by quantitative PCR (Dumont et al. 1998).

Regulatory Significance of the AO

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References

1. Heidt, S., Roelen, D. L., Eijsink, C., Eikmans, M., van Kooten, C., Claas, F. H. and Mulder, A. (2010). Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help. Clinical and experimental immunology. 159(2): 199-207.
2. Sakuma, S., Kato, Y., Nishigaki, F., Magari, K., Miyata, S., Ohkubo, Y., and Goto, T. (2001b). Effects of FK506 and other immunosuppressive anti-rheumatic agents on T cell activation mediated IL-6 and IgM production in vitro. International Immunopharmacology 1(4): 749-57.
3. Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H. and Imanaka, H. (1987). FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. Journal of antibiotics. 40(9): 1249-1255.
4. Ulrich, P., Paul, G., Perentes, E., Mahl, A., and Roman D. (2004). Validation of immune function testing during a 4-week oral toxicity study with FK506. Toxicology Letters 149(1-3): 123-31.
5. Dumont, F.J., Staruch, M.J., Fischer, P., DaSilva, C. and Camacho, R. (1998). Inhibition of T cell activation by pharmacologic disruption of the MEK1/ERK MAP kinase or calcineurin signaling pathways results in differential modulation of cytokine production. Journal of immunology 160 (6): 2579-89.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1017: Interference, nuclear localization of NFAT leads to Reduction, NFAT complex formation (https://aopwiki.org/relationships/1017)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

CN-NFAT system functions in common among mammalian species including human and rodents. Indeed, FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene might be in common among mammalian T cells including humans and rodents (Flanagan et al. 1991).

Key Event Relationship Description

The nuclear factor of activated T cells (NFAT) is a substrate of calcineurin (CN) (Rao et al. 1997).

A NFAT has a nuclear localization signal (NLS) a nuclear export signal (NES) among or adjacent to an N-terminal with a plurality of SP motifs rich in serine and proline. When SP motifs are dephosphorylated by activated CN to expose NLS and cover NES, thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

T-cell receptor (TCR)-mediated stimulus increases the intracellular concentration of calcium and activates CnB, which subsequently induces CnA phosphatase activation, leading to dephosphorylation of NFAT followed by nuclear localization (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

Nuclear localization of NFAT results in the NFAT binding with AP 1 at the IL-2 promoter region, (Schreiber and Crabtree 1992; Jain et al. 1992) and induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

Once CN phosphatase activity is inhibited, then, dephosphorylation of NFAT and following nuclear localization of NFAT decreases, which results in a decrease of NFAT complex formation at the cytokine promoter sites (Rao et al. 1997).

Evidence Supporting this KER

Biological Plausibility

As has been mentioned, NFAT has NLS and NES among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, NES is exposed while NES is hidden, which leads translocation of NFAT into the nucleus (Matsuda and Koyasu 2000).

It is well known from the experiments using CN inhibitors (CNIs) that interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).

In contrast to T cells, B-cell receptor (BCR)-mediated increases in intracellular concentration of calcium in B cells leads to NFAT nuclear localization, thereby producing some classes of cytokines in the same manner as T-cells (Bhattacharyya et al.2011). However, there has been no report of any evidence that CNI acts directly on B cells to effect antibody production.

Expression of IL-2 receptors in dendritic cells and NKT cells is also reported to be regulated by this CN-NFAT system (Panhans-Gross A et al. 2001; Kim et al. 2010), but there is no report showing that CNIs suppress TDAR through the changes in IL-2R expression in these cells.

Empirical Evidence
The relationship of the interfered nuclear localization of NFAT leading to reduced NFAT complex formation is well known, while there have been no reports on the direct measurement of NFAT/AP-1 complex bound at the promoter sites of cytokine genes in the presence of CNIs; however, the amounts of NFAT/AP-1 complexes and the transcribed mRNA levels are expected to be the alternative parameters (Rao et al. 1997, Jain et al. 1992, Jain et al. 1993).

Concentration-dependent reduction of in vitro nuclear localization of NFAT by FK506 was evident at the maximum concentration of 1μM (Maguire et al. 2013). NFAT/AP-1 complex formation is inhibited by CNI (Rao et al. 1997, Rao et al. 1997, Jain et al. 1992).

Uncertainties and Inconsistencies
Nothing especially

References

1. Bhattacharyya, S., Deb, J., Patra, A.K., Thuy Pham, D.A., Chen, W., Vaeth, M., Berberich-Siebelt, F., Klein-Hessling, S., Lamperti, E.D., Reifenberg, K., Jellusova, J., Schweizer, A., Nitschke, L., Leich, E., Rosenwald, A., Brunner, C., Engelmann, S., Bommhardt, U., Avots, A., Müller, M.R., Kondo, E. and Serfling, E. (2011). NFATc1 affects mouse splenic B cell function by controlling the calcineurin-NFAT signaling network. The Journal of experimental medicine 208 (4): 823-39.
2. Flanagan, W.M., Corthésy, B., Bram, R.J. and Crabtree, G.R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 352 (6338): 803-7.
3. Jain, J., McCaffrey, P. G., Valge-Archer, V. E. and Rao, A. (1992). Nuclear factor of activated T cells contains Fos and Jun. Nature. 356(6372): 801-4.
4. Jain, J., Miner, Z. and Rao, A. (1993). Analysis of the preexisting and nuclear forms of nuclear factor of activated T cells. Journal of immunology. 151(2): 837-48.
5. Kim, T., Kim, N. and Kang, H. J. (2010). FK506 causes cellular and functional defects in human natural killer cells. Journal of leukocyte biology. 88:1089-1097.
6. Maguire O, Tornatore KM, O'Loughlin KL, Venuto RC and Minderman H. (2013) Nuclear translocation of nuclear factor of activated T cells (NFAT) as a quantitative pharmacodynamic parameter for tacrolimus. Cytometry A. 83(12):1096-104.
7. Matsuda, S., Koyasu, S. (2000). A second target of cyclosporin A and FK506. Tanpakushitsu kakusan koso. 45(11): 1823-31.
8. Panhans-Gross, A., Novak, N., Kraft, S., and Bieber, T. (2001). Human epidermal Langerhans' cells are targets for the immunosuppressive macrolide tacrolimus (FK506). Journal of Allergy and Clinical Immunology 107(2): 345-52.
9. Rao, A., Luo, C., and Hogan, PG. (1997). Transcription factors of the NFAT family: regulation and function. Annual Review of Immunology 15: 707-47.
10. Schreiber, SL., and Crabtree, GR. (1992). The mechanism of action of cyclosporin A and FK506. Immunology Today 13(4): 136-42.
11. Zhu, J. and McKeon, F. (1999). NF-AT activation requires suppression of Crm1-dependent export by calcineurin. Nature. 398(6724): 256-60.

Relationship: 1509: Reduction, NFAT complex formation leads to Suppression, IL-2 and IL-4 production (https://aopwiki.org/relationships/1509)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

In addition to data on suppression of cytokine production by CNI in rodents, the following is known from the use of human cells. That is to say, FK506 inhibited expression of both IL-2 and mRNA in human anti-CD3/PMA-activated cells (Dumont et al. 1998). Also, FK506 suppresses expression of IL-2R (CD25) and costimulatory molecules CD80 (B7.1)/CD40 in human Langerhans cells (Panhans-Gross A et al. 2001). And FK506 suppresses IL-2 responsive proliferation and cytokine production as well as lowers cytotoxicity directed toward K562 tumor cells in human NK cells (Kim et al. 2010).

Key Event Relationship Description

Nuclear localization of NFAT results in the NFAT binding with AP-1 at the IL-2 promoter region (Schreiber and Crabtree 1992; Jain et al. 1992) and induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13. There are reports that production of IL-2 after activation of T cells is suppressed by CN inhibitor (CNI) treatment in vitro and in vivo as the result of interference to nuclear translocation of NFAT (Dumont et al. 1998). Production in T cells of IL-4 and other classes of cytokines is also suppressed in the same manner as IL-2.

Evidence Supporting this KER

Biological Plausibility
It is generally accepted that NFAT, translocated to the nucleus after T cell stimulation, binds with AP-1 to the promoter regions of the cytokine genes to mount transcription and following production of these T cell -derived cytokines. Among such cytokines, IL-2 and IL-4 promote proliferation, maturation and class-switching of B cells to enhance TDAR.

It is also supported by sufficient evidence that CNI -induced decrease in T cell - derived cytokine productions is mediated through suppressed nuclear localization of NFAT with resultant decrease in the amount of NFAT/AP-1 complex binding to the promoter regions of T cell -derived cytokines.

When stimulated with ovalbumin, CnA-/- mice produce less IFN-g, IL-2, and IL-4 than wild- type mice. However, primary antibody response in CnA-/- mice is normal in response to TNP-ovalbumin (Zhang et al. 1996).

The following phenotypes are observed in NFAT knockout mice: moderate hyperproliferation with splenomegaly; moderately enhanced B- and T-cell responses, with bias towards Th2- cell responses; decreased IFN-γ production in response to TCR ligation; reduced proliferative responses by T cells; impaired repopulation of the thymus and lymphoid organs; impaired Th2-cell responses and IL-4 production; grossly impaired T-cell effector functions, with profound defects in cytokine production and cytolytic activity; B-cell hyperactivity; impaired development of CD4 and CD8 single-positive cells, with increased apoptosis of double-positive thymocytes; and mild hyperactivation of peripheral T cells (Macian, 2005). These findings reflect the fact that NFAT has multiple roles in different immune cells in collaboration with different types of transcription factors including AP-1.

FK506 binds to the cytosolic FK506 binding protein (FKBP12). This complex prevents the activation of the calcium-dependent serine/threonine phosphatase CN. Decreased CN phosphatase activity leads to diminished dephosphorylation of the transcription factor NF-ATp, inhibiting its translocation to the nucleus. Because NF-ATp is an essential transcription factor regulating the IL-2 gene, FK506 ultimately blocks the T-cell response by inhibiting IL-2 transcription (Panhans-Gross A et al. 2001).

FK506 inhibited IL-2 mRNA expression in anti-CD3/PMA-activated cells (Dumont et al. 1998).

Suppression of mRNA levels of cytokines can be measured using an RNase protection assay in vitro and ex vivo. Inhibition of IL-2, IL-3, IL-4, IL-5, GM-CSF, IFN-γ, TNF-α production, and secretion are measurable using the Sandwich ELISA kit (Dumont et al. 1998).

This can be understood to indicate that, although NFAT is widely involved in the function of T cells, the effect of CNIs is to suppress production of some classes of T cell-derived cytokines, suggesting that, based on what is known from CN knockout mice, this is primarily due to suppression caused by CN..

Empirical Evidence
Empirical support of the KE2 leading to KE3 is strong.

Rationale

- It is well established that inhibition of NFAT/AP-1 complex formation at the promoter sites reduces the production of T cell -derived cytokines including IL-2 and IL-4, which are mainly involved in T cell -dependent antibody response.
- NFAT/AP-1 complex formation is inhibited by CNI.
- In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4 and IFN-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA in a dose-dependent (10 nM) manner (Dumont et al. 1998).

Uncertainties and Inconsistencies
In addition to NFAT/AP-1 complexes, NFAT forms complexes at the site of IL-3 and IL-4 enhancers with avian musculoaponeurotic fibrosarcoma oncogene homolog (MAF), early growth response 1 (EGR1), early growth response 4 (EGR4), interferon-regulatory factor 4 (IRF4), octamer-binding transcription factor (OCT), and other transcriptional partners to induce transcription of a variety of cytokines (Macian 2005). The production of cytokine induced by these transcriptional partners also suppressed by CNI.

FK506 suppresses expression of IL-2R (CD25) and costimulatory molecules CD80 (B7.1)/CD40 in Langerhans cells (Panhans-Gross A et al. 2001).

In human NK cells, FK506 suppresses IL-2 responsive proliferation and cytokine production as well as lowers cytotoxicity directed toward K562 tumor cells (Kim et al. 2010). FK506 suppresses IL-2 production of NKT cell line DN32.D3 induced by stimulus from phorbol 12- myristate 13-acetate (PMA)/calcium -ionophore (van Dieren et al. 2010).

The relationship between the above mechanisms and NFAT is unclear.

References

1. Dumont, F.J., Staruch, M.J., Fischer, P., DaSilva, C. and Camacho, R. (1998). Inhibition of T cell activation by pharmacologic disruption of the MEK1/ERK MAP kinase or calcineurin signaling pathways results in differential modulation of cytokine production. Journal of immunology 160 (6): 2579-89.
2. Kim, T., Kim, N. and Kang, H. J. (2010). FK506 causes cellular and functional defects in human natural killer cells. Journal of leukocyte biology. 88:1089-1097.
3. Macian, F. (2005) NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol. 5(6): 472-84.
4. Panhans-Gross, A., Novak, N., Kraft, S., and Bieber, T. (2001). Human epidermal Langerhans' cells are targets for the immunosuppressive macrolide tacrolimus (FK506). Journal of Allergy and Clinical Immunology 107(2): 345-52.
5. van Dieren, J.M., Lambers, M.E.H., Kuipers, E.J., Samsom, J.N., van der Woude, C.J. and Nieuwenhuis, E.E.S. (2010). Local immune regulation of mucosal inflammation by tacrolimus. Digestive diseases and sciences 55(9): 2514-19.
6. Zhang, BW., Zimmer, G., Chen, J., Ladd, D., Li, E., Alt, FW., Wiederrecht, G., Cryan, J., O'Neill, EA., Seidman, CE., Abbas, AK., Seidman, JG. (1996). T cell responses in calcineurin A alpha-deficient mice. J Exp Med. 183(2): 413-20.

Relationship: 1510: Suppression, IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response (https://aopwiki.org/relationships/1510)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

The effects of FK506 on serum concentration of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over four weeks and immunized with KLH (Ulrich et al. 2004). The effects of FK506 and CsA on antigen-specific plaque-forming splenocytes have been demonstrated in mice treated with FK506 or CsA for 4 days and immunized with SRBC (Kino et al. 1987b).

The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (Heidt et al, 2009, Sakuma et al, 2001).

The effects of FK506 and CsA on production of IL-2 and IL-4 have been demonstrated using mice and human cells (Kino et al. 1987a, Dumont et al. 1998).

These facts suggest that there are no species differences between humans and rodents in inhibitions of IL-2 and IL-4 production and TDAR induction.

Key Event Relationship Description

Experiments have shown that IL-2 mRNA expression in T cells stimulated with anti-CD3/ anti-CD28 antibodies decrease after treatment with FK506 (Dumont et al. 1998). Additionally other experiments have shown that mRNA levels of IL-2, IL-4, and other B cell stimulatory cytokines decreases in T cells stimulated with anti-CD3/anti-CD28 antibodies after treatment with FK506. IL-2 and IL-4 stimulate B cells to proliferate and differentiate; therefore, these results suggest that FK506 and cyclosporin A (CsA) is a potent inhibitor of T cell - dependent antibody production (Heidt, S. et al. 2009). Thus, after treatment with FK506, production of IL-2, IL-4, and other cytokines decreases in T cells, reducing stimulation of B cells as well as proliferation, activation, and class switching, and leading to impairment of TDAR.

Evidence Supporting this KER

Biological Plausibility

In humoral immunity, calcineurin inhibitors (CNIs) do not affect B cells directly but rather indirectly through T cells. That is, FK506 and CsA is capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but FK506 and CsA fails to inhibit immunoglobulin levels when pre-activated T cells are use to stimulate B cells. Hence, the inhibition of B cell response by FK506 and CsA appears due solely to inhibition of T helper cells (Heidt et al, 2009). FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2009). It is established that IL-2 stimulates B cells to proliferate through the surface IL-2 receptors and that IL-4 stimulates B cells to proliferate, to induce class switch, and to differentiate into plasma and memory cells.

Several in vivo studies in rodents showed decreased TDAR by the treatment of FK506 (Kino et al. 1987b, Ulrich et al. 2004). In in vitro tests examining antibody production in blood samples obtained from blood-bank donors, PBMC treated with FK506 and CsA suppressed the production of immunoglobulin (Ig) M and G antibodies to T-cell dependent antigens (Heidt et al, 2009).

Empirical Evidence

Empirical support of the suppression, IL-2 and IL-4 production leads to impairment, T-cell dependent antibody response is strong.

Rationale

- In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998).
- FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a).
- After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL of FK506 or 50 and 100 ng/mL of CsA (Heidt et al, 2009).
- After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL of FK506 or 0.1 to 1000 ng/mL of CsA (Sakuma et al, 2001).
- Rats were treated with FK506 for over four weeks and immunized with KLH, after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004).
- Mice were treated with FK506 or CsA for 4 days, and immunized with SRBC, after which antigen-specific plaque-forming splenocytes reduced at the dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).

Uncertainties and Inconsistencies

IL-2 affects multiple populations of immune cells expressing IL-2 receptors, while IL-4 mainly acts on B cells. Therefore, reduced production of both IL-2 and IL-4 might certainly induce suppression of TDAR with some possibility of additional suppression of other immune functions.

References

1. Dumont, F.J., Staruch, M.J., Fischer, P., DaSilva, C. and Camacho, R. (1998). Inhibition of T cell activation by pharmacologic disruption of the MEK1/ERK MAP kinase or calcineurin signaling pathways results in differential modulation of cytokine production. Journal of immunology 160 (6): 2579-89.
2. Heidt, S., Roelen, D. L., Eijsink, C., Eikmans, M., van Kooten, C., Claas, F. H. and Mulder, A. (2010). Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help. Clinical and experimental immunology. 159(2): 199-207.
3. Kino, T., Hatanaka, H., Miyata, S., Inamura, N., Nishiyama, M., Yajima, T., Goto, T., Okuhara, M., Kohsaka, M. and Aoki, H. (1987a). FK-506, a novel immunosuppressant isolated from a Streptomyces. II. Immunosuppressive effect of FK-506 in vitro. Journal of antibiotics. 40(9): 1256-1265.
4. Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H. and Imanaka, H. (1987b). FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. Journal of antibiotics. 40(9): 1249-1255.
5. Sakuma, S., Kato, Y., Nishigaki, F., Magari, K., Miyata, S., Ohkubo, Y., and Goto, T. (2001b). Effects of FK506 and other immunosuppressive anti-rheumatic agents on T cell activation mediated IL-6 and IgM production in vitro. International Immunopharmacology 1(4): 749-57.
6. Ulrich, P., Paul, G., Perentes, E., Mahl, A., and Roman D. (2004). Validation of immune function testing during a 4-week oral toxicity study with FK506. Toxicology Letters 149(1-3): 123-31.

Relationship: 1508: Inhibition, Calcineurin Activity leads to Interference, nuclear localization of NFAT (https://aopwiki.org/relationships/1508)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculoides	Mus musculoides	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=60742)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells, and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all have PPIase activity (Wang P et al. 2005).

CN is broadly distributed throughout the body, including T- and B-cells, and the structure of CnA and CnB is highly conserved from yeasts to humans. Also highly conserved are the amino acid sequences of the catalytic and regulatory domains of CnA isoforms from different organisms (Kincaid. 1993).

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

Key Event Relationship Description

The phosphatase activity of calcineurin (CN) is well known to be inhibited by CN inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) through complex formation with immunophilins.

Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity, such as FK506-binding protein (FKBP) or cyclophilin (Barik. 2006). FKBP and cyclophilin bind with CNIs FK506 and CsA to form complexes, which inhibit CN activity (Barik. 2006).

While FKBP12, FKBP12.6, FKBP13, and FKBP52 are all part of the FK506-binding FKBP family, FKBP12 has a significant involvement in the mechanism of action for FK506-induced immunosuppression (Siekierka et al. 1989, Kang et al. 2008).

FKBP12 is a 12-kDa protein localized in cytoplasm and has been isolated from Jurkat T- cells as a receptor that binds with the FK506 (Bram et al. 1993). FKBP12 has an FK506-binding domain (FKBD) that comprises 108 amino acids, and is expressed in T cells, B cells, langerhans cells and mast cells (Siekierka et al. 1990, Panhans-Gross et al. 2001, Hultsch et al. 1991).

Cyclophilin and FKBP both exhibit PPIase activity, but no structural similarities have been found between them. Additionally, while immunophilin complexes formed with either substance do inhibit CN phosphatase activity, the PPIase activity and the inhibition of activity that they indicate are unrelated to CN regulation.

CN is a heterodimer that comprises a catalytic subunit (CnA) and a Ca-binding regulatory subunit (CnB). CnA handles phosphatase activity as well as calmodulin binding, and CnB regulates intracellular calcium and CnA (Klee et al. 1988, Zhang et al. 1996). CnA is a 59kDa protein with a serine-threonine phosphatase domain. A FK506-FKBP complex binds directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which in turn inhibits phosphatase activity of CN (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991). Cyclophilin- CsA complexes also function in the same manner, binding directly to CnA in the cell, which in turn inhibits CN phosphatase activity.

The nuclear factor of activated T cells (NFAT) is a substrate of CN (Rao et al. 1997). When CN activates through stimulus from outside of the cell,

it binds directly to the N-terminal of NFAT in cytoplasm, after which dephosphorylation of SP motifs exposes nuclear localization signal (NLS) and covers nuclear export signal (NES), thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999). When T-cell activation takes place, T-cell receptor (TCR)-mediated stimulus increases the intracellular concentration of calcium and activates CnB, which subsequently induces CnA phosphatase activation, leading to dephosphorylation of NFAT followed by nuclear localization.

When CN activity is inhibited by the binding of immunophilin complexes, dephosphorylation does not occur in NFAT, thereby interfering with nuclear localization.

Evidence Supporting this KER

Biological Plausibility

The molecular structures and functions of CN and NFAT are evident based on sufficient scientific findings. The well-known mechanisms for inhibition of CN phosphatase activity by CNIs such as FK506 and CsA is initiated by their complex formations with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPIase activity, but modification of these functions is unrelated to inhibition of CN activity and thought to arise in the molecular structure of the complexes (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

It is also well known that inhibition of CN phosphatase activity interfere the dephosphorylation of NFAT leading to suppression of its nuclear localization.

Empirical Evidence

Many experimental data support the inhibition of CN activity induced by CNI -immunophilin complexes and following suppression of nuclear localization. In addition, CN phosphatase activity is inhibited by CNI of FK506 with IC50 values of 0.5 – 30nM (Maguire et al. 2013, Fruman et al.1995) and 80% suppression at 30 μM, and concentration- dependent reduction of in vitro nuclear localization of NFAT was evident at the maximum concentration of 1μM (Maguire et al. 2013).

Uncertainties and Inconsistencies

CN and NFAT are expressed in T cells and other immune cells including B cells, DC and NKT cells, and cytokine productions from these immune cells and expression of IL-2 receptors (IL-2R) in DCs are lowered due to the inhibition of CN phosphatase activity by CNI treatment. Among them, reduced production of IL-2 and IL-4 from T cells plays a major role in suppression of TDAR as a result of lowed proliferation, differentiation and class switching of B cells, and there have been no reports showing that CNI-induced reduction of cytokines other than IL-2 and IL-4 as well as reduced expression of IL-2R resulted in TDAR suppression.

FKBP12, a specific immmunophilin that bind with FK506, is also an accessory molecule that bind to IP3 and Ryanodine receptors, both of which are Ca channel located on the membrane of endoplasmic reticulum and participating in the regulation of intracellular Ca concentration. When binding with FK506, FKBP12 leaves from these receptors to increase the influx of Ca2+ from the endoplasmic reticulum to cytoplasm, which is expected to increase CN activity; however, FK506 treatment suppresses NFAT nuclear localization. In addition, FKBP12-knock out mice show no changes in immune functions including T cell functions. These facts suggest that inhibition of CN-NFAT system induced by FK506 treatment result from direct inhibition of CN phosphatase activity by FK506-FKBP12 complex and not by affecting Ryanodine and IP3 receptors associated with FKBP12.

References

1. Barik, S. (2006). Immunophilins: for the love of proteins. Cellular and Molecular Life Sciences 63(24): 2889-900.
2. Bierer, B.E., Holländer, G., Fruman, D. and Burakoff, S.J. (1993). Cyclosporin A and FK506: molecular mechanisms of immunosuppression and probes for transplantation biology. Current opinion in immunology 5 (5): 763-73.
3. Bram, R.J., Hung, D.T., Martin, P.K., Schreiber, S.L. and Crabtree, G.R. (1993). Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: roles of calcimeurin binding and cellular location. Molecular and cellular biology 13 (8): 4760-9.
4. Flanagan, W.M., Corthésy, B., Bram, R.J. and Crabtree, G.R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 352 (6338): 803-7.
5. Fruman, D. A., Klee, C. B., Bierer, B. E. and Burakoff, S. J. (1992). Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. Proceedings of the National Academy of Sciences of the United States of America. 89(9):3686-90.
6. Fruman, D. A., Bierer, B. E., Benes, J. E., Burakoff, S. J., Austen, K. F. and Katz, H. R. (1995). The complex of FK506-binding protein 12 and FK506 inhibits calcineurin phosphatase activity and IgE activation-induced cytokine transcripts, but not exocytosis, in mouse mast cells. Journal of Immunology.154(4):1846-51.
7. Hultsch, T., Albers, M. W., Schreiber, S.L. and Hohman, R. J. (1991). Immunophilin ligands demonstrate common features of signal transduction leading to exocytosis or transcription. Proceedings of the national academic science of the United States of America. 14: 6229-6233.
8. Kang, C. B., Hong, Y., Dhe-Paganon, S. and Yoon, H. S. (2008). FKBP family proteins: immunophilins with versatile biological functions. Neurosignals. 16: 318-325.
9. Kincaid, R .L. (1993). Calmodulin-dependent protein phosphatases from microorganisms to man. A study in structural conservatism and biological diversity. Adv Second Messenger Phosphoprotein Res. 27:1-23.
10. Klee, C. B., Draetta, G. F. and Hubbard, M. J. (1988). Calcineurin. Advances in enzymology and related areas of molecular biology. 61:149-200.
11. Liu, J., Farmer, J. D. Jr., Lane, W. S., Friedman, J., Weissman, I. and Schreiber, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell. 66(4): 807-815.
12. Liu, J (https://www.ncbi.nlm.nih.gov/pubmed/?term=Liu%20J%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Albers, M. W (https://www.ncbi.nlm.nih.gov/pubmed/?term=Albers%20MW%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Wandless, T. J (https://www.ncbi.nlm.nih.gov/pubmed/?term=Wandless%20TJ%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Luan, S (https://www.ncbi.nlm.nih.gov/pubmed/?term=Luan%20S%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Alberg, D. G (https://www.ncbi.nlm.nih.gov/pubmed/?term=Alberg%20DG%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Belshaw, P. J (https://www.ncbi.nlm.nih.gov/pubmed/?term=Belshaw%20PJ%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Cohen, P

(https://www.ncbi.nlm.nih.gov/pubmed/?term=Cohen%20P%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., MacKintosh, C (https://www.ncbi.nlm.nih.gov/pubmed/?term=MacKintosh%20C%5BAuthor%5D&cauthor=true&cauthor_uid=1373650)., Klee, C. B (https://www.ncbi.nlm.nih.gov/pubmed/?term=Klee%20CB%5BAuthor%5D&cauthor=true&cauthor_uid=1373650). and Schreiber, S.L (https://www.ncbi.nlm.nih.gov/pubmed/?term=Schreiber%20SL%5BAuthor%5D&cauthor=true&cauthor_uid=1373650).. (1992). Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. Biochemistry. (https://www.ncbi.nlm.nih.gov/pubmed/1373650) 31(16):3896-901.

13. Liu, J. (1993). FK506 and cyclosporin, molecular probes for studying intracellular signal transduction. Immunology today. 14(6): 290-305.
14. Maguire O, Tornatore KM, O'Loughlin KL, Venuto RC and Minderman H. (2013) Nuclear translocation of nuclear factor of activated T cells (NFAT) as a quantitative pharmacodynamic parameter for tacrolimus. Cytometry A. 83(12):1096-104.
15. Matsuda, S., Koyasu, S. (2000). A second target of cyclosporin A and FK506. Tanpakushitsu kakusan koso. 45(11): 1823-1831.
16. Panhans-Gross, A., Novak, N., Kraft, S. and Bieber, T. (2001). Human epidermal Langerhans' cells are targets for the immunosuppressive macrolide tacrolimus (FK506). Journal of Allergy and Clinical Immunology 107(2): 345-52.
17. Rao, A., Luo, C. and Hogan, PG. (1997). Transcription factors of the NFAT family: regulation and function. Annual Review of Immunology 15: 707-47.
18. Schreiber, SL. and Crabtree, GR. (1992). The mechanism of action of cyclosporin A and FK506. Immunology Today 13(4): 136-42. >
19. Siekierka, JJ., Hung, SH., Poe, M., Lin, CS. and Sigal, NH. (1989). A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature 341(6244): 755-57.
20. Siekierka, JJ., Wiederrecht, G., Greulich, H., Boulton, D., Hung, SH., Cryan, J., Hodges, PJ. and Sigal, NH. (1990). The cytosolic-binding protein for the immunosuppressant FK-506 is both a ubiquitous and highly conserved peptidyl-prolyl cis-trans isomerase. Journal of Biological Chemistry 265(34): 21011-5.
21. Wang, P. and Heitman, J. (2005) The cyclophilins. Genome Biology 6 (7):226.
22. Zhang, B.W., Zimmer, G., Chen, J., Ladd, D., Li, E., Alt, F.W., Wiederrecht, G., Cryan, J., O'Neill, E.A., Seidman, C.E., Abbas, A.K. and Seidman, J.G. (1996). T cell responses in calcineurin A alpha-deficient mice. Journal of experimental medicine 183(2): 413-20.
23. Zhu, J. and McKeon, F. (1999). NF-AT activation requires suppression of Crm1-dependent export by calcineurin. Nature. 398(6724): 256-60.