Organisation for Economic Co-operation and Development

Adverse Outcome Pathway External Review Report

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

Short name: Immunosuppression

This document is the final AOP154 review report elaborated after author's revisions of the AOP.

Initial title: Adverse Outcome Pathway on binding of FK506-binding protein 12 (FKBP12) by calcineurin inhibitors leading to immunosuppression.

This document has been prepared by Mr Jean-Baptiste FINI (fini@mnhn.fr), consultant for the OECD Secretariat.

It compiles the views and comments of the reviewers and explains how the authors of the AOP have addressed these comments.

It provides the basis to EAGMST for determining if AOP 154 has been adequately revised by their authors following the review and if it can be released to the Working group of the National Coordinators of the Test Guidelines Programme and to the Working Party on Hazard Assessment for endorsement.

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1. Introduction and background to specific AOP

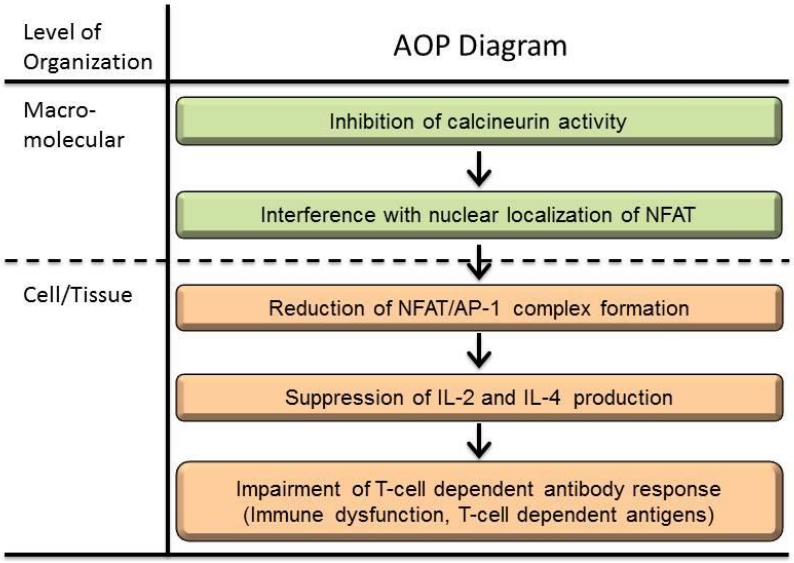
Calcineurin (CN) is a type of protein phosphatase that interferes with immune function when phosphatase activation is inhibited. Immunosuppressants that inhibit CN have been developed based on knowledge of the relationship between CN and the immune function. The relationship between CN and the immune function is well documented, and immunosuppressants that act by inhibiting CN have been developed.

CN inhibitors (CNIs) inhibit the activity of CN phosphatase to suppress many types of immune functions and have been used to prevent hyper immune reactions such as rejection and graft-versus-host disease (GVHD), and to treat autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CNI are reported to induce adverse effects derived from immunosuppression, such as increased frequency and/or severity of infections and increased incidence of tumours. CNI may affect certain types of T-cell-derived immune functions. **Among the immune functions affected, the T-cell dependent antibody response (TDAR) is the most important factor in resisting infection and is considered the most useful endpoint for assessing the immunotoxicity of chemicals**. Therefore, AOP describes the relationship between inhibition of CN activity (as the Molecular Initiating Event MIE) and alteration of TDAR (as the Adverse Outcome AO).

CN activity is inhibited (MIE) when stressors acting as CNI bind to the CN with their respective immunophilins, interfering with the nuclear localisation of nuclear factor of activated T cells (NFAT), a CN substrate (KE1). As a result, reduction of formation of functional NFAT complexes with activator protein 1 (AP-1) which binds to the site of IL-2, IL-4 and other T-cell-derived cytokine promoters is observed (KE2), thereby suppressing the production of these cytokines (KE3). Of the affected cytokines in each of the subsets of helper T cells, the reduced production of IL-2 and IL-4 affects the proliferation and differentiation of B cells to suppress TDAR (AO).

This AOP is based on the understanding of inhibition of calcineurin activity, mainly caused by FK506 and FKBP12 complexes, on which a significant body of scientific literature has been published. It is worth noting that the previous version of this AOP was: “The Adverse Outcome Pathway on binding of FK506-binding protein 12 (FKBP12) by calcineurin inhibitors leading to immunosuppression”.

***Figure 1*: Graphical representation of AOP 154**



1.1. AOP 154 authors

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1.2. External Reviewers

This AOP was reviewed in December 2019/ January 2020 by a panel of four reviewers (see Annex 1). Selection to the panel was driven by the candidates' expertise in immunotoxicity and toxicology. The first selection criterion used was reviewers' skills to assess the AOP. The secondary criteria taken into consideration were those allowing for the balancing, within the panel, of gender, origin (private or public) or geographical distribution.

2. Synthesis of main issues of the review

This section provides an overview of issues raised by the four external reviewers (reviewers' details are provided in Annex 1).

Reviewers, were asked to reply to the following charge questions regarding different aspects of the AOP:

1. Scientific quality:
   -Does the AOP incorporate the appropriate scientific literature?
   -Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

2. Weight of evidence:
   -Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP, justified, i.e. consistent with the considerations outlined in the Users' Handbook?

3. Additional observations:
   -What do you consider to be critical data gaps, if any, and how to fill these gaps?

The version used was the snapshot provided by the OECD Secretariat and accessible at https://aopwiki.org/aopwiki/snapshot/pdf_file/154-2019-12-13T12:46:09+00:00.pdf

A summary of the reviewers' answers to the charge questions for each point is accessible below, with quotations organised point by point under each question. The complete reviews organised by reviewer are accessible in Annex 2 of this report. The replies made by the authors are accessible in Annex 2.

2.1. Scientific quality

Does the AOP incorporate the appropriate scientific literature?

The AOP is generally supported by an accurate and relevant peer-reviewed literature that describes the pathway from inhibition of the calcineurin activity (MIE) through a series of supported key events (KEs) up to cell/tissue effects. However all reviewers agreed that inclusion of additional or updated literature would refine and strengthen the AOP with additional details from the more recent knowledge.

Need for additional information: Many reviewers felt that the bibliography provided in the AOP was insufficient in certain sections, especially bibliography related to MIE, K2 and KE3 and to the methods used to measure the KEs. Some reviewers also suggested expanding the stressor list for the Molecular Initiating Event (MIE) and Key Event Relationships (KERs).

In particular more than one reviewer found that the scientific literature incorporated into the AOP fails to address that non-pharmacological agents may also contribute to alterations in the T-cell dependent antibody response (TDAR).

It was indicated that the paradoxical effect of the use of tacrolimus and cyclosporin A and their association with allergic diseases and elevated IgE in several transplant populations deserves a mention (Kawamura et al., 1997).

More missing literature was highlighted in some sections. The User's Handbook specifies that references must be provided in the Biological Plausibility section which is lacking currently. References should also be provided in the Essentiality of Key Events and for KE3 => AO in the Empirical Support table. References should be included in the Weight of Evidence Summary.

Scope of the AOP: The domain of applicability of this AOP was not clear to some reviewers, especially whether the AOP could be applied to other stressors than two well-known therapeutic agents (cyclosporin and tacrolimus). Another reviewer was wondering if the AOP was not too finely focused and questioned its consequent regulatory significance.

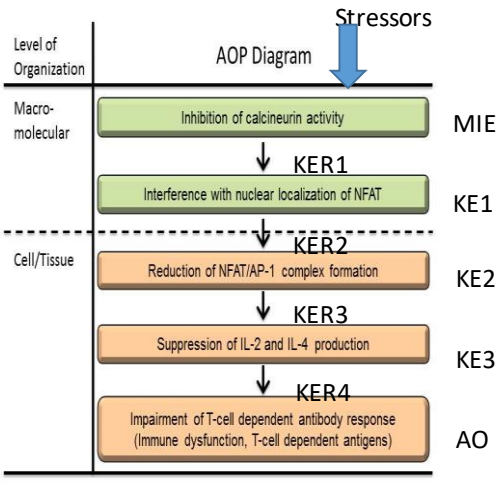
Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

Overall, the scientific content is acknowledged to reflect current knowledge on the subject. However, as stressed in the paragraphs above, some recent literature should be cited.

Moreover, two reviewers are questioning if the sole measurement of IL-2 and Il-4 is sufficient to connect with the KE3. These reviewers highlight the existence of multiple other cytokines described since the 1990's, as acknowledged and cited by the authors such as IFN gamma.
A suggestion was made to cite the recent paper showing that exposure to Cyclosporin A leads to reduction of IL-2 secretion *in vitro* (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6763276/).

2.2. Weight of evidence

Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified ?



Weight of evidence is most of the time appropriate clear and accurate. All authors agreed that the vast majority of the KEs and KERs are correctly weighted.
Some precisions are needed for KER1, KER4 and KE3.

KER1: The authors assigned a value of "high" confidence for quantitative understanding in the Key Event Relationships table (page 2 of 32) or referred to the evidence as "strong" in the Weight of the Evidence Summary (page 5 of 32). The reviewers considered the evidence call should be adjusted to "medium/moderate" for KER1. This adjustment is important because KE1 is a logical target for potential methods development activities. Test method developers need also to understand the degree of confidence in this relationship.
There is no evidence for stressors at this step and this should be added.

KER4:

Reviewers questioned if the suppression of the sole interleukins IL2 and IL4 would be leading to impaired TDAR while other cytokines exists. Even if they have a prominent role in this process, it cannot be ruled out that the other cytokines could also play a role.

One reviewer also highlighted that although the immediate receptors (immunophilins) of cyclosporin A and FK506 are distinct, their similar mechanisms of inhibition of cell signaling indicate that their associated immunophilin complexes interact with the common target, NF-AT (Liu et al., 1991).

Therefore, reviewers suggested re-evaluating weight of evidence from high to moderate.

KE3 needs more work. Literature citation should be updated and completed.
In particular as the KE name is "IL2 and IL4 production" there is a need to make sure there is support for its essentiality and that measured effects on IL-2 and IL-4 are sufficient to lead to the AO.

2.3. Critical gaps in the AOP 154

-*What do you consider to be critical data gaps, if any, and how to fill these gaps*

More than one reviewer found that the applicability domain of this AOP is very narrow (i.e., founded on the action of only two closely related therapeutic agents) which leads to question of the regulatory applicability of the AOP.

In its current form, the AOP provides no guidance related to its potential application. Recognising the complexity of the TDAR and the apparently limited applicability domain, it would be best if statements were included regarding its use as part of an Integrated Approaches to Testing and Assessment (IATA).

One other weakness stressed by two reviewers is that authors rely on IgE antibody production in KER4 as an evidence that the calcineurin inhibition is the MIE in the pathway for suppression of the TDAR. The TDAR also is a response that produces other types of immunoglobulins (i.e., IgM, IgG) whose production depends on which receptors are activated. Authors should therefore elaborate on this point (see section 3.2).

Finally, one reviewer highlighted a data gap on B cell differentiation which can also been linked with CN inhibition.
While cyclosporin A and FK506 are both direct inhibitors of B cell activation, as assessed by proliferation and plasma cells differentiation, they do not affect immunoglobulin release in the absence of T cells (De Bruyne et al., 2015). At present, only few reports have investigated the role of calcineurin inhibition on B-cell differentiation and antibody production in detail, representing a data gap. Thus, I totally agree that inhibition of calcineurin activity will impair T-cell dependent antibody response and so TDAR, but this AOP cannot be used as an alternative to TDAR measurement.

- *Additional observations*

According to the User's Handbook, the Essentiality of the Key Events section is supposed to be organised in "tabular" form, but the authors opted for paragraph form. Ideally, this section would be re-organised in a table with clear, scientifically supported evidence statements (i.e., high, medium, low).

2.4. Conclusion

Although this AOP is considered well written, the reviewers agreed that some work is required to improve the AOP before going forward in the submission process.

Authors were expected to improve the description of some Key Events and Key Events Relationships prior to its broader use. Reviewers identified some gaps listed in the previous parts which needed to be discussed at the TC with both authors and reviewers.

3. Summary record of the teleconference

3.1. TC agenda

Two teleconferences were organised during the period January to February 2020.

1) Only reviewers and review manager attended the first teleconference.
It took place mid-January and aimed at defining the role of the reviewers. In particular, we discussed the main issues that the reviewers should focus on and what was expected from them in terms of the initial AOP review. All reviewers attended the TC.

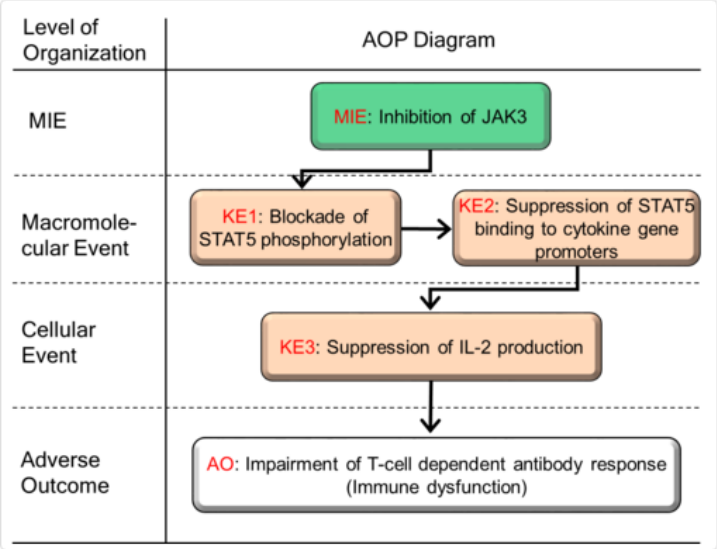
2) The end-of-review teleconference was organised on February 19th 2020 at 1.30 pm CET.
Takao Ashikaga, Hata Sinko, Kushima Kiyoshi, Shigeru Hisada, Yasuhiro Yoshida, Ohishi Takumi, the four reviewers and the review manager attended the TC.
The review manager and the authors thanked the reviewers who devoted significant amount of their time to provide constructive comments, editorial changes and additional literature. All the materials cited have been made available to the authors.
The authors replied to the different comments before the TC and agreed on most of the changes required by the authors prior to the teleconference.
The agenda of the TC was as follows:

- Brief reminders of what we can be expected from an AOP and what an AOP is not.
- Brief reminders on AOP 154, the different Key Events (KE) and Key Event Relationship (KER), the review process and questions asked to the reviewers.
- Discussion of issues raised by the reviewers and answers provided by the authors.
- Other Issues
- Conclusions and elements on the upcoming events in the AOP process.

3.2. Main issues and responses during the call

Authors were asked to incorporate missing literature in the MIE, KE3 and KERs 1 and 4. Authors agreed.
The KE3 that applies specifically to IL2 and IL4 was discussed because of the existence of other cytokines; more discussion is needed to establish future applicability of the AOP. As an example, the authors mention IFN-gamma that is also required for delayed-type hypersensitivity (DTH).
Authors mentioned the fact a more comprehensive view of the biological activity would be built thanks to others AOPs that they are developing. For example, the AOP315 shares KE3 and AO with the AOP154 as shown in the figure below.

Figure 2: AOP 315 - Inhibition of JAK3 leading to impairment of T-Cell dependent Antibody response.



The reviewers acknowledged the amount of work that has gone into this AOP and sibling AOPs that will build a comprehensive AOP network (e.g. AOP 315 – see figure 2).

Changes to the weight of evidence calls of KER1 and KER4 were discussed. It was suggested that calls attributed to these two KERs, currently declared high, should be reduced to a moderate level, taking into account the comments made by the reviewers (cf section 2.2). The authors agreed to take these modifications on board.

Finally, regarding the shortcomings of the AOP, the authors were asked to elaborate on the potential application of the AOP. The authors agreed to develop such section, recognising the use of this AOP, together with other AOPs being developed with same AO, as part of an IATA.

Data gaps on calcineurin inhibition in B cells differentiation - authors made the point that the B cell differentiation was not in the AOP but agreed to incorporate missing literature.

Overall, the authors agreed to implement the suggested changes by updating and making changes to specific sections of this AOP.

All the reviewers appreciated the authors' willingness to improve and revise the AOP in the upcoming weeks following the end of review TC.

There was consensus among the reviewers on the issues raised from the reviewing process. Once the changes proposed to address these issues are implemented, the reviewers would support that this AOP gets submitted for approval and be subsequently published. The authors are expected to concretise actions arising from the review and the teleconference and store this information in the AOP-Wiki discussion pages.

4. Summary of planned revisions

In addition to the minor comments formulated by the reviewers (summarised in section 2 and all accessible in annex 2), authors were asked to complete the literature cited in several places. The required changes are listed below:

1) MIE: Incorporate literature if any on external agents

2) Change the statement " numerous stressors" in the introduction into another more balanced term as only cyclosporine and tacrolimus are quoted in the AOP

3) KE3: Incorporate literature on the different cytokines and mention the development of the AOP 315 with the building of an AOP network.

4) Add information on the others immunoglobulin affected by the CN inhibition.

5) Modify weight of evidence of KER1 and KER4 from high to moderate

6) Elaborate on the potential application of the AOP, since this sequence of events has been clarified for two "model" drugs, FK-506 and cyclosporin-A. Add statements regarding uses as part of an IATA.

5. Further discussion

No further discussion was required as all issues were resolved in the teleconference

6. Outcome of the external review

All the reviewers of this AOP felt that the authors had to improve the AOP. The reviewers though acknowledged the significant contribution of the authors through the development of this AOP to the AOP-Knowledgebase. The reviewers also acknowledged the amount of work that has gone into sibling AOPs that will build a comprehensive AOP network.

The reviewers devoted significant amount of their time to provide constructive comments, editorial changes and additional literature. All these materials have been made available to the authors. There was consensus among the reviewers on the issues raised from the reviewing process. The authors replied to the different comments and agreed to implement most of the suggested changes by updating and making changes to specific sections of this AOP.

All the reviewers appreciated the authors' willingness to improve and revise the AOP in the upcoming weeks following the end of review TC and felt that once the AOP has been modified according to the reviewers' recommendations it would be appropriate that this AOP is submitted for approval and subsequently published. The authors were expected to concretise actions arising from the reviews and the teleconference and store this information in the AOP-Wiki discussion pages.

The authors updated AOP 154 and a new version was made available in the AOP-Wiki on April 6th 2020 (see changes in Annex 3). From a brief overview of the revisions made, the review manager considers these modifications adequately address the comments from the panel. None of the reviewers indicated concerns, although they had not been specifically asked to review the changes.

Annex 1: Reviewers' name

1. Jamie DE WITT (US) : DEWITTJ@ecu.edu
2. Rob VANDEBRIEL (Netherlands) : rob.vandebriel@rivm.nl -
3. Emanuela CORSINI (Italy) : emanuela.corsini@unimi.it
4. David LEHMANN (US) : Lehmann.David@epa.gov

Note that the order of the reviewers given above does not correspond to the order of the complete reviews accessible in Annex2.

Annex 2: Individual reviewers' comments and authors' responses

Written response from the authors to the reviewers comments were provided (in red) in preparation for the end of review Teleconference.

Reviewer #1

1. *Scientific quality:*

   – Does the AOP incorporate the appropriate scientific literature?

   The AOP appears to incorporate scientific literature that supports the MIE and KE outlined within the AOP. Most of the literature appears to be based in basic immunology, transplantation immunology, and pharmacology with very little, if any, literature linking the MIE and/or KEs to immunotoxicological outcomes associated with non-pharmacological exogenous agents such as environmental contaminants. The authors of the AOP reference the International Council for Harmonisation (ICH) guideline for Immunotoxicology Studies (ICH S8) for the evaluation of immunotoxicological outcomes that may be associated with pharmacological agents. This guideline recommends that the T-cell dependent antibody response (TDAR) be conducted when immunotoxicological evaluations are warranted, unless results of standard toxicity studies indicate affected cells do not participate in the TDAR. However, numerous cell types and signaling pathways participate in the TDAR and non-pharmacological exogenous agents may produce deficits in the TDAR via myriad MIEs. The scientific literature incorporated into the AOP therefore fails to address that other MIEs stemming from exposure from non-pharmacological agents may contribute to alterations in the TDAR.

   We agree that non-pharmacological agents should be added as the Stressors. We have not found appropriate non-pharmacological agents, however will try to search and update the Stressors accordingly.

   – Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

   As with the lack of scientific literature relevant to non-pharmacologic exogenous agents, the scientific knowledge contained within this AOP is only relevant to exogenous agents that impact the TDAR through the calcineurin pathway. For example, IL-1, IL-5, and IL-6 also are involved in signaling cascades in the TDAR as are secretory process for release of antibodies as well as metabolic processes involved in production of antibodies. Therefore this AOP is applicable only to those agents that can suppress the TDAR through calcineurin and not via any other MIE.

   We agree with reviewer's comments that many type of cell and signaling pathways involved in the impaired TDAR. However, it is very difficult to develop an AOP involving all the pathways leading to suppression of TDAR, because an AOP should focus on one MIE which leads to the AO with selected pathway. As the future plan, we will build a network of AOPs the associate with impaired TDAR as AO. It seems to be appropriate for us to develop the AOP as a part of AOP network leading to suppression of TDAR. In parallel with the development of this AOP, we are developing another AOP that the AO is also impaired TDAR (AOP315). You can find the draft AOP315 in AOP-Wiki. We understand that AOP should start at one MIE. Therefore, we consider that we need to develop several AOPs that could be a part of the network. Thus, we are now developing AOP as a part of AOP network, focusing on CN inhibition as the MIE

2. *Weight of evidence:*

   – Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

The weight-of-evidence summary for KEs, KERs, and the overall AOP appear to be fairly well justified and the authors attempt to establish biological plausibility. The one weakness appears to be that the authors rely on IgE antibody production in KER4 as evidence that the calcineurin pathway is the MIE for suppression of the TDAR. The TDAR also is a response that produces other types of immunoglobulins (i.e., IgM, IgG) whose production depends on which receptors are activated. As with the weaknesses highlighted under "Scientific Quality," the AOP is sufficient only for exogenous agents that modulate the calcineurin pathway and insufficient for exogenous agents that modulation the TDAR via other pathways.

We agree with reviewer's comments that decreases in only IgE antibody production in KER4 as a result of IL-4 inhibition is a weak point. At present, we could not have found changes in immunoglobulin production other than Ig E subclass caused by suppression of IL-4, and the suppression of IgE production only is a formation we could find in the literature search on the effect of antibody production caused by suppression of IL-4. We would like to continue further literature search. If we find the appropriate literature that indicate that IL-4 suppression affect the other types of immunoglobulins (IgM, IgG), we will update the AOP.

3. *Additional observations:*

– What do you consider to be critical gaps, if any, and how to fill these gaps?

It is critical that the authors modify the AOP along one of two lines of thought. First, if the authors intend that this AOP is applicable across a broad range of exogenous agents, including non-pharmacologic agents, they need to provide evidence from the scientific literature that non-pharmacologic exogenous agents can affect the TDAR via impacts on calcineurin. This evidence is not currently contained in this version of the AOP. Second, if the authors intend that this AOP will only be applicable to a fairly narrow range of exogenous agents, they need to elaborate on which types of agents are likely to be operable via this pathway (i.e., pharmacologic agents like cyclosporin).

We believe that this AOP could be applicable to non-pharmacological agent that affects calcineurin, however we have not find the appropriate agents other than the current 2 stressors. We understand that the additional non-pharmacological agents need to be added to expand the usefulness of this AOP. We will have an effort to add the effects of non-pharmaceutical compounds on CN activities.

An additional point that is not contained within the AOP is consideration of life stage differences that may alter the applicability of the AOP. The authors indicate that the proposed AOP is not dependent on life-stage and appears to be applicable to all life-stages. However, they provide no evidence that it IS applicable to all life-stages. When measured in immunologically immature mammals, for example, the TDAR is not fully functional and appears to be suppressed compared to mammals of an age of full immunocompetency. The authors provide no evidence that the AOP could be applied to mammals that are not fully immunocompetent and therefore provide no evidence that the AOP is applicable to all life-stages.

As the reviewer indicated, we have not provided any evidence of the life stage the. Therefore, in terms of risk management, we showed that every life stage have a risk. However, we will reconsider the life stage applicability considering that TDAR is not well evaluated in juvenile animals.

Reviewer #2

External review charge questions

1. Scientific quality:

-Does the AOP incorporate the appropriate scientific literature?

The foundation of AOP154 is that inhibition of calcineurin activity (molecular initiating event/MIE) leads to impaired T-cell dependent antibody response. The authors are established scientists with appropriate background and experience in in the area of immunotoxicology. Overall, the AOP describes what is known and identified knowledge gaps in the pathway related to key event relationship (KER) 1 and KER 2.
The AOP is generally supported by peer-reviewed literature that describes the pathway from inhibition of the MIE through a series of supported key events (KEs) up to cell/tissue effects. However, the authors indicate that there are "numerous stressors" that inhibit CN phosphatase activity, but do not provide any examples other than two well-known therapeutic agents (cyclosporin and tacrolimus). Additional (literature-supported) examples are needed to support the foundation of the AOP and to demonstrate its potential utility. Otherwise, the AOP is too finely focused and doesn't appear to have much regulatory significance.
As the reviewer indicated, we show only two stressors. We would like to try to find other stressors that inhibit CN phosphatase activity for literature.

There are also several places where in text references are missing. For example, the User's Handbook specifies that references must be provided in the Biological Plausibility section. References should also be provided in the Essentiality of Key Events and for KE3 => AO in the Empirical Support table on page 7 of 32. References should be included in the Weight of Evidence Summary. The lack of in text references makes it much more difficult for the reader to confirm the validity of statements.
According to the reviewer's comment, we would like to search and add a lack of information.

-Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

Yes. The authors could consider citing another recent paper (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6763276/) showing that exposure to CsA leads to reduction of IL-2 secretion *in vitro*.
Thank you for sharing very helpful information. We would like to cite and add descriptions.

2. Weight of evidence:

-Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP, justified, i.e. consistent with the considerations outlined in the Users' Handbook?

I agree with the weight of evidence designations assigned to MIE, KEs and, except for KER2, the KERs. With respect to KER2, the authors indicated that there is not a "clear quantitative relationship" between inhibition of CN phosphatase activity and nuclear translocation of NFAT. However, they assigned a value of "high" confidence for quantitative understanding in the Key Event Relationships table (page 2 of 32) or referred to the evidence as "strong" in the Weight of the Evidence Summary (page 5 of 32)). The evidence call should be adjusted to "medium/moderate" for KER2. This adjustment is important because KER2 is a logical target for potential methods development activities and methods developers need to understand the degree of confidence in this relationship.

There isn't an evidence call-out for Stressors in the Summary of the AOP section (page 3 of 32). Similarly, there isn't an evidence call-out for *Rattus norvegicus* in the Domain of Applicability section (page 3 of 32).

It will be easier to interpret the Empirical Support table (page 5 of 32) if a column were added specifically for the evidence call-outs for each KER. Not only would it be more obvious to the reader this way, it would also match the formatting of the preceding table.

We agree with reviewer's comment. With regard to KER that could not be showed a clear quantitative relationship, we will correct the Weight of the Evidence to "medium/moderate".

3. *Additional observations:*

-What do you consider to be critical data gaps, if any, and how to fill these gaps?

Data Gaps –

- Considering that inhibition of CN phosphatase activity is the MIE, the lack of a "clear quantitative relationship" between inhibition of CN phosphatase activity and nuclear translocation of NFAT is a notable data gap (KER1) that, in its current state, could limit the utility of potential predictive tests.
- As written, the applicability domain of this AOP is very narrow (i.e., founded on the action of two closely related therapeutic agents) which leads this Reviewer to question the regulatory applicability of the AOP.
- In its current form, the AOP provides no guidance on how to apply it. Recognizing the complexity of the TDAR and the apparently limited applicability domain, it would be best if statements were included regarding use as part of an IATA.

As mentioned above, we will build a network of AOPs with TDAR as AO. We believe that AOP network including TDAR contribute to IATA of immunotoxicology. Therefore, we will develop some AOPs including TDAR as AO. We will add the statements regarding use as part of and IATA.

Other comments –

- The abstract is confusing in some places. For example, the first sentence would be clearer if it were worded "Inhibition of calcineurin (CN), a protein phosphatase, is known to impair immune function. Also, once a term is abbreviated, the abbreviation should be used in subsequent sections (i.e., CNIs vs CN inhibitors).
- According to the User's Handbook, the Essentiality of the Key Events section is supposed to be organized in "tabular" form, but the authors opted for paragraph form. Ideally, this section will be organized in a table with clear, scientifically supported evidence statements (i.e., high, medium, low).
- The TDAR is certainly a very useful assay, but it is an overstatement to describe it as the "…most important/useful endpoint" in all of immunotoxicity (pages 2 and 8). The AOP is focused on immunosuppression and TDAR should be discussed in that context.
- As written, the Overall Assessment of the AOP section (page 3 of 32) doesn't flow very well and would benefit from language adjustments and correction of typos (e.g., the word "bond" should also be changed to "bind").
- There are several typos (i.e., missing periods, extra periods) in Appendix 2.
- Consistency matters. To minimize confusion, please be sure that any adjustments to evidence calls are made throughout the AOP.

According to the reviewer's comment, we will replace the sentences, organize in tabular form, and correct the typo.

Regarding the usefulness of TDAR, ICH S8 guideline for immunotoxicology recommends that TDAR should be conducted when immunotoxicological evaluations are warranted. Therefore, we focus on TDAR.

Reviewer #3

1. Scientific quality:

• *Does the AOP incorporate the appropriate scientific literature?*

Yes, it does.

• *Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?*

While indeed reduction of the NFAT/AP-1 complex results in suppression of IL-2 and IL-4 production, this knowledge dates back to the early 1990's. In the meantime, have additional cytokines been reported that depend on NF-AT/AP-1 complex formation? The importance of this question comes from the fact that the whole of a TDAR relies on the activity of at least three cell types: dendritic cells, T-cells, and B-cells and therefore the TDAR likely depends on (effects on) a range of cytokines. In fact, the authors mention IFN-gamma themselves (required for DTH!). We need to be sure that measuring effects on IL-2 and IL-4 is sufficient to interrogate KE3.

We agree with reviewer's comments. However, we would like to show other cytokines would involve in the impaired TDAR in other AOP.

2. Weight of evidence:

• *Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?*

Yes, in my opinion they are.

3. Regulatory applicability:

• *Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?*

I have no question on the strength of evidence. However, the authors should elaborate on the potential application of the AOP, since this sequence of events has been clarified for two "model" drugs, FK-506 and cyclosporin-A. Again, these observations were made in the early 1990's and possibly, in the meantime chemicals and (additional) drugs have been identified that act via this sequence of events.

According to the reviewer's comment, we would like to search additional stressors.

4. Conclusion:

• *What are your overall conclusions of the assessment of this AOP?*

The underlying mechanisms are solid, KE3 needs more work, possibly its application is limited.

Reviewer #4

. Scientific quality:

-Does the AOP incorporate the appropriate scientific literature?

-Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

In the description of the AOP, the most salient papers of the many works published on the subject were cited. Overall, the scientific content of the AOP reflect current scientific knowledge on this specific topic. Perhaps the authors could have cited the paradoxical effect of the use of tacrolimus and cyclosporin A and their association with allergic diseases and elevated IgE in several transplant populations deserves a mention (Kawamura et al. ,1997).

It is relevant to mention that since the writing of this AOP, at least one very relevant paper has been published in 2019, on the response to influenza vaccination (Gangappa et al., 2019), which showed lower levels of influenza vaccine-specific antibody response in renal transplant recipients under immunosuppressive regimen including tacrolimus, further supporting the AOP.

Thank you for sharing very helpful paper. We would like to cite it and add descriptions.

Specific comments on the scientific content:

- although psoriasis and allergy both involve the immune system, the causes are different, and psoriasis is an autoimmune disorder. I suggest to write: …treat autoimmune and allergic disorders…
- in the Background, I will not limit the AOP to FK506 and FKBP12. Although the immediate receptors (immunophilins) of cyclosporin A and FK506 are distinct, their similar mechanisms of inhibition of cell signaling indicate that their associated immunophilin complexes interact with the common target, NF-AT (Liu et al., 1991). In many parts of the text, reference is made to calcineurin inhibitors in general, and the fact of mentioning in some parts only FK506 / FKBP12 is limiting. I will write …caused by FK506 and cyclosporin A… The AOP should be rewritten accordingly.

FK506-FKBP12 complex binds to calcineurin (CN), a Ca2+-calmodulin-dependent protein phosphatase, suppressing the interleukin2-dependent proliferation of T cells. FKBP12 itself, however, does not play an important role in T cells or in immune responses. On the contrary, FKBP12 participate in neurotransmitter release, neural nitric oxide production, nerve growth, signal transduction via the type 1 receptor for transforming growth factor-β and intracellular Ca2+ release from the sarcoplasmic and endoplasmic reticulum, via the ryanodine receptor and the inositol 1,4,5-trisphosphate receptor.

- TDAR is considered a gold standard in immunotoxicology assessment in general not just for drugs, it also applies to chemicals and, for example, it is mandatory for the registration of pesticides at US-EPA. Text needs to be corrected accordingly.
- maybe the effect of calcineurin inhibitors on T follicular helper cells and other CD4 T cell subsets should be expanded to further support the adverse outcome (decreased antibody production).

According to the reviewer's comment, we would like to correct.

We agree with reviewer's comment that TDAR is considered a gold standard in immunotoxicology assessment in general.

2. Weight of evidence:

-Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP, justified, i.e. consistent with the considerations outlined in the Users' Handbook?

Overall, the AOP is fully justified by published data, and clear key event relationships exist. The proposed AOP is biological plausible: in general, humoral immune responses depend critically upon T cell help. Thus, interfering with calcineurin will affect Th cells activation, compromising acquired immune responses toward T cell dependent antigens, including antibody production.

3. Additional observations:

-What do you consider to be critical data gaps, if any, and how to fill these gaps?

It is evident that calcineurin inhibitors affect the humoral immune response mainly by interfering with T helper signals, but not by targeting B cell antibody release directly. While cyclosporin A and FK506 are both direct inhibitors of B cell activation, as assessed by proliferation and plasmacells differentiation, they do not affect immunoglobulin release in the absence of T cells (De Bruyne et al., 2015). At present, only few reports have investigated the role of calcineurin inhibition on B-cell differentiation and antibody production in detail, representing a data gap. Thus, I totally agree that inhibition of calcineurin activity will impair T-cell dependent antibody response and so TDAR, but this AOP cannot be used as an alternative to TDAR measurement. It can only be used to assess direct T cell immunotoxicants. NF-AT inhibition is one of the mechanism that T cell immunotoxicant can use to compromise the immune response but not the only one.

Thank you for sharing very helpful literature and agreement that the impaired TDAR is lead via T -helper signals. As mentioned above, we will build a network of AOPs with TDAR as AO. We believe that the combination of alternative methods is established by merged some AOPs including TDAR as AO.

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Annex 3: Modifications in the AOP done by April 6th

Please note that addition are visible in red.

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

Inhibition of CN might induce suppression of cytokines production from all the T helper cell subsets as well as other immune functions of other immune cells. Suppression of cell-mediated immunity is involved in the pharmacology of preventing hyper immune reactions such as rejection and GVHD, and treatment of autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CN inhibition might induce immunosuppression-derived adverse outcomes. One of the effects is increased frequency and/or severity of infections. Compromised host might be related with impairment of multiple immune functions; however, impaired TDAR deems to be usually related. Moreover, TDAR is the frequently used measurable endpoint in immunotoxicity testing according the ICH S8 or US EPA OPPTS 870.7800 immunotoxicity testing guideline. Therefore, the present AOP focus on CN inhibition-induced impairment of TDAR.

Domain of applicability

The proposed AOP regarding inhibition of CN activity leading to impaired TDAR is not dependent on life stage, sex, or age. Since tacrolimus (FK506) ointment (Protopic) is approved for pediatric atopic dermatitis, the MOA for immunosuppression appears to be applicable to all life stages. The applicable state is considered supported by the draft FDA guidance for immunotoxicology that was recently issued (2020) indicating that example of immunotoxicology testing could included TDAR; to address the concern of immunotoxicity in offspring in juvenile animal studies.

Since FK506 or Cyclosporine A (CsA)-induced outcomes in humans are mimicked by similar responses in a variety of animal models including non-human primates and rodents, immunosuppression induced by inhibition of CN activity is considered to occur across a variety of mammalian species.

In addition to the drugs, it is known that CN activity is suppressed by alkeylbenzene sulfonate (dodecylbenzene sulfonate) extracted from an acrylonitrile butadiene rubber (Ito et al. 2013) suggesting that the proposed AOP would be applicable to non-pharmacological agents.

For the chemicals such as pesticide, TDAR is also recommended in the US EPA OPPTS 870.7800 immunotoxicity testing guideline.

Essentiality is supported by several knockout animals as follows.

Stage	Essentiality	Evidence	Supported by literatures
MIE and later	CnA-KO mice	Strong	The CN molecule consists of two regions, CnA and CnB, of which CnA exhibits phosphatase activity. In CnA-KO mice, T-cell proliferation in response to ovalbumin stimulation is lower than that for wild-type mice and is not complemented by normal antibody producing cells. In addition, when stimulated with ovalbumin, CnA-KO mice produce less IFN-γ, IL-2, and IL-4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to TNP-ovalbumin, which means that CnA deficiency affects only on T cell-dependent antibody response (TDAR) (Zhang et al. 1996).
KE1 and later	NFAT-KO mice	Strong	The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly, moderately enhanced B- and T-cell responses, with bias towards Th2-cell response, decreased IFN-γ production in response to T-cell receptor (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, and mild hyperactivation of peripheral T cells. Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by CN. Suppression of T-cell-derived cytokines is noted both in CnA-knockout and NFAT-knockout mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system (Macian, 2005).
Stressor	FKBP12-KO mice	Moderate	FK506 induces suppression of immune responses; however, there is no literature showing a relationship of a relationship between FKBP12 knockout and the immune system in the FKBP12-KO mouse model. Steric structure of FKBP12/FK506 complex is considered the key factor for inhibition of CN phosphatase activity, but not for the enzymatic activities of FKBP12

Weight of Evidence

N inhibition to interference, NFAT nuclear translocation
Moderate

KER2: Interference, nuclear localization NFAT to reduction, NFAT/AP-1 complex formation
NFAT has NLS and NES among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS is exposed and the NES is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000)
CNIs interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 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).

KER3:
T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015)
Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001)
Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012)

KER4:
CsA is known one of the calcineurin inhibitiors. CsA-treatment is reported to suppresses the productions of IL-2 and IL-4 and result in the reduction of the productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida et al. 2015)
Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody results in the decrease in T cell dependent antibody production.

Th2 cell produces cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 from Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells.

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cell. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016)

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, 2010).

Empirical support

KER1:

CN phosphatase activity is inhibited by CNI of FK506 with IC50 values of 0.5 nM (FK506) and 5nM (CsA) after 1 hours treatment (Fruman et al.1992)

Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the concentration from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 μM (1000 nM) under the conditions of 2 hours treatment of tacrolimus (Maguire et al. 2013)

Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991)

KER2:

Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the concentration from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 μM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013)

Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991).

The experiment of gel mobility shift assay using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1µM (Jain et al. 1992)

Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991)

KER3:

Reduction, NFAT/AP-1 complex formation leads to suppression, IL-2 and IL-4 production

In NFATp- and NFAT4-deficient mice, cultured splenocytes bound anti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998)

In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 μg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 μg/mL (Yoshida et al. 2015.

In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 μM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 μM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM for 4 h (Checker et al. 2012).

Gel mobility shift assay revealed that treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders NFAT nuclear translocation and following formation of NFAT/AP-1 complexes in the nucleus (Flanagan et al. 1991)

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 after 22 to 24 hours culture 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)

Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018)

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010)

Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

KER4:
Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida et al. 2015)

In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4weeks (Sanofi K.K. 2018)

In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines such as IL-4 and IL-5 on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Taiho Pharmaceutical 2013)

1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010)

K506 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)

In CD3/phorbol 12-myristate-13-acetate-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)
Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (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 sheep red blood cells (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.

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 (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2010).

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 (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001

Quantitative Considerations

KER4

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida et al. 2015).

Inhibition of IL-4 production in mice treated with oral administration of suplatast tosilate suppresses antigen-specific IgE production with a dose-dependent manner (Taiho Pharmaceutical 2013). In the inhibition of IL-4 production in human cell culture by suplatast tosilate at the concentration of 10 μg/mL for 10 days, antigen specific IgE production was suppressed from 56 to 72% and IL-4 production was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies. T (Owens T, 1991).

The draft FDA guidance of nonclinical safety evaluation for immunotoxicology is recently issued (2020) and recommends TDAR assay. Because TDAR is a common secondary assay that requires functionality of several key immune cell subtypes (e.g., antigen-presenting cells, T-helper cells, B cells)
For the assessment for pesticides, US EPA&OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using sheep red blood cells.

As a part of an IATA of immunotoxicology, the present AOP could be used to predict whether or not a compound that potentially acts on T cells could also affect TDAR. On the other hand, it would be inappropriate to use the present AOP alone as an alternative to TDAR measurement in the ICH S8 or US EPA&OPPTS 870.7800 immunotoxicity testing guideline

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