

**PMC Ag WG project:
New developmental immunotoxicity evidence from Babu et al. (2016) study**

Review of Babu et al. (2016)^a / SWOT Analysis

Linked to a one-generation reproductive toxicity study (OGRTS) on silver acetate^b (AgAc), Babu and co-workers at the US FDA Center for Food Safety and Applied Nutrition have separately published findings from an assessment of various immunotoxicology parameters in offspring.

The outcomes may be briefly summarised as follows: Rats were orally exposed via drinking water to silver acetate (AgAc) at treatment levels corresponding to 0, 0.4, 4 and 40 mg/kg bw/day (0, 0.26, 2.6 or 26 mg ionic Ag/kg bw/day). Splenic and thymic lymphocyte subsets from postnatal day (PND) 4 and 26 pups were assessed by flow cytometry for changes in phenotypic markers. Natural killer cell (NK) activity and mitogen-induced lymphocyte proliferation were selected as immune system function markers. PND 4 pups had lower percentages of splenic CD8+ (cytotoxic) T cells in the 4 and 40 mg AgAc/kg bw/day groups (2.6 or 26 mg ionic Ag/kg bw/day). Changes in phenotypic markers in splenocytes from PND 26 pups in the same groups included significantly lower TcR+ cells, and also an elevated B lymphocyte population at 40 mg AgAc/kg bw/day (26 mg ionic Ag/kg bw/day). Evidence of immune function disturbance was also presented in the paper, viz. mitogen-induced^c lymphocyte proliferation was apparently depressed in all three AgAc-treated groups at PND 4. However, no effects on the thymus, including on thymocyte phenotypic markers, were detected even though tissue exposure to Ag+ was evident. Innate immune response (splenic NK activity) was unaffected by treatment. In overview, these investigators reported that exposure to ionic Ag had a clear impact on rat splenic development (mainly in the early lactation period) during a OGRTS, although thymic development was unaffected. An important outcome was the inability to define a no observed (adverse) effect level for the immune function marker of the Concanavalin A response (mitogen-stimulated lymphocyte proliferation) in the F₁ generation.

Equivalent findings to those above have not previously been reported for ionic silver, and are therefore novel, and could: (i) ultimately have regulatory significance; (ii) bear on related aspects of the previously submitted PMC Testing Proposal for an Extended One Generation Reproductive Toxicity study [EOGRTS] for silver. Therefore, a SWOT Analysis^d of the Babu et al. publication has been conducted:-

1 - Significance/Strengths:

The parallel OGRTS (reported by Sprando et al., 2017) is ranked as robust (KL1/2), and its satellite immunotoxicology evaluations (Babu et al., 2016) would no doubt be regarded as relevant by the regulators. Justification to set aside the findings of Babu et al. is lacking (though it is not optimised in all technical aspects; see also '2 – Weaknesses'). For the specific methodologies utilised, e.g. in the immunophenotyping by flow cytometry, assay conduct appears generally acceptable (based on the details provided in the paper).

The Babu et al. publication relates to what previously represented an area of conflicting evidence and/or data gaps for ionic Ag.

The reduced splenic mitogen-induced lymphocyte proliferation response appears to be the clearest treatment-related effect, including possessing the greatest effect magnitude compared to control group values. The absence of a demonstrable NOAEL in offspring for this functional immune system parameter is a linked concern. Hence, the lowest observed effect level could be considered to be 0.26 mg ionic Ag equivalent/kg bw/day.

^a Babu US, Balan KV, Bigley E, Pereira M, Black T, Olejnik N, Keltner Z, Sprando RL (2016) Effects of maternal silver acetate exposure on immune biomarkers in a rodent model. Food Chem Toxicol. 98: 195-200.

^b Sprando RL, Black T, Keltner Z, Olejnik N, Ferguson M (2017) Silver acetate exposure: Effects on reproduction and post natal development. Food Chem Toxicol. 106: 547-557.

^c Concanavalin A-stimulated response.

^d Strengths/Significance – Weaknesses – Opportunities – Threats (SWOT).

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Conclusions/Recommendations: At face value, the study appears to be significant to developmental immunotoxicity (DIT) considerations for ionic Ag, and does impact on related aspects of the previous PMC EOGRTS TP. See also Table 1.

DIT study design and interpretation is a specialised area, and it is recommended that PMC refer these aspects to a recognised subject matter expert for a cross-checking opinion. It would be prudent to integrate all recent mammalian toxicology publications/data on Ag which may be relevant to such an opinion (refer to a recent literature survey update).

2 - Weaknesses:

Certain of the specific findings merit further evaluation. Although statistically significant, the magnitude of some of the splenocyte population shifts was not major and a monotonic dose-response was not always evident (e.g. at PND 4). This bears on the interpretation of these detected effects. A few other aspects of the results are puzzling, e.g. though investigators suggest that the reduced splenic Concanavalin A response among PD4 pups could be attributed to decreased CD8+ population, they had to point out that this is discordant with the low-dose group increased CD8+ cell counts.

The study integrates immunotoxicology testing concepts based on Chapter V of the draft FDA Redbook II which date from the early 1990's, and are therefore not necessarily in line with the latest testing approaches, including those integrated into the OECD TG 443 for the EOGRTS. In particular, the functional immunity assays performed were rather limited; and importantly, no integrating T-cell dependent antibody response (TDAR) assay^e was conducted.

It is crucial to differentiate between a primary immunosuppressive effect and indirect effects mediated by stress, or general homeostasis disturbance (e.g. such as may be caused by gut microbiota disturbance due to Ag biocidal effects). Related principles and the means to differentiate primary immunosuppression are mentioned in a review by Moore et al. (2016)^f. Stress hormone measurements, and monitors of certain other confounding influences relevant to Ag, were not included in the FDA OGRS on AgAc.

Some errors are apparent in the publication (e.g. in respect of Fig. 2), though these do not substantively affect its credibility.

Conclusions/Recommendations: Integrate the viewpoint of one or more external experts in DIT testing on the technical approach taken by Babu et al., and on any key deficiencies or gaps. For instance, whilst statistically significant differences in certain splenocyte populations were noted for treated groups, an expert would be able to comment on whether the magnitude of these effects represented an event with serious implications.

^e **Steady state immune system versus evaluation of its responsiveness:** Assessment of 'steady state' immune system competence following exposure to a test substance is undertaken as a core part of an EOGRTS. Splenic cellularity and haematology are evaluated in cohort 1A; spleen and thymus from all F1 adults are weighed; and spleen, thymus, and samples of bone marrow from all F1 adults are taken for possible histopathology. However, these do not evaluate whether the test substance has affected the ability to mount a proper functional response to antigen exposure. Functional response, which integrates several aspects of the immune system, is determined in EOGRTS Cohort 3 with a T-cell dependent antibody response (TDAR) assay to detect substances that suppress adaptive immunity.

^f Moore NP, Beekhuijzen M, Boogaard PJ, Foreman JE, North CM, Palermo C, Schneider S, Strauss V, van Ravenzwaay B, Poole A (2016) Guidance on the selection of cohorts for the extended one-generation reproduction toxicity study (OECD test guideline 443). Regul Toxicol Pharmacol. 80: 32-40.

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3 - Opportunities:

The weakness described in the previous section in respect of functional immune system evaluation represents an area of criticism, and a pressure point for proposing further investigation of whether ionic Ag definitely causes DIT. It represents a justification for a follow-on study seeking to confirm or refute the Babu et al. report (and also the main reproductive toxicity outcomes from Sprando et al., 2017), i.e. justification support of an EOGRTS with a DIT cohort. The EOGRTS TG for DIT features a T-cell dependent antibody response (TDAR) assay which can be supplemented by inclusion of other functional immunity markers in the study protocol. Recent risk assessment guidelines, incorporating recommendations for correct detection of DIT, are clear that TDAR is an important parameter⁹, which has a reference data set for immunotoxic substances accumulated over several decades. As a secondary consideration, TDAR assay is generally accepted by regulatory bodies worldwide, e.g. it features in current US EPA test guidelines for immunotoxicity evaluation in adult animals (OPPTS 870.7800). When two or more assays are used in combinatorial mode (such as TDAR and NK cell assays) the predictive power for immunosuppression/reduced host resistance is generally excellent. In this context, a notable favourable finding from Babu et al. was that NK cell activity appeared unaffected by ionic Ag treatment.

There is a disparity between the outcomes of Babu et al. where effects on the thymus were not evident using objective and sensitive markers, and the findings in 2-generation reproductive toxicity evaluation of silver containing active substances. In the latter instance – as a less specific indicator of possible DIT – thymus weights were reduced in treated groups, e.g. affecting both F₁ and F₂ generations in the studies on silver zinc zeolite and silver sodium hydrogen zirconium phosphate). It might be argued that the presence of constituents other than Ag complicates read-across interpretation, but this disparity between the 2-generation studies and the FDA OGRS could also represent a secondary justification that the DIT picture is unclear, and therefore requires further follow up in a new EOGRTS. In previous decision making on SZZ, RAC did accord some importance to the thymus effects in offspring seen with SCAS.

Conclusions/Recommendations: Progress a technical re-review of the existing PMC TP for an EOGRTS, including DIT aspects. Integrate all recent mammalian toxicology publications/data on Ag which may be relevant. Integrate the viewpoint of one or more external experts in DIT testing on how the TP should be adapted and any justifications for doing so. Assess whether enabling immunotoxicology work would further inform on the viability of proceeding with an EOGRTS.

4 - Threats:

Overall, Babu et al. (2016) represents additional pressure toward a classification of ionic Ag for reproductive toxicity. It is not possible to reliably predict whether ECHA RAC would judge the findings to be of such toxicological significance to accord a Cat. 1B classification rather than Cat. 2, also taking into account the themes covered in a separate report^h on Sprando et al., 2017. It is perplexing that the Babu et al. study was not picked up by Kemi in the drafting of their recent CLH dossiers for SCAS (though background searches indicate that separately EU EFSA have evaluated it, and consider an immunotoxic effect to have been demonstrated).

A risk exists that regulators could conflate the specific spleen lymphocyte findings from Babu et al. with less specific effects evident in 2-generation studies on SCAS – predominantly thymic weight depression seen in F₁ and F₂ generation pups – and conclude that DIT potential of ionic silver is proven. This would be scientifically flawed, given that Babu et al. did not report thymic cell population disturbance, and because indirect effects such as stress may impact on thymic weight. Nevertheless, it does represent a foreseeable threat in terms of regulatory interpretation/classification weight-of-evidence.

⁹ WHO, 2012. Guidance for Immunotoxicity Risk Assessment for Chemicals. Harmonization Project Document No. 10. World Health Organization, Geneva.

^h PMC Ag WG project: SCAS CLH dossier reviews / Sprando et al. (2017) review / Updated Ag literature search, Raffray Biosciences Ltd., 31 August 2017.

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As a further key consideration, the Babu et al. report undermines the PMC TP justification for omission of the optional DIT cohort in the EOGRTS. The PMC TP predated the latest developments in the DIT dataset; triggering justification for DIT cohort inclusion is now considered to be fulfilled – see also Table 1.

If a PMC-sponsored EOGRTS does proceed, budget and complexity will be adversely impacted by these latest data. A worst-case budget assumption would now be prudent, also factoring the F₂ generation costing (due to triggers evident in Sprando et al., 2017).

Conclusions/Recommendations: Obtain a cross-checking viewpoint from a DIT expert on the impacts related to ionic Ag classification, and on the EOGRTS TP. Update other Ag stakeholders on the threats and industry options. Until further expert input and/or regulatory developments are clear, make a budget provision for a worst-case EOGRTS, plus a further provision for enabling immunotoxicity foundation studies to support the EOGRTS.

TABLE 1 Ionic Ag – Immunotoxicity evidence base: trigger points relevant to EOGRTS

The following table provides a synthesis of the available evidence previously reviewed with PMC WG's whilst now also integrating the latest findings from Babu et al. (2016).

Key criterion	Evidence Summation Conclusion	Remarks
SAR or chemical class linked to immunotoxicity	TENTATIVE	Only if Ag viewed as aligned to other immunotoxic/'heavy' metals. Some regulators lean toward this speculative viewpoint.
Evidence of mechanistically-relevant endocrine disruption	NO	
Altered weight or pathology of lymphoid organs	EQUIVOCAL	Some thymic effects, principally organ weight depression were observed in 2-generation studies on SCAS (including an effect in offspring). However, this was <u>not</u> replicated in Babu et al. (2016).
Altered cellularity of bone marrow, spleen, thymus, or lymph nodes	YESⁱ	Babu et al. (2016) is an important study (in respect of spleen cell effects) which does align with previous less reliable evidence.
Altered differential white cell counts	NO	Fragmentary evidence only in humans and animal models.
Functional immunotoxicity in adults	NO	No robust evidence from conventional RDT and 2-generation studies on silver-containing substances. Conflicting reports exist for Ag NP (e.g. i.v. route) of questionable relevance to Ag ⁺ .
Functional immunotoxicity in neonates	NOT PROPERLY ASSESSED	Babu et al. (2016) included only limited evaluation of <u>functional</u> immune system status. For example, a T-cell dependent antibody response (TDAR) assay was not included.

ⁱ In terms of guidance applicable to EOGRTS, this is sufficient to represent an external trigger for inclusion of the developmental immunotoxicity cohort extension to the study.