Standards, based on validated and accepted test methods, can be used to evaluate the accuracy and reliability of other analogous test methods (also referred to as "me-too" tests) that are based on the same or similar scientific principles and that measure or predict the same biological or toxic effects.

Performance Standards should be provided by the Management Team of a validation study, and, as appropriate, used in the TGs issued for new test methods. The three main elements of Performance Standards are:

a) The essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed mechanistically and functionally similar test method. These components include the unique characteristics of the test method, critical procedural details and quality control measures. Adherence to the essential test method components will help to ensure that a proposed test method is based on the same concepts as the corresponding validated test method.

b) A list of recommended reference chemicals that are used to assess the accuracy and reliability of a proposed mechanistically and functionally similar test method. These chemicals are a representative subset of those used to demonstrate the reliability and the accuracy of the validated method.

c) Accuracy and reliability values, which represent the comparable performance requisites that should be achieved by the proposed test method when evaluated by using the list of reference chemicals.

So far, Performance Standards have been developed only for *in vitro* methods, i.e. for *in vitro* skin corrosion testing (27, 28) and *in vitro* skin irritation testing (29). In both cases, they should be used to evaluate the performance of human skin models which are similar to those that have already been validated.

Background to the LLNA

The LLNA identifies chemicals that have skin sensitising potential (8; 30–32). The assay measures sensitising activity as a function of proliferative responses induced in auricular lymph nodes following the repeated topical exposure of mice to several concentrations of the test chemical. In the standard LLNA, the proliferation of draining lymph node cells (LNCs) is measured by using the incorporation of ^{3}H-thymidine and subsequent β scintillation counting. For this purpose, mice are injected intravenously (via the tail vein) with a source of ^{3}H-thymidine, five days after the initiation of exposure to the test chemical.

This approach to measuring the proliferative activity of LNCs was based on studies in which the sensitivity and specificity of various read-outs for lymph node activation and lymphocyte turnover were compared. Of the endpoints considered, the incorporation of radiolabelled thymidine was found to provide the most robust and most reliable correlation with skin sensitising potential.

Although the standard LLNA, which incorporates this approach for the determination of LNC activation and proliferation, has provided a useful and reliable method for identifying skin sensitising chemicals, it is acknowledged that it would be beneficial to have available a version of the LLNA that does not require the use of radioisotopes. For this reason, there has been interest in exploring other relevant read-outs for the assay, including alternative strategies for the measurement of LNC turnover.

Among the approaches that have been explored are:

a) the direct measurement of changes in draining lymph weight and/or cellularity (33, 34);

b) the measurement of other endpoints, such as induced changes in the concentration of ATP, that can serve as surrogates of altered lymph node cellularity;

c) the measurement of induced changes in the relative number of lymphocyte phenotypes found in draining lymph nodes, e.g. alterations in B lymphocytes ($B220^{+}$) number or in the representation of discrete T-lymphocyte sub-sets (CD62L/CD44; 35–37);

d) the characterisation of the elaboration by LNCs of cytokines, such as interleukin-2 (IL-2), a T-lymphocyte growth factor (38–40);

e) the use of non-radioactive methods for the determination of cell turnover in draining lymph nodes, e.g. the use of BrdU (41–43);

f) the use of radioisotypes other than ^{3}H-thymidine, such ^{125}I-uridine (44); and

g) the use of *in vitro*, rather than *in vivo*, radiolabelling of LNCs (45).

However, the challenge is to ensure that such alternative approaches, employing novel read-outs, have sensitivity, specificity, and overall accuracy and reliability, comparable to the standard LLNA. It is for this purpose, and for verifying the acceptability of the performance characteristics of modified assays, that the proposed Performance Standards described here were developed. However, it must be

emphasised that such Performance Standards are intended for providing an accelerated validation only for versions of the LLNA that involve only minor modifications to the standard assay as described in OECD TG 429 (1).

Both ECVAM and ICCVAM (46) have been working on developing Performance Standards for the LLNA. Their two draft documents were presented and discussed at the ECVAM workshop.

Against this background, the workshop participants debated and suggested the criteria and a list of reference chemicals that should be considered in developing Performance Standards for the LLNA. Consideration was given, not only to the performance of the test as it was assessed in the peer review of the LLNA, but also to the experience gained from the use of the test during the years that have followed that evaluation.

The proposed Performance Standards, as outlined in this workshop, were subsequently considered by ECVAM and ICCVAM as a contribution to the ongoing process of harmonisation of the two documents.

Proposed Performance Standards

Essential test method components

For these Performance Standards to apply, the modified LLNA must comply fully with the OECD TG 429 (1), with the exception of the way in which the lymph node cell proliferation is measured. Ideally, there should be no alteration to the prediction model, but it is accepted that this may be necessary, dependent on how the proliferation is measured. Significant changes to the prediction model may, of course, trigger the need for a more substantial assessment than is provided for by this proposed set of Performance Standards.

Recommended reference chemicals

Reference chemicals should be used to assess whether the performance of modified LLNA methods is comparable with that of the standard LLNA. A set of 20 chemicals, which comprise 13 sensitisers, 5 non-sensitisers, 1 false positive and 1 false negative in the standard LLNA, are suggested for the purpose (Table 1). For the selection of the relevant substances, the following criteria have been considered:

a) The chemicals should be readily available from one or more commercial suppliers.

b) Ideally, for each of the selected chemicals, LLNA and guinea-pig data (from either the GPMT or the Buehler test) should be available, as well as evidence for the elicitation of contact sensitisation in humans.

c) For the majority of the chemicals, there should be a clear consensus about the presence or absence of skin sensitising potential, where the results of all the existing predictive test results match the human data. The exceptions to this are sodium lauryl sulphate (SLS) and nickel sulphate, which have been selected as being false positive and false negative, respectively, in the standard LLNA.

d) There should be no doubt that the skin sensitising activity is attributable to the defined chemical structure, rather than to a contaminant.

e) Defined EC3 values should be available for the selected chemicals.

f) The selected chemicals should not be unstable or require unusual storage or transport conditions.

Within the pool of chemicals selected, attempts have been made to represent the variety of chemical classes and properties associated with skin sensitisation, although it must be recognised that, with a limited number of chemicals, not all the variables can be fully represented.

Furthermore, the list of chemicals has been selected to reflect the range of skin sensitisation potencies which are known to exist, but with emphasis on those chemicals considered to show weak or moderate potency, since these will provide the best assurance of the retention of test sensitivity, commensurate with the absolute minimum of additional animal testing.

In additional, in any evaluations of this type, it is important to define, in advance, whether a chemical is sufficiently sensitising to be formally classified according to the current regulations as a skin sensitiser. Only if this is the case, should it be identified as a positive in a predictive test, with the consequence that the existence of positive human data (e.g. in clinical case reports) is not, of itself, sufficient to mean that a chemical should be regarded as a substance which should test positive in a predictive assay.

The number of chemicals proposed should be sufficient to permit an assessment of the performance of modified versions of the LLNA, while minimising the number of animals required for such an evaluation.

Performance requisites: predictive capacity and reliability

Predictive capacity is defined as the capacity of a method to predict the accepted reference values

Table 1: Reference chemicals recommended for the evaluation of modified LLNA methods for the identification of skin sensitisation hazard

Chemical name	CAS No.	Physical form	Vehicle	EC3 LLNA	Value	n	Reference
Benzoquinone	106-51-4	Solid	AOO	+	0.01[a]	1	Gerberick *et al.* (54)
2,4-Dinitrochlorobenzene	97-00-7	Liquid	AOO	+	0.04[b]	13	Basketter *et al.* (20)
4-Phenylenediamine	106-50-3	Solid	AOO	+	0.11[b]	10	Basketter *et al.* (20)
Isoeugenol	97-54-1	Liquid	AOO	+	1.5[b]	31	Basketter *et al.* (20)
2-Mercaptobenzothiazole	149-30-4	Solid	DMF	+	1.7[a]	1	Gerberick *et al.* (54)
Diethyl maleate	141-05-9	Liquid	AOO	+	3.9[b]	2	Basketter *et al.* (55) Ryan *et al.* (56)
α-Hexyl cinnamic aldehyde	101-86-0	Liquid	AOO	+	9.9[b]	15	Basketter *et al.* (20)
Eugenol	97-53-0	Liquid	AOO	+	10.1[b]	4	Basketter *et al.* (20)
Citral	5392-40-5	Liquid	AOO	+	13[a]	1	Gerberick *et al.* (54)
Phenyl benzoate	93-99-2	Solid	AOO	+	20[a]	1	Gerberick *et al.* (54)
Cinnamic alcohol	104-54-1	Solid	AOO	+	21[a]	1	Gerberick *et al.* (54)
Imidazolidinyl urea	39236-46-9	Solid	DMF	+	24[a]	1	Gerberick *et al.* (54)
Ethyl acrylate	140-88-5	Liquid	AOO	+	32.4[b]	2	Dearman *et al.* (57) Warbrick *et al.* (58)
Methyl salicylate	119-36-8	Liquid	AOO	–			Gerberick *et al.* (54)
Isopropanol	67-63-0	Liquid	AOO	–			Gerberick *et al.* (54)
Salicylic acid	69-72-7	Solid	AOO	–			Gerberick *et al.* (54)
Lactic acid	50-21-5	Solid	DMSO	–			Gerberick *et al.* (54)
Hexane	110-54-3	Liquid	AOO	–			Gerberick *et al.* (54)
Sodium lauryl sulphate	151-21-3	Solid	DMF	False positive			Gerberick *et al.* (54)
Nickel sulphate	10101-98-1	Solid	DMSO	False negative			Basketter & Scholes (59)

[a]*single EC3 values;* [b]*mean EC3 values; AOO = acetone/olive oil (4:1 v/v); DMF = dimethylformamide; DMSO = dimethylsulphoxide. + = sensitiser in the standard LLNA; – = non-sensitiser in the standard LLNA.*

(47). This is also referred to as the accuracy of a test method. The predictive capacity of a proposed test method should, when evaluated by using the recommended reference chemicals, at least meet or exceed that of the validated reference method. For this purpose, the threshold concentration at which a test chemical is positive in a new test method must fall within the range of 0.5–2.0× the published threshold (EC3) values from the standard LLNA in the relevant vehicle.

Reliability refers to the extent to which a test method can be performed reproducibly within and between laboratories and over time, when conducted by using the same protocol (26).

For the assessment of the inter-laboratory reproducibility, it is recommended that, when tested in at least three different laboratories, the threshold concentrations at which both α-hexyl cinnamic aldehyde (HCA) and 2,4-dinitrochlorobenzene (DNCB) are positive, fall within a range of 0.5× and 2.0× the published threshold (EC3) values in the standard LLNA.

Intra-laboratory reproducibility should be demonstrated by using HCA on four separate occasions over at least a 12-week period. Again, the threshold concentration should fall between 0.5× and 2.0× that of the expected threshold (EC3) value in the standard LLNA.

Alternative Endpoints to the Standard LLNA: Examples

This section describes three examples of alternative endpoints that are currently being developed for the LLNA.

1. A non-radioactive (non-RI) method, in which ATP content measurement is used as the endpoint

Principle

ATP is the principal energy source for all living organisms, and the amount of ATP determined is

known to correlate with the number of living cells (48, 49). Therefore, ATP content is considered to be a possible alternative endpoint to ^{3}H-thymidine incorporation as an index of cell number. An alternative LLNA involving the use of this endpoint as a non-RI method, termed the LLNA-DA (LLNA modified by Daicel, based on ATP content), has been developed (by Daicel Chemical Industries Ltd, Niigata, Japan; 50, 51). Measuring ATP contents has several advantages. The procedure for determining ATP content is easy and rapid, with a wide dynamic range for its stimulation index (SI).

Description of the method

In the protocol for the LLNA-DA, groups of female CBA/JNCrlj mice are treated by the topical application of 25μl of the test chemical (or the vehicle control) to the dorsum of both ears on days 1, 2, 3, and 7. Pretreatment with 1% SLS is carried out 1 hour before each application. On day 8, the auricular lymph nodes are excised. After recording the lymph node weight, single cell suspensions in phosphate-buffered saline are immediately prepared for each individual animal. The ATP content is determined by a luciferin–luciferase assay after appropriate sample dilution. For the determination of the ATP content, several measurement kits are commercially available (e.g. the Via Light™ HS Kit [Lonza Rockland, Inc., Rockland, ME, USA] and CheckLite™ 250 Plus [Kikkoman Corporation, Chiba, Japan]). A SI relative to the concurrent vehicle control is derived, and a SI of 3 is set as the cut-off value.

Summary of results

Thirty-one well-known chemicals were tested by the lead laboratory, and clear dose–response results were observed for weak sensitisers, as well as for strong or extreme sensitisers. The performance of this method showed good agreement with that of the standard LLNA or other test systems (Table 2). The accuracy of LLNA-DA compared to the standard LLNA was 93% (28/30), LLNA-DA *versus* guinea-pig tests was 80% (20/25), and LLNA-DA *versus* human tests was 79% (15/19). Similarly to the LLNA, the LLNA-DA uses a SI of 3 to discriminate between positive and negative responses. The EC3 values of the LLNA-DA, calculated from the positive tested chemicals, were compared with those of the original LLNA (Figure 1). This demonstrated that the EC3 values obtained with LLNA-DA were very similar to those obtained with the standard LLNA.

To evaluate inter-laboratory reproducibility, two large scale inter-laboratory validation studies were conducted in Japan. The results from 17 laboratories, with 14 chemicals under blind conditions in the two studies, showed that this method is sensitive, with little intra-laboratory variation. In the first study, acceptably small inter-laboratory variations in SI values were obtained for 10 chemicals, with the exception of two metallic salts. In the second study, with five chemicals, including three metallic salts, acceptably small variations for all the chemicals were obtained.

Conclusions

Compared with the standard LLNA, the LLNA-DA not only has a modified endpoint measurement, but also requires an adjustment to the dosing schedule and a pretreatment with SLS — changes which are necessary to enable the SI cut-off value of the LLNA-DA to retain that of standard the LLNA. As a consequence, this method cannot be considered to be a minor modification of the standard LLNA. However, as described above, the evidence concerning its sensitivity and reliability suggests that this approach looks promising.

2. A non-RI modification of the LLNA, based on BrdU incorporation

Principle

This modification of the LLNA has been developed as an alternative to the standard LLNA. It is based on BrdU (5-bromo-2′-deoxyuridine) incorporation in place of ^{3}H-thymidine incorporation, to measure lymph node cell proliferation (41).

Description of the method

The method based on BrdU incorporation is practically identical to the standard LLNA methodology, apart from the use of BrdU and colorimetric detection, for which a single intraperitoneal injection (5mg/mouse per injection) of BrdU was made on day 4. This administration schedule was found to be the most effective labelling protocol for yielding maximum SI values, based on preliminary study data with several different protocols. Approximately 24 hours after the BrdU injection, the auricular lymph nodes were removed, weighed, and stored at –20°C until analysed by using an enzyme-linked immunosorbent assay (ELISA) to measure the level of BrdU incorporation. In the current form of the test, cellular proliferative responses are measured by using a commercial BrdU detection

Table 2: Comparison of the concordances for 31 chemicals

Chemicals	LLNA-DA	LLNA*	GPMT/BA*	HMT/HPTA*
2,4-Dinitrochlorobenzene	+	+	+	
p-Phenylenediamine	+	+	+	+
Toluene diisocyanate	+	+		
Glutaraldehyde	+	+		
$K_2Cr_2O_7$	+	+	+	+
Phthalic anhydride	+	+	+	
Trimellitic anhydride	+	+		
Formaldehyde	+	+	+	+
Cinnamic aldehyde	+	+	+	+
Isoeugenol	+	+	+	+
Cobalt chloride	+	+	+	+
Eugenol	+	+	+	+
Resorcinol	+	+	–	+
Benzocaine	+	+/–	+	+/–
Abietic acid	+	+	+	+
α-Hexyl cinnamic aldehyde	+	+	+	
Mercaptobenzothiazole	–	+	+	+
Citral	+	+	+	+
Hydroxycitronellal	+	+	+	+
Imidazolidinyl urea	+	+	+	+
SLS	+	+	–	–
$NiSO_4$	–	–	+	+
Benzalkonium chloride	+	–	–	+
Propyl paraben	–	–	–	+/–
Diethylphthalate	–	–		
1-Bromobutane	–	–		
Methylsalicylate	–	–	–	–
Chlorobenzene	–	–	–	
Lactic Acid	–	–	–	
Hexane	–	–		–
Isopropanol	–	–	–	

*GPMT = guinea-pig maximisation test; BA = Buehler assay; HMT = human maximisation test; HPTA = human patch test allergen. *Results of the LLNA, GPMT/BA and HMT/HPTA are taken from references 18, 20, 54 and 60. + = sensitiser; – = non-sensitiser; +/– = ambiguous results.*

kit (e.g. one provided by Roche Diagnostics, Indianapolis, IN, USA; Cat. No. 11647229001). To perform the BrdU ELISA, the lymph nodes were crushed, passed through a #70 nylon mesh, and individual LNCs were suspended in 15ml of physiological saline. The cell suspension was added to the wells of a flat-bottomed microplate, in triplicate. After fixation and denaturation of the LNCs, anti-BrdU antibody was added to each well, and after rinsing, a substrate solution containing tetramethylbenzidine (TMB) was added and allowed to produce the chromogen. Absorbance at 370nm, with a reference wavelength of 492nm, was defined as the BrdU labelling index (41).

Summary of results

This method can display clear dose-related responses, and the potency class prediction is obtained with similar concentrations of test chemicals to those required in the standard LLNA (42, 43).

However, the potential lower sensitivity of non-RI alternative methods was also recognised as an issue. To evaluate the assay performance of this non-RI LLNA, 23 well-known chemicals, categorised as human contact allergens from class 1 to class 5, were tested, and the results were analysed to identify the best prediction model (Table 3).

Consequently, the conditions that set the cut-off point as SI > 1.5, with statistical significance between the treated and concurrent vehicle control or the cut-off point as SI > 1.5 with > 3 SD of the mean SI of the concurrent vehicle control, were adopted as the best endpoints for this non-RI LLNA method. By using these decision criteria, the highest concordance and lowest number of false negatives in the non-RI LLNA were obtained, as compared with the standard LLNA (52).

In addition, a novel approach to predicting the sensitisation potency of chemicals by comparison with known human contact allergens has been proposed as a useful application of this method (Table 4; 43). In this approach, nine well-known chemicals, categorised as human contact allergens from class 1 to class 5, were tested by the non-RI LLNA, with the reference allergens, 2,4-dinitrochlorobenzene (DNCB) as the class 1 human contact allergen, isoeugenol as the class 2 human contact allergen, and HCA as the class 3 human contact allergen. All the chemicals were assigned to the correct or adjacent classes.

Conclusions

The results suggested that this new strategy for a non-RI LLNA could provide both hazard identification and sensitisation potency data, which are prerequisites for judging the sensitisation risk for humans represented by new chemical products. These results indicate that a non-RI LLNA based on the BrdU incorporation, may be one of the promising alternatives to the standard LLNA.

3. The measurement of cell numbers and ear thickness as alternative non-radioactive endpoints in the LLNA

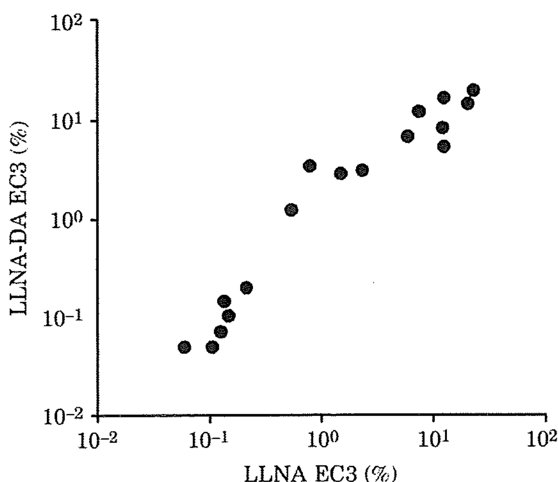
Principle

In the LLNA, lymphocyte proliferation in the auricular lymph node is measured by analysing ^{3}H-thymidine incorporation in the lymph node as a whole, after intravenous injection in the test mice. Based on the notion that the process of proliferation is induced within the lymph node, and

Table 3: Positive (+) and negative (–) classifications by different SI cut-off values in the non-RI LLNA based on BrdU incorporation

	Cut-off SI value																		
Chemical name	**3.0**	**2.9**	**2.8**	**2.7**	**2.6**	**2.5**	**2.4**	**2.3**	**2.2**	**2.1**	**2.0**	**1.9**	**1.8**	**1.7**	**1.6**	**1.5**	**1.4**	**1.3**	**RI**
2,4-Dinitrochlorobenzene	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1,4-Benzoquinone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Diphencyclopropenone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glutaraldehyde	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1,4-Phenylenediamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2-Mercaptobenzothiazole	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
Cinnamic aldehyde	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Isoeugenol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1,3-Aminophenol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-(4-Isopropylphenyl) isobutyraldehyde	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
Citral	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eugenol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydroxycitronellal	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
α-Hexyl cinnamic aldehyde	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Isopropyl myristate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4-Chloroaniline	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aniline	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–
2-Hydroxypropylmethacrylate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glycerol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Isopropanol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phthalic acid diethyl ester	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Propylene glycol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Dimethyl isophthalate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Figure 1: Correlation of EC3 values in the standard LLNA and the LLNA-DA



that the proliferation of lymphocytes results in increased numbers of lymphocyte after application of the sensitiser on the ear, assessment of lymphocyte numbers rather than lymphocyte proliferation as the read-out, has been proposed (33, 34). Many studies show that the ear-draining lymph nodes increase in size after the application of sensitisers on the ear. In addition, the number of lymphocytes is also increased after such treatment. There is good correlation between lymph node cell number counts and ^{3}H-thymidine incorporation (Figure 2).

Description of the method

This alternative non-RI test is based on the standard LLNA described in OECD TG 429 (1). Instead of assessing proliferative reactions directly, the method involves the removal of the lymph nodes, the preparation of single cell suspensions according to routine procedures, and the subsequent counting of cell numbers by using automated cell-counting devices. However, the proposed test also involves a number of other deviations from the standard LLNA: the use of BALB/c or NMRI mice, the excision of lymph nodes at day 4 instead of day 6, and the use of DAE433 as a vehicle. Also, a cut-off at a stimulation index of 1.4, subject to the provision of statistical significance, is used.

Some chemicals that have irritant capacity, also appear to be capable of inducing cell proliferation, and thus increase numbers of cells in the lymph nodes draining the ears on to which the test chemical is applied. Therefore, an additional endpoint on irritancy, i.e. the measurement of ear thickness, was added to this non-RI approach. Ear thickness can be measured in the same mice in which the modified LLNA is carried out. At the same time as when the lymph nodes are removed, punch biopsies of the ears are made and weighed (53). An increment in weight amounting to 1.2-times the control value, and which is statistically significant, is considered a positive result. The classification of chemicals is then based on both types of information (Table 5).

Summary of results

In a collaborative study, carried out by nine laboratories in Europe with 12 chemicals (including four irritants and one fully negative control) and BALB/c mice, the proposal to use lymph node cell numbers as the read-out for assessing the sensitising capacities of chemicals was put to the test (33, 34). The majority of the laboratories correctly identified the chemicals that were characterised as sensitisers, on the basis of statistically-significant increases in cell numbers. The results for HCA are shown in Table 6. The results from a sin-

Table 4: Classification criteria for the sensitisation potency of chemicals tested by the non-RI LLNA based on BrdU incorporation

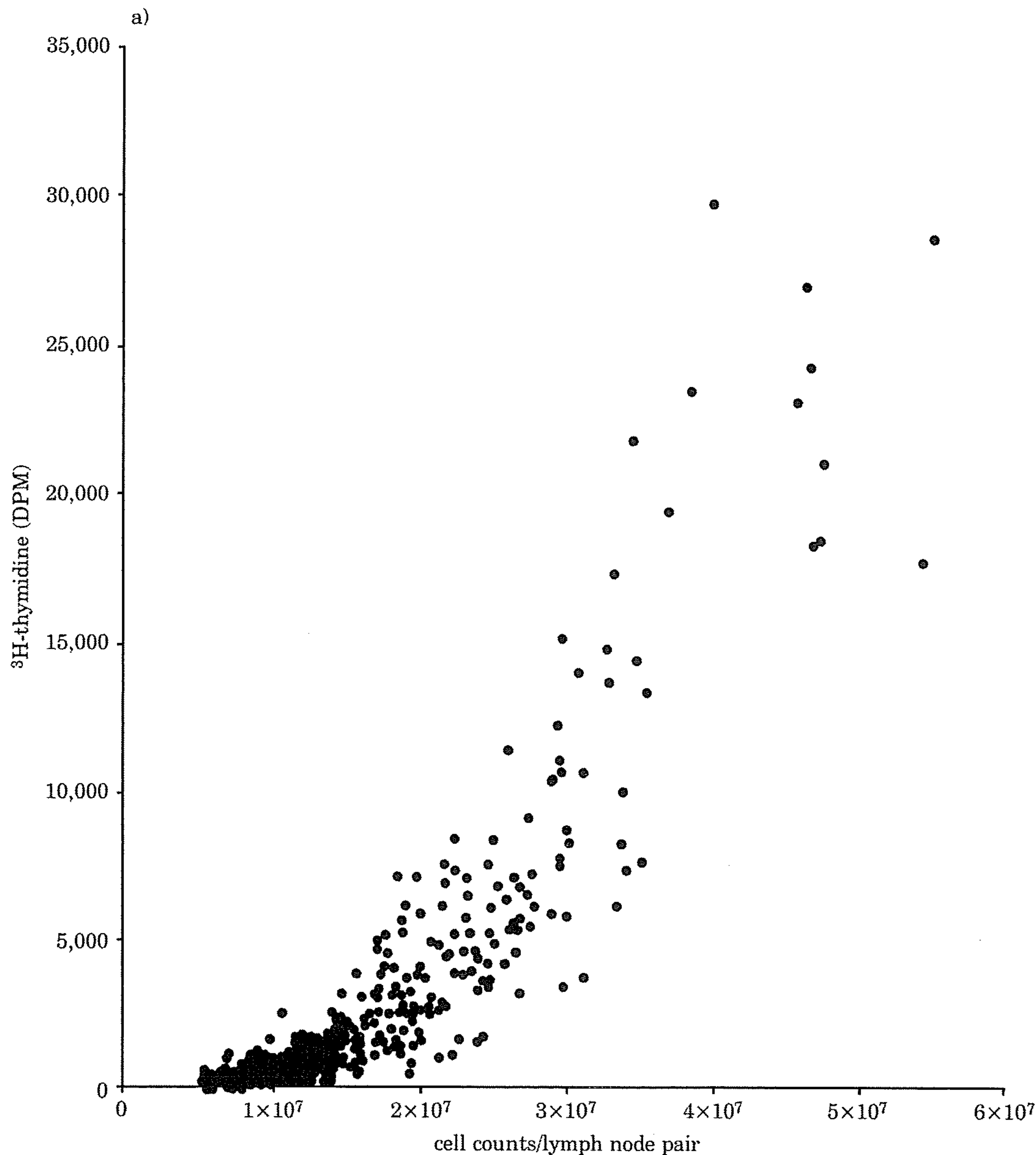
Human class	Requirements	Sensitisation class
1	SI for 2% test chemical ≥ SI for 2% DNCB	Strong sensitiser
2	SI for 2% test chemical < SI for 2% DNCB SI for 10% test chemical ≥ SI for 10% isoeugenol	Moderate sensitiser Moderate sensitiser
3	SI for 10% test chemical < SI for 10% isoeugenol SI for 50% test chemical ≥ SI for 50% HCA	Weak sensitiser Weak sensitiser
4–5	SI for 50% test chemical < SI for 50% HCA	Extremely weak or non-sensitiser

gle laboratory indicated that the assessment of potency by using this method was reproducible.

Conclusions

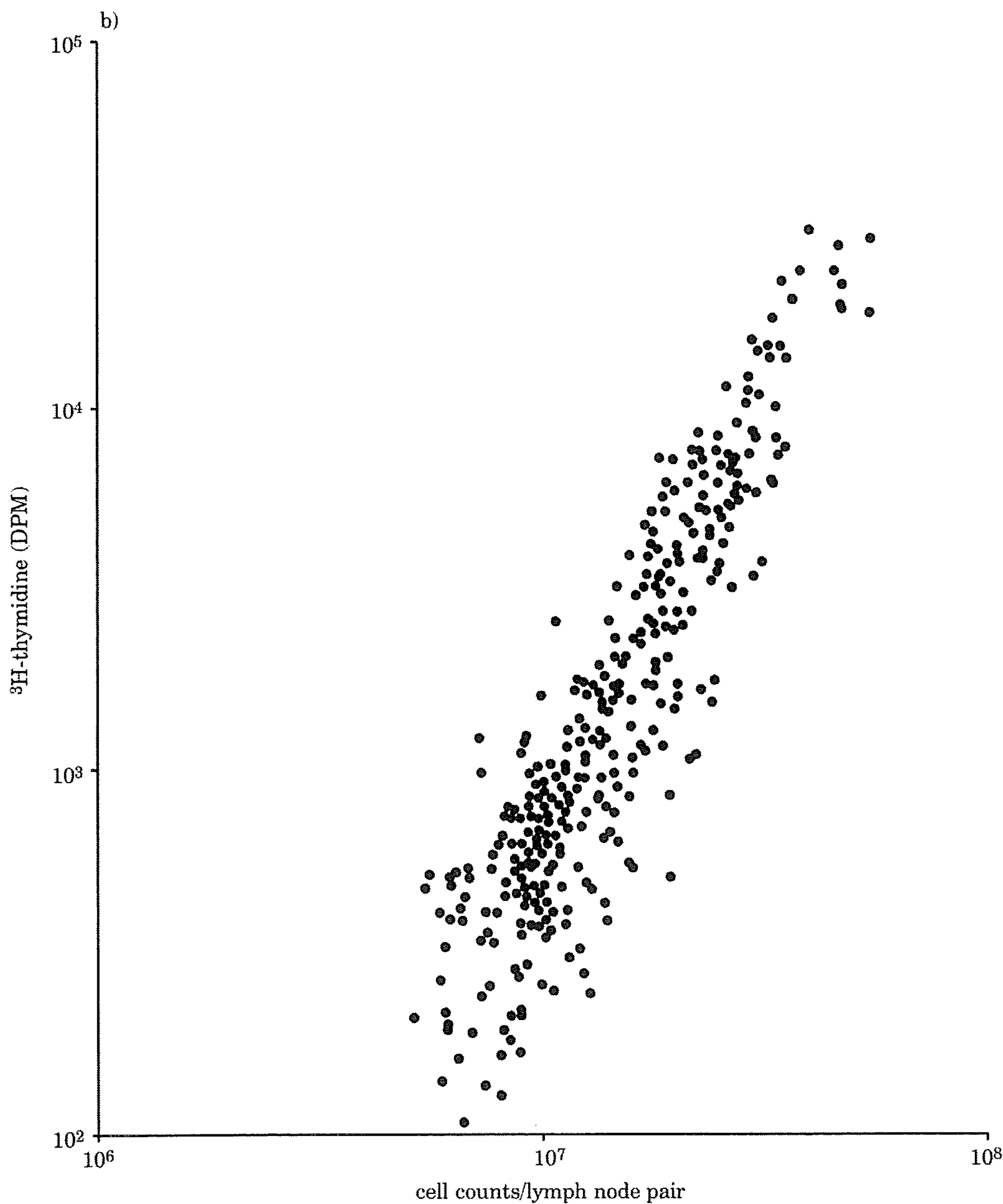
This proposed non-RI LLNA uses cell number as a correlate of cell proliferation, but, as other modifications to the standard LLNA were also made, the method constitutes a major change. There is value in the proposed test, in that it would, if validated, eliminate the use of a radioactive label. Moreover, the measurement of ear thickness may provide a means of providing further information that can help to discriminate true sensitisers from chemicals that induce increase lymphocyte numbers or proliferation in lymph nodes for other reasons. With regard to the counting of cell numbers,

Figure 2: Lymph node cell proliferation: cell counts compared with ^{3}H-thymidine uptake values



Data kindly provided by BGIA, Bonn, Germany. (b) shows an expansion of the data points in the bottom-left area of graph (a).

Figure 2: continued



Data kindly provided by BGIA, Bonn, Germany. (b) shows an expansion of the data points in the bottom-left area of graph (a).

further experience needs to be gained with this approach, in a setting where there are no other deviations from the standard LLNA, and formal comparisons of the same endpoints in the same animals are performed. Further experience in the interpretation of ear thickness measurements is also required, so that criteria can be set for judging when a positive ear swelling response can reverse the conclusion of sensitisation, based on proliferation of cells (numbers) in the LLNA. It should be noted that the additional endpoint of measuring ear thickness could also be used in combination with other (non-radioactive) modifications of the LLNA.

Table 5: A classification scheme for evaluating tests based on cell counting and the measurement of ear thickness

Endpoints	Results							
Lymph node weight	+	+	+	+	–	–	–	–
Lymph node cell counts	+	+	–	–	+	+	–	–
Assessment I	A/I	A/I	I	I	A	A	–	–
Acute skin reaction	–	+	+	–	–	+	+	–
Assessment II	–	I	I	–	–	I	I	–
Overall assessment	A	A/I	I	I?	A	A/I	I	–

+ = statistically-significant increase; A = allergen; I = irritant; Assessment I = evaluation on the basis of lymph nodes only; Assessment II = evaluation on the basis of ear thickness only.

Summary and Conclusions

The LLNA is becoming the skin sensitisation assay of first choice for many regulatory authorities. Consequently, there is an increasing drive to replace its radiolabel-based endpoint. A number of alternatives to the standard LLNA, which do not employ radiolabel, have already been developed. However, several of these efforts involve major changes to the standard LLNA protocol, so a substantial validation process will be required, to ensure that they retain the degree of sensitivity and specificity afforded by the standard method (1). Where the changes to the LLNA are only minor, retaining the same endpoint measure (proliferation in draining auricular lymph nodes), ECVAM and some other authorities (e.g. ICCVAM) have decided to consider the establishment of Performance Standards to allow for a streamlined validation process.

The ECVAM draft Performance Standards have been reviewed in this workshop, and recommendations have been made, which cover both strategic issues, such as the harmonisation of standards between validation authorities, and suggestions for the selection of chemicals and how the modified LLNA protocols and associated prediction models should perform in relation to them. In addition, three examples of modified LLNAs which avoid the use of radiolabelling have been considered (but not reviewed in detail). These have been presented in a common format, to display the principles on which they are founded, the current status of their evaluation, and brief conclusions on their performances. Whilst some of them may be relatively close to acceptability for use, the workshop participants agreed that none of them have yet been adequately validated as substitutes for the standard LLNA, either because they do not fit the criteria of "minor change" or because their data package is at present insufficient.

Recommendations

1. Wherever possible, Performance Standards should be harmonised between validation authorities.

2. Minor modifications to the assay can be assessed by using Performance Standards such as those recommended in this report.

Table 6: Comparisons of observed stimulation indices between different laboratories, for HCA in the non-RI LLNA based on cell counting

	Lab. 1	Lab. 2	Lab. 1	Lab. 2	Lab. 3	Lab. 4	Lab. 5	Lab. 6	Lab. 7
Vehicle	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3% HCA	1.05	1.20	0.92	1.03	1.21	1.06	1.05	1.30	0.98
10% HCA	1.11	**1.90**	**1.69**	1.15	**1.53**	1.29	1.24	**1.64**	**1.64**
30% HCA	**1.59**	**2.01**	**1.57**	**2.02**	**2.37**	**1.90**	**2.03**	**2.10**	**3.10**

Statistically-significant stimulation indices are marked in bold; laboratories 1 and 2 performed repeat experiments.

3. Minor modifications to the OECD TG 429 should be assessed by using a carefully selected set of chemicals and against a clear set of criteria, both of which have been detailed in this report.

4. For modifications to the OECD TG 429 which are not regarded as minor, it may also be the case that the information needed would not be as comprehensive as for a completely new test. Such data requirements should be judged on a case-by-case basis.

5. The progress that has been made in developing realistic alternative read-outs for the standard LLNA should be welcomed, and there should be further investment in research in this area.

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Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells

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IL-17-producing T helper cells (Th17) have been recently identified as a previously undescribed subset of helper T cells. Here, we demonstrate that aryl hydrocarbon receptor (Ahr) has an important regulatory function in the commitment of Th17 cells. Ahr was robustly induced under Th17-polarizing conditions. Ahr-deficient naïve T cells showed a considerable loss in the ability to differentiate into Th17 cells when induced by TGF-β plus IL-6. We were able to demonstrate that Ahr interacts with Stat1 and Stat5, which negatively regulate Th17 development. Whereas Stat1 activation returned to its basal level in Ahr wild type naïve T cells 24 h after stimulation with TGF-β plus IL-6, Stat1 remained activated in Ahr-deficient naïve T cells after stimulation. These results indicate that Ahr participates in Th17 cell differentiation through regulating Stat1 activation, a finding that constitutes additional mechanisms in the modulation of Th17 cell development.

dioxin receptor | IL-17 | IL-6 | ROR | regulatory T cells

Interleukin 17 (IL-17)-producing T helper cells (Th17) are a new subset of T helper cells. It has been demonstrated that these Th17 cells are associated with autoimmune conditions, such as experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) (1–3). Th17 differentiation is regulated by various cytokines. Th17 differentiation was induced by TGF-β and IL-6 in mice, and IL-1β but not TGF-β, has been shown to participate in the development of Th17 cells together with IL-6 in humans (2, 4). The development of Th17 cells is regulated negatively by IFN-γ, IL-27, and IL-2, the signals of which are dependent on Stat1 (IFN-γ and IL-27) and Stat5 (IL-2), respectively (5–7). The orphan nuclear receptors, retinoid-related orphan receptor γ (RORγ) and RORα, have been identified as the key transcription factors that determine the differentiation of Th17 lineage (8, 9). More recently, two groups have reported that the aryl hydrocarbon receptor (Ahr) activated by its ligand regulates Treg and Th17 cell development (10, 11). However, it is not clear how Ahr participates in the development of Th17 cells. In this paper, we demonstrate that Ahr is involved in the differentiation of Th17 cells by regulating Stat1 activation, which suppresses Th17 cell differentiation, under Th17-polarizing conditions.

Ahr, also known as dioxin receptor, is a ligand-activated transcription factor that belongs to the basic-helix–loop–helix-PER-ARNT-SIM family (12, 13). Ahr is present in the cytoplasm, where it forms a complex with heat shock protein (HSP) 90, Ahr-interacting protein (AIP), and p23 (14–16). Upon binding with a ligand, Ahr undergoes a conformation change, translocates to the nucleus, and dimerizes with Ahr nuclear translocator (Arnt). Within the nucleus, the Ahr/Arnt heterodimer binds to a specific sequence, designated as the xenobiotic responsive element (XRE), which causes a variety of toxicological effects (17–20). Interestingly, it has been recently reported that Ahr is a ligand-dependent E3 ubiquitin ligase (21), implying that Ahr has dual functions in controlling intracellular protein levels, serving both as a transcriptional factor to promote the induction of target proteins and as a ligand-dependent E3 ubiquitin ligase to regulate selective protein degradation. It has been reported that Ahr activated by ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) regulates the generation of regulatory T cells (Treg) and modulates the Th1/Th2 balance (22, 23). However, little is known about the molecular mechanism of how Ahr is involved in immune regulation. In this study, we demonstrated that Ahr induced by IL-6 and TGF-β, as well as ligand-activated Ahr, participates in Th17 cell differentiation and acts as a regulator of Stat1 activation under Th17-inducing conditions.

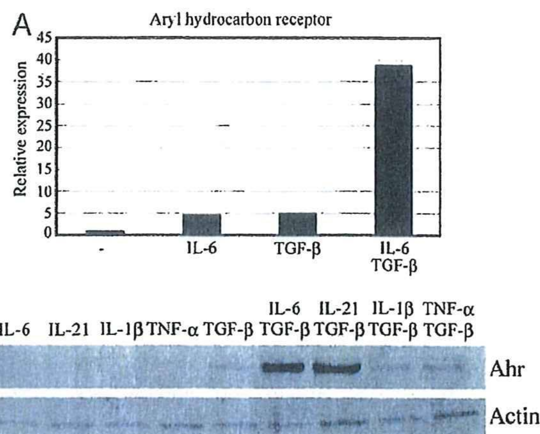

Fig. 1. Ahr is specifically expressed in Th17 cells. Isolated naïve T cells were cultured with anti-CD3/CD28 beads and the indicated cytokines for 2 days. (*A*) Gene expression profiles in nonstimulated and stimulated naïve T cells were compared by DNA microarray. (*B*) The indicated cells were lysed and subjected to Western blot analysis for the expression of Ahr and actin. Data are from one representative of three experiments.

Results

Ahr Is Induced Under Th17-Polarizing Conditions. To identify as yet unknown factors that participate in the differentiation of Th17 cells, we first used a DNA microarray for naïve T cells stimulated with IL-6 and TGF-β added either alone or in combination. This gene expression profiling analysis demonstrated that Ahr was highly expressed in naïve T cells stimulated by TGF-β plus IL-6, but not by either of these alone (Fig. 1*A*). Next, we used Western

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The authors declare no conflict of interest.

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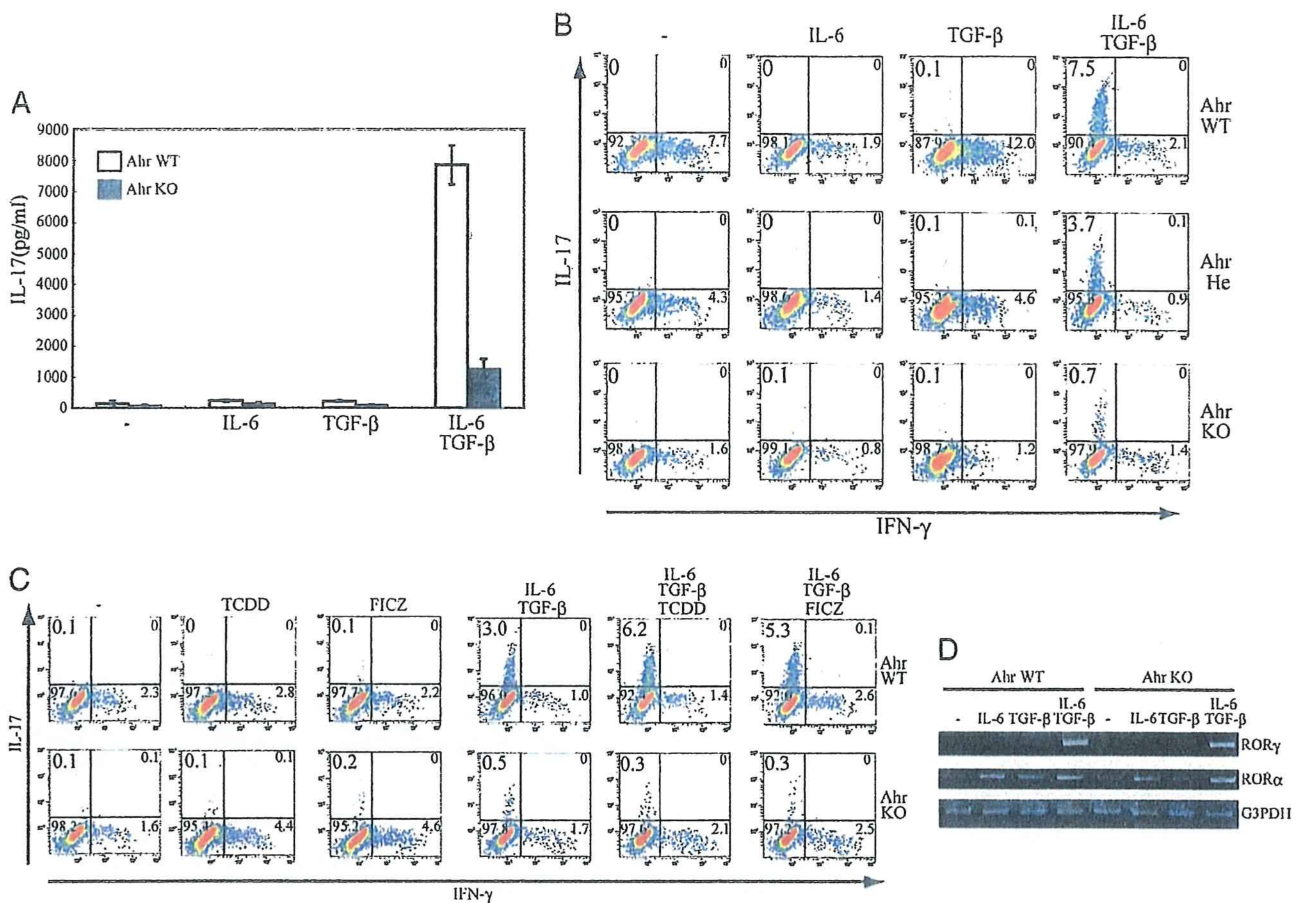

Fig. 2. Ahr deficiency reduces IL-17 production in naïve T cells. (*A*) Purified naïve T cells were stimulated with anti-CD3/CD28 beads in the presence of IL-6 or TGF-β, either alone or combined. Supernatants were collected 4 days after stimulation, and IL-17 production was measured by means of ELISA. Data show means ± SE of three independent experiments. (*B* and *C*) Dot plots show intracellular staining for IFN-γ and IL-17. (*B*) Isolated naïve T cells from Ahr WT, He, and KO splenocytes were cultured with anti-CD3/CD28 beads and the indicated cytokines for 4 days. (*C*) Naïve T cells isolated from Ahr WT and KO mice were stimulated with anti-CD3/CD28 beads and TGF-β plus IL-6 in the presence or absence of TCDD or FICZ for 3 days. (*D*) Naïve T cells isolated from Ahr WT and KO mice were stimulated with anti-CD3/CD28 beads and the indicated cytokines for 2 days. Total RNA and cDNA were prepared as described in Methods. RORγ and RORα induction was examined by using RT-PCR. (*B–D*) These results are representative of three independent experiments.

blot analysis to examine the expression of Ahr in naïve T cells under Th17-polarizing conditions. As shown in Fig. 1*B*, we confirmed the robust expression of Ahr under Th17-differentiating conditions. It has been reported that IL-21, like IL-6, also can initiate Th17 differentiation combined with TGF-β (24), and we also detected expression of Ahr induced by TGF-β plus IL-21 (Fig. 1*B*). Thus, Ahr is selectively induced under experimental conditions promoting Th17 cell development. However, other proinflammatory cytokines such as TNF-α and IL-1β did not induce Ahr expression even in combination with TGF-β.

Ahr Is Involved in the Differentiation of Th17 Cells. We next used Ahr deficient (KO) mice to examine whether Ahr indeed participates in Th17 development. Naïve T cells were isolated from Ahr WT and KO mice and stimulated by IL-6 or TGF-β alone or in combination. After stimulation, IL-17 production was measured with ELISA, and, as shown in Fig. 2*A*, the secretion of IL-17 was found to be drastically reduced in Ahr-deficient naïve T cells in comparison with WT naïve T cells under optimal conditions for Th17 cell development. Flow cytometry (FACS) analysis also revealed that Th17 cell differentiation was partially impaired in Ahr heterozygous (He) naïve T cells and significantly suppressed in Ahr KO naïve T cells in comparison with WT cells (Fig. 2*B*). Because TCDD (dioxin) and 6-formylindolo[3,2-b]carbazole (FICZ), which are exogenous and endogenous ligands, respectively, can bind and activate Ahr (10), we next investigated how these ligands influence Th17 cell development in Ahr WT and KO naïve T cells. TCDD or FICZ alone could not induce Th17 cell development, whereas their addition increased the percentage of IL-17-secreting cells induced by TGF-β plus IL-6 in WT cells (Fig. 2*C*). On the other hand, Ahr KO naïve T cells did not exhibit any increase in the generation of Th17 cells even in the presence of these ligands (Fig. 2*C*). Taken together, these data strongly indicate that Ahr is involved in Th17 development.

It has been reported that RORα and RORγ are required for the induction of Th17 cells (8, 9). We analyzed whether Ahr regulates their expression under Th17-polarizing conditions. Naïve T cells from Ahr WT and KO mice were stimulated with IL-6 and TGF-β, either alone or combined, followed by examination of RORα and RORγ induction by means of reverse transcriptase-PCR (RT-PCR). There was no difference in the induction of RORα and RORγ by TGF-β plus IL-6 between Ahr WT and KO naïve T cells (Fig. 2*D*). This suggests that the suppression of Th17 cell differentiation by Ahr deficiency is not because of its negative effect on the expression of RORα and RORγ.

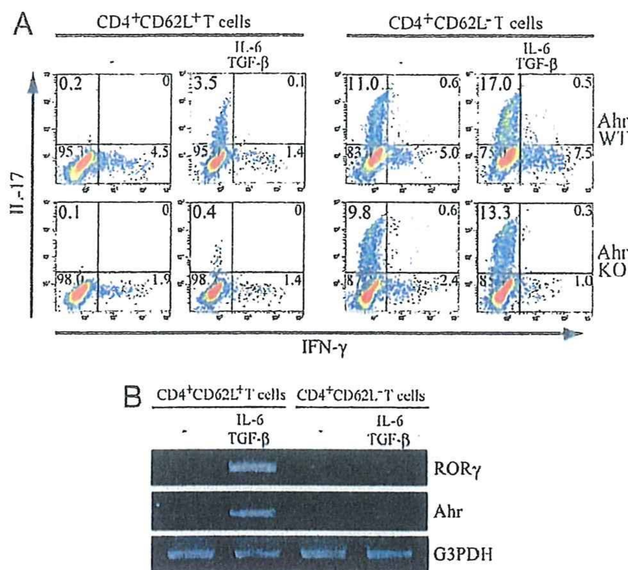

Fig. 3. Different pattern of IL-17 production between CD4+CD62L− and CD4+CD62L+ cells. CD4+CD62L− and CD4+CD62L+ cells isolated from WT mice were stimulated with anti-CD3/CD28 beads and TGF-β plus IL-6. (*A*) Three days after stimulation, cells were re-stimulated with PMA and ionomycin for 5 h and with GolgiStop (final 2 h), and then subjected to intracellular cytokine staining. Dot plots show intracellular staining for IFN-γ and IL-17. (*B*) Two days after stimulation, total RNA and cDNA were prepared as described in Methods. RORγ and Ahr induction was examined by using RT-PCR. These results are representative of three independent experiments.

IL-17 Is Produced in CD4+CD62L− Cells Without TGF-β Plus IL-6 Treatment. In contrast to our results, a recently reported study found that CD44loCD25−CD4+ T cells from Ahr KO mice can differentiate into Th17 cells, but lack the expression of IL-22 (11). In our study, we separated CD4+ T cells into CD4+CD62L− (4–6% in the spleen cell population) and CD4+CD62L+ (15–20% in the spleen cell population) T cells and used CD4+CD62L+ T cells as naïve T cells. In contrast, Stockinger *et al.* used CD4+ T cells including CD62L− fractions. We found that CD4+CD62L− cells spontaneously produced IL-17 without TGF-β plus IL-6, and their addition promoted IL-17 production (Fig. 3*A*). Ahr and RORγ were not expressed in CD4+CD62L− cells in the presence or absence of TGF-β plus IL-6 (Fig. 3*B*), suggesting that CD4+CD62L− cells that produce IL-17 are distinct from a definitive Th17 cell subset. Additionally, even CD4+CD62L− cells from Ahr KO mice could produce IL-17 with or without Th17-polarizing stimuli (Fig. 3*A*). These data collectively indicate that CD4+ T cells, including CD4+CD62L− cells, neither require Th17-polarizing stimuli nor the expression of Ahr and RORγ for IL-17 production.

Ahr Deficiency Partially Impairs Treg Development. Because Ahr was slightly induced by TGF-β alone (Fig. 1*B*), we investigated whether Ahr regulates the differentiation of Treg cells by TGF-β. We used FACS to measure Foxp3 expression in Ahr WT and KO naïve T cells stimulated by TGF-β. Compared with Ahr WT naïve T cells, Foxp3 induction was partially but significantly inhibited in Ahr KO naïve T cells (Fig. 4). Although TCDD or FICZ alone could not induce Foxp3 expression, its induction was enhanced when they were combined with TGF-β in WT cells, but not in Ahr KO cells (Fig. 4). Thus, Ahr participates in the generation of Treg cells.

Ahr Participates in Th17 Cell Development by Regulating Stat1. It was previously reported that the Stat family is essential for Th17 development, and that RORα and RORγ are induced in a Stat3-dependent manner by treatment with IL-6 and TGF-β (6, 25). On the other hand, Stat1 activation induced by IFN-γ or IL-27 inhibits Th17 polarization (5–7). Moreover, it has been demonstrated that IL-2 signaling interferes with Th17 differentiation through the activation of Stat5. Consistent with these findings, we previously reported that the combination of IL-6 and TGF-β could maintain activation of Stat3, but not of Stat1, 24 h after stimulation and that the suppressive effect of IL-27 and IFN-γ on the induction of Th17 cells is exerted through the maintenance and prolongation of Stat1 activation after IL-6 and TGF-β stimulation (26). In the current study, we investigated the relationship between Ahr induction and Stat regulation to gain a better understanding of the role of Ahr in Th17 cell differentiation. We first examined whether Ahr would bind with members of the Stat family under Th17-polarlizing conditions. Naïve T cells were stimulated with IL-6, TGF-β, or TGF-β plus IL-6, and the interaction between Ahr and the Stat family members was measured with the aid of immunoprecipitation and Western blotting. The results demonstrated that Ahr interacted with Stat1 and Stat5, but not with either Stat3 or Stat6 (Fig. 5*A*). We speculated that Ahr might participate in Th17 cell development by regulating Stat1 and Stat5. To validate this hypothesis, we next compared the inhibitory effect of IFN-γ on Th17 induction in Ahr WT and He naïve T cells, because it is known that IFN-γ serves to limit the generation of Th17 cells in a Stat1 activation-dependent manner. Because Th17 cell differentiation is significantly impaired in Ahr-deficient naïve T cells, it is not possible to examine the inhibitory effect of IFN-γ on Th17 development in Ahr-deficient naïve T cells. We, therefore, used Ahr-He naïve T cells to compare the inhibitory effect of IFN-γ with that in WT naïve T cells. As shown in Fig. 5*B*, IFN-γ suppressed Th17 cell

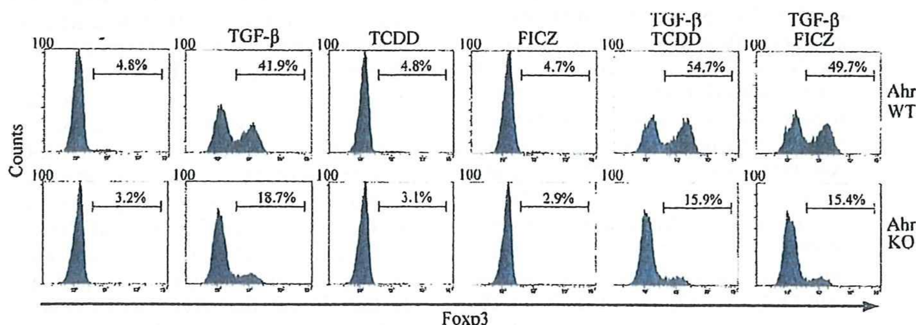

Fig. 4. Ahr partially participates in the generation of Treg cells by TGF-β. Naïve T cells isolated from Ahr WT and KO mice were stimulated with anti-CD3/CD28 beads and TGF-β with or without Ahr ligands for 2 days. Foxp3 expression was determined by staining with anti-mouse Foxp3 antibody. These data are representative of three independent experiments.

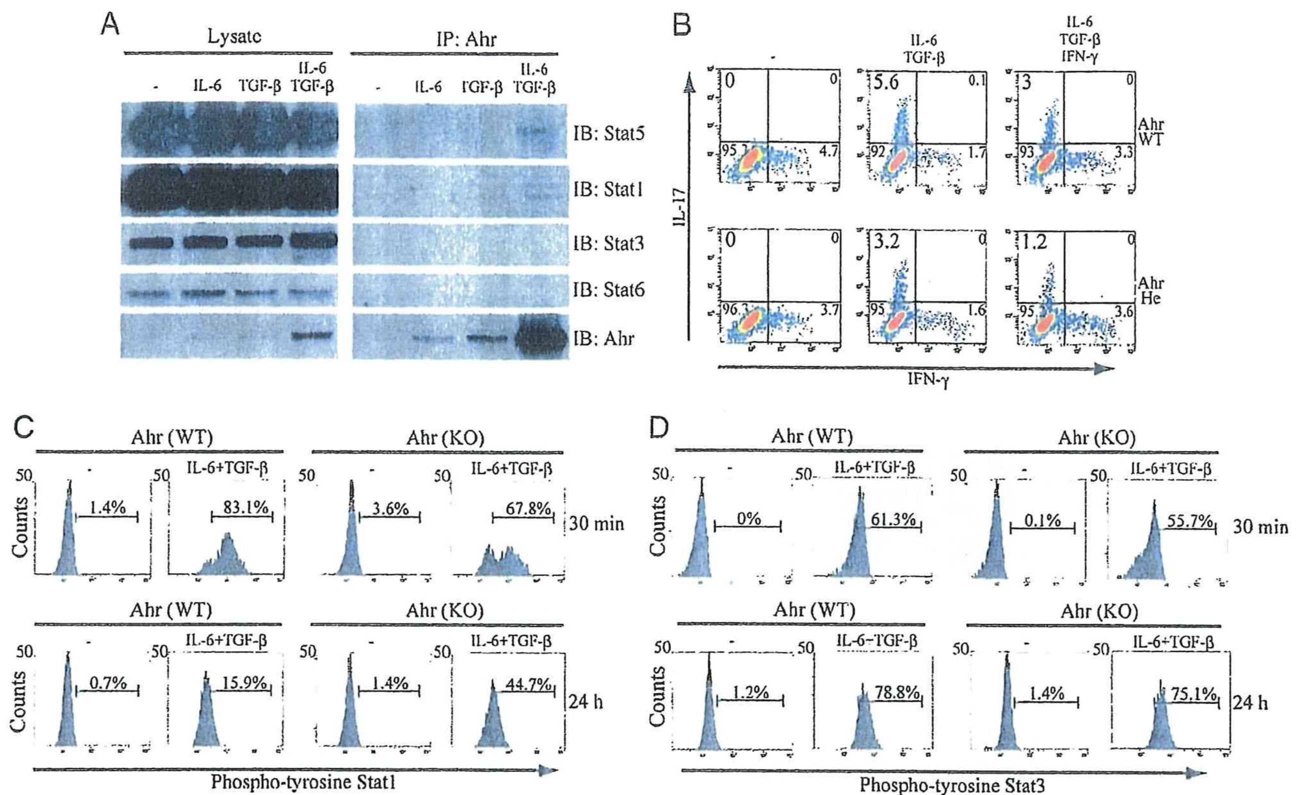

Fig. 5. Ahr regulates the activation of Stat1 in Th17 cell development. (*A*) MACS-sorted naïve T cells were cultured with anti-CD3/CD28 beads and stimulated with IL-6 or TGF-β, either alone or combined, for 2 days. Whole cell lysates were immunoprecipitated with anti-Ahr antibody, after which Stat1, Stat3, Stat5, Stat6, and Ahr were detected with Western blotting. IP, immunoprecipitation; IB, immunoblot. (*B*) Naïve T cells isolated from Ahr WT and He mice were stimulated with anti-CD3/CD28 beads and TGF-β plus IL-6 in the presence or absence of IFN-γ for 3 days, followed by re-stimulation with PMA and ionomycin for 5 h and with GolgiStop (final 2 h), and then staining for intracellular cytokines. Dot plots show intracellular staining for IFN-γ and IL-17. (*C* and *D*) Naïve T cells isolated from Ahr WT and KO splenocytes were stimulated with anti-CD3/CD28 beads and TGF-β plus IL-6 for 30 min or 24 h, fixed and permeabilized in 90% methanol, and finally stained with Alexa Fluor 488-conjugated phospho-Stat1 and PE-conjugated phospho-Stat3. Intracellular levels of phospho-Stat1 (*C*) and Stat3 (*D*) were measured by means of flow cytometry. These results are representative of three independent experiments.

development to a higher degree in Ahr-He naïve T cells (inhibitory effect: 62.5%) than in WT cells (inhibitory effect: 46.4%). Given that IFN-γ inhibits the generation of Th17 cells via activation of Stat1, it is possible that the higher degree of inhibition of Th17 cell development by IFN-γ in Ahr-He naïve T cells is because of enhanced Stat1 activation compared to that in WT naïve T cells.

We previously reported that Stat3 remained activated under Th17-culturing conditions, whereas Stat1 activation was relatively transient and returned to the basal level during 24 h of the experimental period (26). In the current study, we compared the activation of these Stats under Th17-polarizing conditions in Ahr WT and KO naïve T cells to confirm that Ahr affects the state of the activation of Stats. Naïve T cells isolated from Ahr WT and KO mice were stimulated with IL-6 and TGF-β, and 30 min or 24 h after stimulation, Stat1 and Stat3 activation in both types of naïve T cells was measured by using intracellular staining. Stat1 was activated at a similar intensity in both Ahr WT and KO naïve T cells 30 min after IL-6 and TGF-β stimulation (Fig. 5*C*). Consistent with a previous finding (26), Stat1 activation was not maintained 24 h after stimulation in Ahr WT naïve T cells. In contrast, Stat1 remained activated 24 h after stimulation in Ahr-deficient naïve T cells (Fig. 5*C*). On the other hand, there was no difference in Stat3 activation 30 min or 24 h after stimulation between Ahr WT and KO naïve T cells (Fig. 5*D*). These results indicate that Ahr selectively regulates the activation of Stat1, but not of Stat3, under Th17-polarizing conditions.

Discussion

Th17 cells, known as a previoulsy undescribed lineage of Th cells, are associated with autoimmunity. Although it has been recently demonstrated that RORα and RORγ are key transcription factors in Th17 cells (8, 9), the mechanism of Th17 cell differentiation is not yet well understood. We previously demonstrated that IL-27 and IFN-γ suppressed the generation of Th17 cells without significant effects on the expression of RORγ (26). In this study, we confirmed that RORα, like RORγ, was expressed under Th17-polarizing conditions even in the presence of IL-27 or IFN-γ (data not shown). These results strongly suggest that regulatory molecules other than RORα and RORγ may play an important role in the development of Th17 cells. In support of this hypothesis, it has been more recently reported that Ahr, activated by its ligand, controls Treg and Th17 cell differentiation (10, 11), and we found in the current study that Ahr is markedly induced by TGF-β plus IL-6 and participates in the generation of Th17 cells in the absence of its exogenous ligand.

Ahr was induced specifically under Th17-polarizing conditions such as TGF-β plus IL-6 or TGF-β plus IL-21, but not by other inflammatory cytokines combined with TGF-β or under Th1-polarizing conditions (IL-12 and anti-IL-4) (data not shown). We further found that Ahr was expressed also in Stat1-deficient naïve T cells treated with TGF-β plus IL-6 [supporting information (SI) Fig. S1], indicating that Ahr induction is independent of Stat1. Although the exact molecular mechanism of Ahr expression in Th17 development is not clear at this point, Ahr

induction may be regulated downstream of Stat3 by IL-6 and TGF-β, similar to the induction of RORα and RORγ as reported elsewhere (8, 9). We demonstrated that Ahr deficiency significantly impaired Th17 development induced by IL-6 and TGF-β even though RORs are expressed, similar to the case of treatment with IL-27 and IFN-γ, which also indicated that Th17 development requires other regulatory mechanisms in addition to regulation by RORs. Recent studies have demonstrated that ligand-activated Ahr regulates Th17 cell development (10, 11). Stockinger *et al.* showed that $CD44^{lo}CD25^{-}CD4^{+}$ T cells from Ahr WT and KO mice can develop Th17 cells with TGF-β plus IL-6, whereas FICZ, one of the Ahr ligands, promotes the generation of Th17 cells induced by the combined usage of the two cytokines in Ahr WT $CD44^{lo}CD25^{-}CD4^{+}$ T cells, but not in Ahr KO $CD44^{lo}CD25^{-}CD4^{+}$ T cells (11). In our study, however, we could demonstrate that Th17 cell development is impaired in Ahr-deficient naïve T cells under Th17-polarizing conditions in either the presence or absence of Ahr ligands. We speculated that the reason for this discrepancy might be related to the difference in the sorted naïve T cell fractions used in the two studies. We have found that $CD4^{+}CD62L^{-}$ cells from Ahr WT and KO mice spontaneously produce IL-17 regardless of the presence or absence of TGF-β plus IL-6, despite the fact that neither Ahr nor RORγ was expressed in those cells. This may explain the discrepancies in our results and those of Stockinger *et al.*, because they used $CD4^{+}$ T cells including $CD4^{+}CD62L^{-}$ cells. Because effector memory $CD4^{+}$ T cells are characterized by $CD45RB^{low}CD44^{high}CD62L^{-}$, our isolated $CD4^{+}CD62L^{-}$ cells may belong to the effector memory $CD4^{+}$ T cell family. However, it is currently unknown whether effector memory $CD4^{+}$ T cells can produce IL-17 by anti-CD3 plus anti-CD28. Further analysis is required to develop the characteristics of this population in Th17 cell differentiation.

Th17 differentiation is positively regulated by IL-6 or IL-21 in combination with TGF-β and negatively regulated by IFN-γ or IL-27, which are controlled by Stat3 and Stat1, respectively (2, 5, 6, 7, 25). Given that Stat1 can bind with the IL-17 promoter and serve as a repressor (7), the maintenance of its activation may inhibit the interaction between ROR proteins and the IL-17 promoter by masking their binding sites. In our study, we found that Ahr binds to Stat1 and Stat5, but not to other tested members of the Stat family, raising the possibility that Ahr may regulate the generation of Th17 cells by modifying the activation of Stat1 and Stat5, which negatively regulate Th17 generation. Indeed, we found that Ahr deficiency prolonged Stat1 activation 24 h after stimulation with TGF-β plus IL-6, whereas its activation was relatively transient and returned to the basal level in WT naïve T cells during that period. On the other hand, Stat3 activation was maintained equally in both Ahr WT and KO naïve T cells. Consistent with the finding of a previous report (7), we confirmed that Th17 cell development is enhanced under Th17-polarizing conditions in the presence of neutralizing antibodies for IL-2 (data not shown), indicating that Th17 differentiation is inhibited by endogenous IL-2 secreted from naïve T cells cultured under Th17-polarizing conditions. Interaction of Ahr with Stat5 also leads us to speculate that Ahr downregulates the activation of Stat5 by IL-2 produced in naïve T cells through binding with Stat5, like Stat1, resulting in the induction of Th17 cells. At present, it is not yet understood how Ahr interacts with Stat1 and Stat5 and negatively regulates their activation in Th17 cell differentiation. It has been reported that nuclear receptors such as peroxisome proliferator-activated receptor γ (PPARγ) and estrogen receptor (ER) negatively modulate Stat3 activated by IL-6 (27). When PPARγ is activated by its ligand, the resultant PPARγ-ligand complex directly interacts with IL-6-activated Stat3 and suppresses its transcriptional activity. Although in our study, Ahr interacted with Stat1 independently of its ligand, there may be an as yet unidentified endogenous Ahr ligand that determines the interaction between Ahr and Stat1 (Stat5) in Th17 cell development by forming a complex with Ahr.

Ahr is known to have dual functions in controlling intracellular protein levels, serving both as a transcriptional factor and as a ligand-dependent E3 ubiquitin ligase (21). It also is possible that Ahr regulates the activation of Stat1 through the degradation of activated Stat1 by functioning as a ligand-dependent E3 ubiquitin ligase in the generation of Th17 cells.

At this point, we cannot exclude the possibility that Ahr may have mechanisms other than regulating the activation of Stat1 in Th17 cell differentiation. Therefore, it is important to determine the molecular basis of the interaction of Ahr with members of the Stat family and the regulation of their activation.

We were able to show that Treg induction by TGF-β was inhibited partially but significantly in Ahr-deficient naïve T cells. It has been reported that Treg differentiation is negatively regulated by IFN-γ in a Stat1-dependent manner (28). We confirmed that IFN-γ partially inhibits Treg cell development by TGF-β and that IFN-γ blocking by its neutralizing antibodies enhances Treg differentiation (Fig. S2), which suggests that the induction of Treg as well as of Th17 was disrupted under Stat1-activating conditions. Because Ahr can be slightly induced by TGF-β alone, it is expected that TGF-β–induced Ahr may regulate Treg development through the suppression of Stat1 activation by endogenous IFN-γ secreted from naïve T cells cultured under Treg-inducing conditions. We found that Treg induction by TGF-β was enhanced when Ahr was activated by TCDD or FICZ. However, Weiner *et al.* reported that FICZ inhibited Treg cell development by TGF-β, whereas Treg was induced by TCDD alone even in the absence of TGF-β (10), thus contradicting our data. At the present time, we cannot explain the reason for this major discrepancy between their results and ours.

In summary, our findings demonstrate that Ahr is essential for Th17 development through the interference of Stat1 activation under Th17-polarizing conditions. Because Ahr controls the activation of Stat1 by forming a previously undescribed complex, Ahr/Stat1, Ahr may be involved in various immune systems, including innate immunity, via Stat-dependent pathways.

Materials and Methods

Mice. C57BL/6 wild-type mice were obtained from CLEA Japan Inc., and Ahr KO mice on the C57BL/6 background were provided by Dr. Yoshiaki Fujii-Kuriyama (University of Tsukuba, Tsukuba, Japan). All mice were maintained under specific, pathogen-free conditions. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of the Graduate School of Frontier Bioscience, Osaka University.

Isolation of Naïve T Cells and T Cell Differentiation. Naïve T cells were purified from spleens of C57BL/6, Ahr WT, He, or KO female mice by using the $CD4^{+}$ T cell Isolation Kit and CD62L MicroBeads (Miltenyi). Purified naïve T cells were stimulated with the Dynabeads Mouse CD3/CD28 T cell Expander (Invitrogen) for 3 days. As indicated, cultures were supplemented with recombinant cytokines: Mouse IL-6 (20 ng/ml; R&D Systems), mouse IL-21 (100 ng/ml; R&D Systems), mouse IL-1β (20 ng/ml; R&D Systems), mouse TNF-α (100 ng/ml; R&D Systems), or human TGF-β1 (2 ng/ml; R&D Systems), alone or combined. Additionally, recombinant mouse IFN-γ (20 ng/ml; R&D Systems), FICZ (100 nM; kindly donated by Dr. Yoshiaki Fujii-Kuriyama, University of Tsukuba), or TCDD (160 nM; Cerilliant) was added to some samples.

DNA Microarray. Naïve T cells were cultured with anti-CD3/CD28 beads and indicated cytokines for 2 days. cRNA was synthesized and hybridized to Murine Genome 430 2.0 microarray chips (Affymetrix). Microarray data were analyzed by Gene Spring (Agilent).

IL-17 ELISA. Naïve T cells purified from Ahr WT and KO splenocyte populations were stimulated with anti-CD3/CD28 beads and indicated cytokines. After 4 days, mouse IL-17 from the supernatants was measured by means of ELISA according to the manufacturer's instructions (R&D Systems).

Intracellular Cytokines and Foxp3 Staining. T cells were stimulated with 50 ng/ml PMA (Calbiochem), 800 ng/ml ionomycin (Calbiochem) for 5 h and GolgiStop (BD PharMingen) for the final 2 h, followed by fixation and permeabilization with Cytofix/Cytoperb (BD PharMingen). Cells were stained intracellularly with Phycoerythrin (PE)-conjugated anti-IL-17 (BD PharMingen) and FITC-labeled anti-IFN-γ (eBioscience). For Foxp3 staining, T cells were fixed and permeabilized with the Fixation/Permeabilization buffer (eBioscience) for 30 min at 4°C before intracellular staining with FITC-conjugated anti-Foxp3 (eBioscience). Flow cytometric analysis was performed with a Cytomics FC500 (Beckman Coulter).

Immunoprecipitation and Western Blotting. Purified naïve T cells were cultured with indicated cytokines for 2 days, and cells were lysed with a lysis buffer [1% Nonidet P-40, 20 mM Tris-HCl (ph 7.5), 150 mM NaCl, 10 mM Na_2VO_4, 0.5 mM DTT, and 1/100 protease inhibitor]. Ahr was immunoprecipitated with anti-Ahr (BIOMOL) and then subjected to SDS/PAGE. Whole cell lysates and the immunocomplex were analyzed with Western blotting by using anti-Stat1 (BD Transduction Laboratories), anti-Stat3 (BD Transduction Laboratories), anti-Stat5 (C-17; Santa Cruz Biotechnology), anti-Stat6 (BD Transduction Laboratories), or anti-Ahr (BIOMOL).

Reverse Transcriptase-PCR (RT-PCR). Total RNA was prepared by using RNeasy (Qiagen), and cDNA was prepared as described elsewhere (26). Reaction conditions consisted of a 45-s denaturation step at 94°C, a 30-s annealing step at 58°C, and a 30-s extension step at 72°C for 25 cycles (G3PDH), 35 cycles (RORγ), or 37 cycles (RORα). The specific primers were as follows: RORγ, sense 5′-GCGGAGCAGACACACTTACA-3′ and antisense 5′-TTGGCAAACTCCACCACATA-3′; RORα, sense 5′-AGTTTGGTCGGATGTCCAAG-3′ and antisense 5′-AGCTGCCACATCACCTCTCT-3′; G3PDH, sense 5′-TCCACCACCCTGTTGCTGTA-3′ and antisense 5′-ACCACAGTCCATGCCATCAC-3′.

Flow Cytometric Analysis of Phospho-Stat1 (Y701) and Phospho-Stat3 (Y705). Naïve T cells were cultured with TGF-β plus IL-6 for 30 min or 24 h. Cells were fixed with Fixation Buffer (BD PharMingen) for 10 min at 37°C and then permeabilized in 90% methanol for 30 min on ice. Cells were washed twice in Stain Buffer (BD PharMingen), and stained with Alexa Fluor 488-conjugated phospho-Stat1 (Y701) antibody or PE-conjugated phospho-Stat3 (Y705) antibody for 1 h at room temperature (BD PharMingen). Flow cytometric analysis was performed with a Cytomics FC500 (Beckman Coulter).

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Benzene-induced hematopoietic toxicity transmitted by AhR in wild-type mouse and nullified by repopulation with AhR-deficient bone marrow cells: Time after benzene treatment and recovery

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Mice

ABSTRACT

Previously, we found an aryl hydrocarbon receptor (AhR)-transmitted benzene-induced hematotoxicity; that is, AhR-knockout (KO) mice did not show any hematotoxicity after benzene exposure [Yoon, B.I., Hirabayashi, Y., Kawasaki, Y., Kodama, Y., Kaneko, T., Kanno, J., Kim, D.Y., Fujii-Kuriyama, Y., Inoue, T., 2002. Aryl hydrocarbon receptor mediates benzene-induced hematotoxicity. Toxicol. Sci. 70, 150–156]. Furthermore, our preliminary study showed a significant attenuation of benzene-induced hematopoietic toxicity by AhR expression, when the bone marrow (BM) of mice was repopulated with AhR-KO BM cells [Hirabayashi, Y., Yoon, B.I., Li, G., Fujii-Kuriyama, Y., Kaneko, T., Kanno, J., Inoue, T., 2005a. Benzene-induced hematopoietic toxicity transmitted by AhR in the wild-type mouse was negated by repopulation of AhR deficient bone marrow cells. Organohalogen Comp. 67, 2280–2283]. In this study, benzene-induced hematotoxicity and its nullification by AhR-KO BM cells were further precisely reevaluated including the duration of the effect after benzene treatment and recovery after the cessation of exposure. Exposure routes, namely, intraperitoneal (*i.p.*) injection used in our previous study and intragastric (*i.g.*) administration used in this study, were also compared in terms of their toxicologic outcomes. From the results of this study, mice that had been lethally irradiated and repopulated with BM cells from AhR-KO mice essentially did not show any benzene-induced hematotoxicity. The AhR-KO BM cells nullified benzene-induced toxicities in notably different hematopoietic endpoints between the *i.p.* treatment and the *i.g.* treatment; however, the number of granulo-macrophage colony-forming unit *in vitro* (CFU-GM) was a common target parameter, the benzene-induced toxicity of which was nullified by the AhR-KO BM cells.

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1. Introduction

Recent studies have shown that the aryl hydrocarbon receptor (AhR) in primitive cells transmits negative signals for the proliferation of such cells (Hirabayashi et al., 2003; Garrett and Gasiewicz, 2005). This observation may require further detailed studies, because previous *in vitro* studies showed that AhR promotes cellular proliferation on one hand (Ma and Whitlock, 1996; Shimba et al., 2002), but rather suppress on the other hand (Fong et al., 2005). As we previously reported, AhR-knockout (KO) mice showed an increase in number of primitive hematopoietic progenitor cells; on the other hand, a decrease in number of relatively mature progenitor cells in a homeostatic manner (Hirabayashi et al., 2003). Therefore, there are two possibilities: one is the hierarchic positional effect of cellular differentiation and the other is a particular cell-proliferative gene alteration in *in vitro* cell lines.

We have reported that benzene-induced hematopoietic toxicity is transmitted by AhR (Yoon et al., 2002). We also found that cytochrome P450 2E1 (CYP2E1) that is, related to benzene metabolism is also up-regulated following benzene exposure in the bone marrow (BM) (Yoon et al., 2003). Therefore, it is of interest to hypothesize the important role of BM cells in hematopoietic toxicity with respect to AhR function. Accordingly, on the basis of the latest studies presented at the 25th International Dioxin Symposium, benzene-induced hematopoietic toxicity was evaluated in wild-type (Wt) mice after whole-body irradiation at a lethal dose followed by repopulation with BM cells that lack AhR or, *vice versa*, in AhR-KO mice after repopulation with Wt BM cells. As for the results, a one-day examination on day 12 after benzene exposure

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