

Gold- and silver-induced murine autoimmunity – requirement for cytokines and CD28 in murine heavy metal-induced autoimmunity

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Summary

Treatment with gold in the form of aurothiomaleate, silver or mercury (Hg) in genetically susceptible mouse strains (H-2^s) induces a systemic autoimmune condition characterized by anti-nuclear antibodies targeting the 34-kDa nucleolar protein fibrillarin, as well as lymphoproliferation and systemic immune-complex (IC) deposits. In this study we have examined the effect of single-gene deletions for interferon (IFN)- γ , interleukin (IL)-4, IL-6 or CD28 in B10.S (H-2^s) mice on heavy metal-induced autoimmunity. Targeting of the genes for IFN- γ , IL-6 or CD28 abrogated the development of both anti-fibrillarin antibodies (AFA) and IC deposits using a modest dose of Hg (130 μ g Hg/kg body weight/day). Deletion of IL-4 severely reduced the IgG1 AFA induced by all three metals, left the total IgG AFA response intact, but abrogated the Hg-induced systemic IC deposits. In conclusion, intact IFN- γ and CD28 genes are necessary for induction of AFA with all three metals and systemic IC deposits using Hg, while lack of IL-4 distinctly skews the metal-induced AFA response towards T helper type 1. In a previous study using a higher dose of Hg (415 μ g Hg/kg body weight/day), IC deposits were preserved in IL-4^{-/-} and IL-6^{-/-} mice, and also AFA in the latter mice. Therefore, the attenuated autoimmunity following loss of IL-4 and IL-6 is dose-dependent, as higher doses of Hg are able to override the attenuation observed using lower doses.

Keywords: autoimmunity, CD28, cytokines, gold, silver

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Introduction

The archetypal systemic autoimmune disease systemic lupus erythematosus is characterized by anti-nuclear autoantibodies, lymphoproliferation and systemic immune-complex (IC) deposits. While linkage studies have provided evidence that multiple genes are involved in systemic autoimmune diseases and interact with each other, candidate genes have been identified mainly by association studies and the use of mouse models [1]. One model for induction of systemic autoimmune conditions includes heavy metals, most notably mercury [2]. Mercuric chloride (Hg) induces serum anti-nuclear autoantibodies, systemic IC deposits [3,4] and lymphoproliferation [5], including a transient polyclonal activation of the immune system [6]. The major autoantibody specificity is the nucleolar 34-kDa protein fibrillarin [7,8], a core component in nucleolar ribonucleoprotein

particles [9], with chromatin as a minor antigen [10]. The susceptibility to develop Hg-induced anti-fibrillarin autoantibodies (AFA) is linked closely to the major histocompatibility complex class II (H-2) region, especially H-2A [10], while renal IC deposits are associated with loci in chromosomes 1 (*Hmr-1*) and 7 [10].

Silver (Ag) [11] and gold (aurothiomaleate: Au) [12] also induce AFA in genetically susceptible strains, H-2A being the most important susceptibility locus for these metals [12,13]. However, the autoimmune response in Ag- or Au-treated mice is focused to fibrillarin, not causing systemic IC deposits and giving rise to weaker lymphoproliferation and polyclonal activation than Hg [12,14].

Studies in recent years have addressed the immunological events in Hg-induced autoimmunity (HgIA), illustrating a strong dependency on T (CD4⁺) cells [15], as well as the co-stimulation factors CD28 and CD40L [16] and ICOS

[17]. The need for the co-stimulatory pathways CD28/B7, CD40/CD40L and ICOS-B7h in HgIA is evidence not only for the involvement of T cells, but also indicates similarity with a conventional immune response. Another feature of HgIA is the increase in cytokines of T helper type 2 (Th2) [interleukin (IL)-4, IL-5] as well as Th1 [interferon (IFN)- γ] type [18]. While IFN- γ is necessary for the autoimmune reaction in HgIA, IL-4 was found to be dispensable [19–21]. However, no studies have been performed to examine the requirement for cytokines and co-stimulatory factors in Ag- and Au-induced autoimmunity, which is the subject of the present study. Using genetically susceptible H-2^s mice with single-gene mutations we found similarities between the three metals with regard to the requirement for cytokines and co-stimulatory molecules in induction of autoimmunity, indicating a similar basic mechanism of action for these metals. Taken together with previous studies, we observed a dose-dependent effect of IL-4 and IL-6 in the metal-induced autoimmune response, higher doses being able to override the effect of the deleted genes, which is in contrast to the absolute need for IFN- γ and CD28 over a wide dose range.

Material and methods

Mice

Female and male B10.S (H-2^s) mice were obtained from (BomMice, Ry, Denmark). B10.S mice with targeted mutations (knock-out: KO) for IL-4, IL-6, IFN- γ or CD-28 were developed as described previously [16,20] at the Animal Facilities of the Scripps Research Institute (La Jolla, CA, USA) under specific pathogen-free conditions. Subsequently the mice were maintained as homozygous knock-outs by brother–sister mating for a period approaching 10 years at Scripps and in the animal facilities of the Faculty of Health Sciences, Linköping. During this time it was necessary to cross individual strains with WT B10.S mice five to seven times to maintain fecundity, followed by crossing of heterozygous mice to rescue homogeneity of the specific gene interruption. The mice were kept in steel-wire cages in a high-barrier unit under 12-h dark–12-h light cycles and given sterilized food pellets (type R36; Lactamin, Vadstena, Sweden) and water *ad libitum*.

Treatment protocol

Treatment consisted of 6 mg/l and 0.5 g/l Hg and Ag (Fluka, Seelze, Germany), respectively, in the drinking water for 10 weeks. Sodium Au (Myocrisin 100 mg/ml; Aventis Pharma AB, Stockholm, Sweden) was given as weekly intramuscular injections for 10 weeks with 0.1 ml containing 0.45 mg aurothiomaleate/mouse (about 22.5 mg/kg body weight). Controls received only tap water. The Local Ethical Committee for Laboratory Animal Experiments approved the study.

Blood sampling

After 10 weeks' treatment blood was obtained from the retro-orbital plexa and the mice were killed. The collected blood was allowed to clot, centrifuged at 500 g for 10 min, and the serum stored at –70°C.

Analysis of anti-nuclear antibodies by immunofluorescence

The presence, pattern and titre of serum anti-nuclear antibodies of the four different IgG isotypes were determined by indirect immunofluorescence (IF) using HEp-2 cells as a substrate [22]. Briefly, sera were diluted 1:80–1:2560 and incubated on slides with monolayer HEp-2 cells (Binding Site Ltd, Birmingham, UK), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig)G, IgG1, IgG2a, IgG2b or IgG3 antibodies (Southern Biotechnology Associates Inc., Birmingham, AL, USA) diluted 1:50. The titre was defined as the highest serum dilution, which gave a specific nuclear staining. No staining at a serum dilution of 1:80 was considered as a negative result (0). The titre was assessed in each serum using a Nikon incident-light fluorescence microscope (Nikon Instech Co. Ltd., Kanagawa, Japan). All observations were conducted with coded samples. A pool of sera from young, individually ANA-negative mice was used as a negative control.

In order to compare the Th1 and Th2 influence on ANoA, a Th cell index was calculated using titre steps [22]. No specific staining on HEp-2 cells using a serum dilution of 1:80 was considered as '0'. Specific staining at a serum dilution of 1:80 was considered as one titre step (+1), at 1:160 as two titre steps (+2), etc. By subtracting the number of titre steps for IgG1 from that of IgG2a in the individual serum sample, a Th cell index of the AFA response was obtained in the individual mouse. Also, mice showing neither ANoA of the IgG1 or IgG2a isotype were included in the calculation. The arithmetic mean of the Th cell index in the different groups was then calculated. A negative value indicates that ANoA of the IgG1 isotype (Th2) prevailed over ANoA of the IgG2a isotype (Th1).

Analysis of anti-nuclear antibodies by immunoblotting

The specificity of the anti-nuclear antibodies in the serum was assessed by immunoblotting as described previously [23], with minor modifications. Briefly, mouse liver nucleoli were isolated [24], and aliquots of boiled nucleoli were sodium dodecyl sulphate–polyacrylamide gel electrophoresis separated using a 12.5% gel. The electrophoretic transfer to 0.45- μ m nitrocellulose membranes (BioRad Laboratories, Hercules, CA, USA) was performed for 1 h at 0.8 mA/cm² under water cooling (Criterion Blotter; BioRad Laboratories). Nitrocellulose strips were blocked in a Tris-

buffered solution (TBS)–5% non-fat dry milk (blotting grade; BioRad Laboratories)–0.05% Tween 20 overnight at 4°C before being incubated with sera diluted 200-fold in TBS–Tween. Bound murine IgG antibody was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern Biotechnology) diluted 1:5000, followed by enhanced chemiluminescence (ECL Western blotting detection reagents; Amersham, Stockholm, Sweden). For human sera bound IgG was detected with HRP-conjugated goat anti-mouse IgG (Southern Biotechnology) followed by ECL, as above. Human reference sera specific for fibrillarin (Binding Site, Birmingham, AL, USA), and Sm/RNP (American Research Products, Belmont, MA, USA) were used.

Renal histology

Pieces of the left kidney and the spleen were examined by IF, as described previously [25], using FITC-conjugated goat anti-mouse IgG and anti-IgM antibodies (Southern Biotechnology) and anti-C3c antibodies (Organon-Technica, West Chester, PA, USA). The titre of mesangial deposits was determined by serial dilution of the antibodies to 1:5120. The end-point titre of the deposits was defined as the highest dilution of antibody at which a specific fluorescence could be detected. When no specific fluorescence was detected at a dilution of 1:40, the result was recorded as '0'. Vessel wall deposits were graded using a fixed antibody dilution: 1:80 for IgG and 1:160 for C3c. Grading was as follows: 0, no deposits; 1, slight deposits; 2, moderate deposits; 3, heavy deposits.

Tissue immune deposits

Kidney tissues were examined histologically using staining with para-aminosalicylic acid and haematoxylin and eosin, as described previously [26]. The assessment was made without knowledge of treatment.

Statistics

Statistical analyses were performed using GraphPad Software Inc. The Mann–Whitney *U*-test was used to study differences in parameters between KO mice, controls and WT mice. The difference between the number of ANoA-positive mice in the different strains was tested by Fisher's exact test.

Results

Heavy metal-induced anti-nuclear antibodies and IC deposits in B10.S^{+/+} (WT) mice

An IgG response with autoantibodies outlining the nucleoli and staining multiple dots in the nucleoplasm (Fig. 1), which

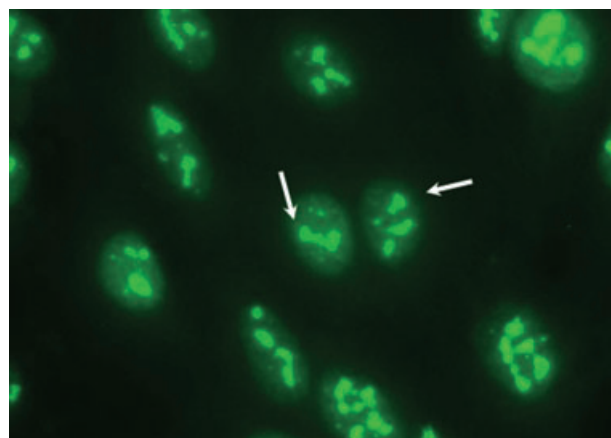


Fig. 1. Serum from a B10.S wild-type mouse treated with aurothiomaleate for 10 weeks, incubated on HEp-2 cells, followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G antibodies. Strong nucleolar staining with nuclear dots.

fulfilled the criteria for the clumpy type of nucleolar staining [27], was seen in female and male B10.S^{+/+} (WT) mice treated with Au, Ag or Hg for 10 weeks.

In female WT mice, the total IgG ANoA response rate varied between 87 and 100% (Table 1). ANoA of each of the different IgG1, IgG2a or IgG2b isotypes was present in 75–100% of the female WT mice treated with any of the three metals. ANoA of the IgG3 isotype with a moderate titre was seen in 87% of Au-treated female WT mice, but significantly less frequent ($P < 0.05$) in mice given Ag or Hg. The titre of IgG1 and IgG2a ANoA was significantly higher in females treated with Au compared with HgCl₂ ($P < 0.01$) (Table 2). The IgG2b ANoA titre was significantly higher after treatment with Au compared with Ag ($P < 0.05$).

The fraction of male WT mice developing IgG ANoA after treatment with Hg, Ag or Au was similar to that in the females (88–100%) (Table 3). However, IgG2a ANoA was present in only 50% of males given Au, but in all Ag- and Hg-treated males, while IgG1 ANoA was seen in 13% of males given Au, and in 75% and 43% of Ag- and Hg-treated males respectively (Table 3). IgG3 ANoA was not found after Au treatment in the males, and in only a low fraction and/or a low titre in males treated with Ag or Hg. The ANoA titre was not significantly different between the groups of male WT mice (Table 4).

Only the Hg-treated WT mice showed a significantly increased titre of both IgG and C3c deposits in the renal glomerular mesangium (Fig. 2a,c) compared with WT controls (Fig. 2b,d) (Table 5). Au-treated WT mice showed a significantly increased titre of mesangial C3c deposits compared with WT control mice. Metal-treated WT mice showed a higher mean titre of IgM deposits in the mesangium (Table 5) compared with untreated controls (Fig. 2e,f),

Table 1. Anti-nucleolar antibody (ANoA) of different immunoglobulin (Ig) G isotypes in female B10.S wild-type and genetically targeted mice after treatment with aurothiomaleate (AuTM), silver nitrate (AgNO₃) or HgCl₂ for 10 weeks.

Treatment	B10.S	<i>n</i>	ANoA				
			Total IgG* (%)	IgG1 (%)	IgG2a (%)	IgG2b (%)	IgG3 (%)
AuTM	WT	7	87	87	87	87	87 [†]
	IL-4 ^{-/-}	7	57	0 [‡]	43 [§]	14 ^{‡,§,¶}	0 [‡]
	IFN- γ ^{-/-}	5	0	0	0	0	0
	IL-6 ^{-/-}	5	0	0	0	0	0
	CD28 ^{-/-}	4	0	0	0	0	0
AgNO ₃	WT	5	100	80	80	100	20
	IL-4 ^{-/-}	5	100	20	100	80	20
	IFN- γ ^{-/-}	7	0	0	0	0	0
	IL-6 ^{-/-}	6	0	0	0	0	0
	CD28 ^{-/-}	9	0	0	0	0	0
HgCl ₂	WT	9	89	78	78	75	13
	IL-4 ^{-/-}	8	100	0**	100	87	0
	IFN- γ ^{-/-}	9	0	0	0	0	0
	IL-6 ^{-/-}	5	0	0	0	0	0
	CD28 ^{-/-}	5	0	0	0	0	0

*Fraction of mice with IgG ANoA of any isotype. [†]Significantly different from wt mice treated with HgCl₂, $P < 0.01$ or AgNO₃, $P < 0.05$, (Fisher's exact test). [‡]Significantly different from AuTM-treated wild-type (WT) mice, $P < 0.01$ (Fisher's exact test). [§]Significantly different from HgCl₂-treated interleukin (IL)-4^{-/-} mice, $P < 0.05$ (Fisher's exact test). [¶]Significantly different from AgNO₃-treated IL-4^{-/-} mice, $P < 0.05$ (Fisher's exact test). **Significantly different from HgCl₂-treated WT mice, $P < 0.01$ (Fisher's exact test); -/-, gene deletion as indicated; IFN, interferon.

although the difference was not statistically significant. The mesangial IgG deposits in Hg-treated B10.S WT mice were dominated by the IgG1 isotype, but also contained IgG2a (data not shown). Renal and splenic vessel wall granular IgG

deposits were present in 33% and 17%, respectively, of Hg-treated female WT mice (Fig. 2a). The male Hg-treated B10.S WT mice showed no deposits in the renal or splenic vessel walls.

Table 2. Reciprocal anti-nucleolar antibody (ANoA) titre of different immunoglobulin (Ig) G isotypes in female B10.S wild-type (WT) and genetically targeted mice after treatment with aurothiomaleate (AuTM), silver nitrate (AgNO₃) or HgCl₂ for 10 weeks.

Treatment	B10.S	<i>n</i>	ANoA				Th cell index [†]
			IgG1*	IgG2a*	IgG2b*	IgG3*	
AuTM	WT	7	1813 $\pm$ 347 [‡]	2453 $\pm$ 627 [‡]	907 $\pm$ 174 [§]	320 $\pm$ 72	0.3
	IL-4 ^{-/-}	7	0 [‡]	667 $\pm$ 347 [‡]	320 $\pm$ 0 [‡]	0	3.3
	IFN- γ ^{-/-}	5	0	0	0	0	—
	IL-6 ^{-/-}	5	0	0	0	0	—
	CD28 ^{-/-}	4	0	0	0	0	—
AgNO ₃	WT	5	1300 $\pm$ 506	1280 $\pm$ 0**	272 $\pm$ 100	80 $\pm$ 0	0.8
	IL-4 ^{-/-}	5	320 $\pm$ 0	640 $\pm$ 0 ^{††}	768 $\pm$ 485 ^{††}	160 $\pm$ 0	3.4
	IFN- γ ^{-/-}	7	0	0	0	0	—
	IL-6 ^{-/-}	6	0	0	0	0	—
	CD28 ^{-/-}	9	0	0	0	0	—
HgCl ₂	WT	9	343 $\pm$ 16	331 $\pm$ 163	400 $\pm$ 113	160 $\pm$ 0	0
	IL-4 ^{-/-}	8	0	290 $\pm$ 84	143 $\pm$ 35	0	2.4
	IFN- γ ^{-/-}	9	0	0	0	0	—
	IL-6 ^{-/-}	5	0	0	0	0	—
	CD28 ^{-/-}	5	0	0	0	0	—

*Mean reciprocal titre $\pm$ standard error of the mean in mice with ANoA. [†]T helper type 1 (Th1)/Th2-deviation of the ANoA response calculated as described in Material and methods, using only mice with serum IgG1 or IgG2a ANoA. [‡]Significantly different titre from HgCl₂-treated WT mice, $P < 0.01$ (Mann-Whitney *U*-test). [§]Significantly different from AgNO₃-treated WT mice, $P < 0.05$ (Mann-Whitney *U*-test). [¶]Significantly different titre from AuTM-treated WT mice, $P < 0.05$ (Mann-Whitney *U*-test). **Significantly different titre from HgCl₂-treated WT mice, $P < 0.01$ (Mann-Whitney *U*-test). ^{††}Significantly different titre from HgCl₂-treated interleukin (IL)-4^{-/-} mice, $P < 0.05$ (Mann-Whitney *U*-test); -/-, gene deletion as indicated; IFN, interferon.

Table 3. Anti-nucleolar antibody (ANoA) of different immunoglobulin (Ig) G isotypes in male B10.S wild-type (WT) and genetically targeted mice after treatment with aurothiomaleate (AuTM), silver nitrate (AgNO₃) or HgCl₂ for 10 weeks

Treatment	B10.S	n	ANoA				
			Total IgG*	IgG1	IgG2a	IgG2b	IgG3
			(%)	(%)	(%)	(%)	(%)
AuTM	WT	8	88	13	50	88	0
	IL-4 ^{-/-}	5	100	0	60	100	0
	IFN- γ ^{-/-}	5	0	0	0	0	0
	IL-6 ^{-/-}	5	0	0	0	0	0
	CD28 ^{-/-}	5	0	0	0	0	0
AgNO ₃	WT	4	100	75	100	75	50
	IL-4 ^{-/-}	5	100	60	100	100	40
	IFN- γ ^{-/-}	5	0	0	0	0	0
	IL-6 ^{-/-}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	CD28 ^{-/-}	7	0	0	0	0	0
HgCl ₂ n.d.	WT	7	100	43	100	71	14
	IL-4 ^{-/-}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	IFN- γ ^{-/-}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	IL-6 ^{-/-}	7	0	0	0	0	0
	CD28 ^{-/-}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

*Fraction of mice with IgG ANoA of any isotype; n.d., not done; -/-, gene deletion as indicated; IL, interleukin; IFN, interferon.

Heavy metal-induced anti-nuclear antibodies and IC deposits in B10.S mice targeted for the IL-4 gene

Targeting of the IL-4 gene resulted in an IgG ANoA response in 57–100% of female mice after 10 weeks' treatment with Au, Ag or Hg (Table 1). The IgG1 ANoA response was abolished totally in Au- and Hg-treated female mice, while a single Ag-treated mouse (20%) showed a moderate IgG1 ANoA titre. The fraction of IgG2a and IgG2b ANoA-positive

mice was significantly lower in IL-4-deficient female mice treated with Au compared with Ag or Hg. Furthermore, the titre of IgG2a and IgG2b ANoA was significantly lower in IL-4^{-/-} females treated with Au, but not Hg or Ag, compared with the corresponding metal-treated WT mice (Table 2). With regard to the Th cell index, targeting of the IL-4 gene caused a distinct Th1 deviation in all metal-treated mice.

Although there were no significant differences in ANoA isotypes between male IL-4^{-/-} and WT mice (Table 3),

Table 4. Reciprocal anti-nucleolar antibody (ANoA) titre of different immunoglobulin (Ig) G isotypes in male B10.S wild-type (WT) and genetically targeted mice after treatment with aurothiomaleate (AuTM), silver nitrate (AgNO₃) or HgCl₂ for 10 weeks.

Treatment	B10.S	n	ANoA				Th cell index [†]
			IgG1*	IgG2a*	IgG2b*	IgG3*	
AuTM	WT	8	160 ± 0	240 ± 135	171 ± 27	0	1.5
	IL-4 ^{-/-}	5	0	133 ± 27	192 ± 32	0	1.0
	IFN- γ ^{-/-}	5	0	0	0	0	–
	IL-6 ^{-/-}	5	0	0	0	0	–
	CD28 ^{-/-}	5	0	0	0	0	–
AgNO ₃	WT	4	347 ± 162	340 ± 115	333 ± 173	60 ± 20	0.8
	IL-4 ^{-/-}	5	133 ± 27	832 ± 274	640 ± 175 [‡]	100 ± 60	2.8
	IFN- γ ^{-/-}	5	0	0	0	0	–
	IL-6 ^{-/-}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	CD28 ^{-/-}	7	0	0	0	0	–
HgCl ₂	WT	7	667 ± 347	571 ± 155	272 ± 100	320 ± 0	1.9
	IL-4 ^{-/-}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	IFN- γ ^{-/-}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	IL-6 ^{-/-}	7	0	0	0	0	–
	CD28 ^{-/-}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

*Mean reciprocal titre ± standard error of the mean in the mice with ANoA. [†]T helper type 1 (Th1)/Th2-deviation of the ANoA response calculated as described in Material and methods, using only mice with serum IgG1 or IgG2a ANoA. [‡]Significantly different from interleukin (IL)-4^{-/-} mice treated with AuTM, *P* < 0.05 (Mann–Whitney *U*-test); n.d., not done; -/-, gene deletion as indicated; IFN, interferon.

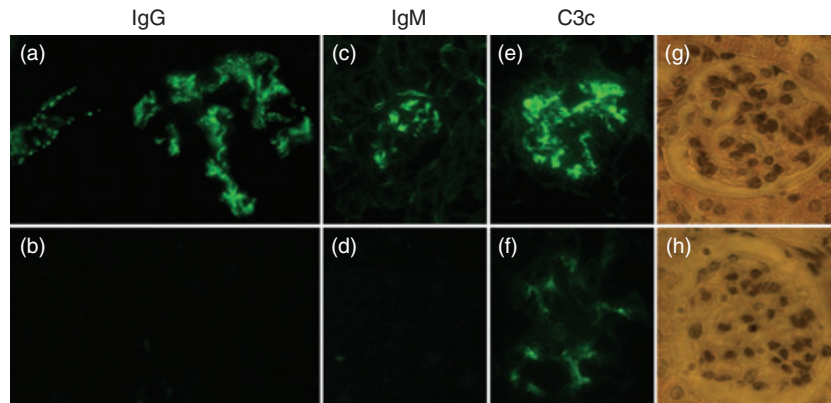


Fig. 2. (a–f) Direct immunofluorescence using fluorescein isothiocyanate-conjugated anti-mouse antibodies to the specified antigens on cryostat sections of representative renal tissue from mice treated with 6 mg mercury (Hg)/l for 10 weeks (a, c, e) and untreated controls (b, d, f). (a, c, e) Granular immune deposits in the renal mesangium, and in (a) also in an arteriole; (b, d) no or minimal glomerular deposits; (f) slight glomerular deposits; (g–h) haematoxylin and eosin-stained sections from mice treated with Hg as above (g); untreated controls (h); (g) modest increase of mesangial cells compared with the untreated controls in (h).

probably because of the partial response of most IgG ANoA isotypes in these mice, the Ag-treated mice showed a Th1-skewed ANoA response (Table 4). The IgG3 ANoA response was infrequent, with a low titre in all groups (Tables 3 and 4).

Deletion of the IL-4 gene caused a large decrease in the mean titre of renal mesangial deposits of IgG, IgM and C3c in metal-treated mice compared with WT mice (Table 5).

Table 5. Tissue immune-complex deposits in wild-type (WT) and genetically targeted B10.S mice after treatment with aurothiomaleate (AuTM), silver nitrate (AgNO₃) or HgCl₂ for 10 weeks.

Treatment	Strain	n	Renal mesangial deposits		
			IgG ^a	C3c ^a	IgM ^b
AuTM	WT	12	120 ± 106	880 ± 220 ^c	1.5 ± 0.2
	IL-4 ^{-/-}	4	9 ± 9	50 ± 30 ^d	1.0 ± 0
	IFN-γ ^{-/-}	7	17 ± 12	90 ± 50 ^e	1.0 ± 0
	IL-6 ^{-/-}	10	24 ± 17	80 ± 30 ^e	1.2 ± 0.1
	CD28 ^{-/-}	8	0	100 ± 30 ^d	1.0 ± 0
AgNO ₃	WT	8	110 ± 80	300 ± 80	1.6 ± 0.2
	IL-4 ^{-/-}	2	0	80 ± 80	1.0 ± 0
	IFN-γ ^{-/-}	0	—	—	—
	IL-6 ^{-/-}	6	430 ± 270	880 ± 530 ^f	1.7 ± 0.3
	CD28 ^{-/-}	9	0	142 ± 30	1.0 ± 0 ^g
HgCl ₂	WT	11	800 ± 225 ^h	742 ± 280 ⁱ	1.8 ± 0.1
	IL-4 ^{-/-}	4	0	0	1.3 ± 0.3
	IFN-γ ^{-/-}	5	0	64 ± 39 ^j	1.0 ± 0
	IL-6 ^{-/-}	10	0	96 ± 26 ^j	1.2 ± 0.1 ^k
	CD28 ^{-/-}	2	0	80 ± 80	2.5 ± 0.5 ^l
Controls	WT	10	40 ± 30	190 ± 60	1.4 ± 0.3
	IL-4 ^{-/-}	6	10 ± 10	110 ± 50	1.0 ± 0
	IFN-γ ^{-/-}	6	0	160 ± 40	1.0 ± 0
	IL-6 ^{-/-}	5	0	100 ± 40	1.0 ± 0
	CD28 ^{-/-}	5	8 ± 8	190 ± 60	1.0 ± 0

^aFigures denote mean reciprocal titre ± standard error of the mean. ^bGrading: 0, no deposits; 1, slight deposits; 2, moderate deposits; 3, heavy deposits. ^c*P* < 0.05 significantly different from untreated WT controls (Mann–Whitney *U*-test). ^d*P* < 0.01 significantly different from AuTM-treated WT mice (Mann–Whitney *U*-test). ^e*P* < 0.05 significantly different from AuTM-treated WT mice (Mann–Whitney *U*-test). ^f*P* < 0.01 significantly different from AgNO₃-treated WT mice (Mann–Whitney *U*-test). ^g*P* < 0.05 significantly different from untreated WT controls (Mann–Whitney *U*-test). ^h*P* < 0.01 significantly different from untreated WT controls (Mann–Whitney *U*-test). ⁱ*P* < 0.05 significantly different from HgCl₂-treated WT mice (Mann–Whitney *U*-test). ^j*P* < 0.05 significantly different from HgCl₂-treated WT mice (Mann–Whitney *U*-test). ^k*P* < 0.05 significantly different from HgCl₂-treated WT mice (Mann–Whitney *U*-test). ^l*P* < 0.01 significantly different from HgCl₂-treated WT mice (Mann–Whitney *U*-test); ^{–/–}: gene deletion as indicated; Ig: immunoglobulin; IL:interleukin; IFN: interferon.

Heavy metal-induced anti-nuclear antibodies and IC deposits in B10.S mice targeted for the IL-6, IFN- γ or CD28 gene

Targeting of the gene for IFN- γ , IL-6 or the co-stimulatory factor CD28 totally abolished the ANoA response of all IgG isotypes to all three metals in all males as well as females studied (Tables 1 and 3).

Deletion of the IFN- γ , IL-6 or CD28 genes abolished or reduced the titre of renal mesangial deposits of IgG, IgM or C3c in all metal-treated mice compared with the WT mice (Table 5). The only exceptions were (a) two Ag-treated IL-6 $^{-/-}$ mice, which showed a substantial titre of mesangial deposits of IgG, IgM and C3c, causing a significantly increased titre of C3c in the group of Ag-treated IL-6 $^{-/-}$ mice compared with the WT mice; and (b) Hg-treated CD28 $^{-/-}$ mice which showed significantly increased IgM deposits compared with WT mice (Table 5).

The renal histology of metal-treated WT and gene-targeted mice

The Hg-treated WT mice, with significantly increased mesangial IgG and C3c deposits compared with control mice, showed a slight global increase of mesangial cells (Fig. 2g) compared with untreated control WT mice (Fig. 2h). No other glomerular alterations were observed. Neither WT mice treated with Ag or Au, nor any of the groups of gene-targeted, metal-treated mice, showed any histologically discernible alteration of the glomeruli compared with the untreated controls. This indicates that the mesangial cell proliferation is associated with the combination of an increase in IgG and C3c.

Specificity of the heavy metal-induced ANoA

Immunoblotting showed that the sera from Ag- and Au-treated B10.S WT mice reacted with a 34-kDa nuclear protein, which was also targeted by the reference serum to the protein fibrillarin (Fig. 3). The reactivity with the 34-kDa band also remained in metal-treated IL-4 $^{-/-}$ mice while targeting of the IFN- γ , IL-6 or CD28 gene abolished the reaction.

Discussion

The role of certain cytokines and co-stimulatory molecules for induction of systemic autoimmunity with the heavy metals Hg, Ag and Au was assessed by studying strains with single-gene deletions on the metal-susceptible B10.S (H-2 ^{s}) background.

Previous studies on the effect of deleting IL-4 [19,20] or IFN- γ [20] showed that IL-4 was dispensable and IFN- γ critical for induction of autoimmunity by Hg. We now

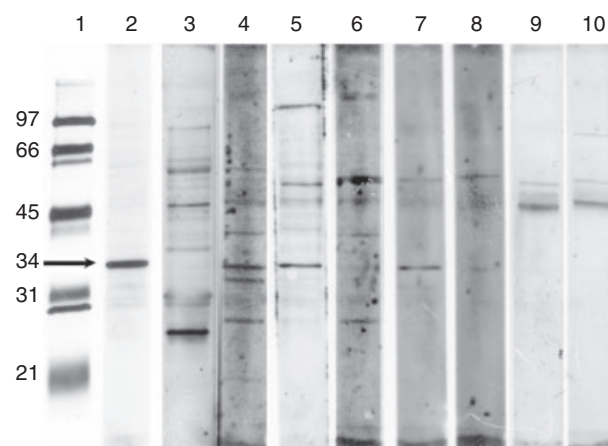


Fig. 3. Immunoblotting of sera using sodium dodecyl sulphate–polyacrylamide gel electrophoresis separated mouse liver nuclei. Lane 1: molecular weight markers (kDa). Lane 2: human reference serum blotting the 34-kDa protein fibrillarin (arrow). Lane 3: human reference serum blotting the Sm/RNP proteins B (28 kDa) and B' (29 kDa). Lanes 4 and 5: B10.S wild-type (WT) mice treated with silver nitrate (AgNO₃) and aurothiomaleate (Au) for 10 weeks respectively. Lane 6: B10.S untreated, control WT mouse. Lanes 7–10: mice treated with Au for 10 weeks with deletion of the interleukin (IL)-4, interferon- γ , IL-6, and CD28 gene respectively. Lanes 4, 5 and 7 show reactivity with a protein of an apparent molecular weight of 34 kDa corresponding to fibrillarin (lane 2), while lanes 6 and 8–10 show no such reactivity.

report that lack of the IL-4 gene virtually abolished the IgG1 ANoA response induced by all three heavy metals, which tallies with the dependency on IL-4 for switching to IgG1 [28]. The titres of IgG2a, IgG2b and IgG3 ANoA declined in IL-4 $^{-/-}$ mice after Au treatment, but were not altered consistently after treatment with Hg or Ag compared with metal-treated WT mice. This is in accordance with previous observations in Hg-treated IL-4 $^{-/-}$ mice on the B10.S [20] and another H-2 ^{s} [19] background. The observed isotype pattern also tallies with the antibody response towards a haptenated foreign antigen, nitrophenyl-chicken γ -globulin in IL-4 $^{-/-}$ mice [28].

However, our results are at variance with the reduction of IgG1 but the increase in IgG2a, IgG2b and IgG3 ANoA isotypes observed using anti-IL-4 monoclonal antibody (mAb) therapy during Hg treatment of H-2 ^{s} mice [21]. IFN- γ and IL-4 are expected to have an antagonizing effect on each other's expression and function, because of transcription factor T-bet expression which promotes Th1 cell differentiation, IFN- γ production and IgG2a class-switching, and the transcription factor GATA binding factor 3 (GATA-3) promoting Th2 cell development, IL-4 production and IgG1/IgE-switching [29]. These antagonizing functions are likely to be preserved following a transient IL-4 deficiency because of mAb therapy, while they may have been lost or attenuated by the chronic, life-long absence of IL-4 in genetically targeted mice.

We observed in the present study that lack of IL-4 totally abolished the renal mesangial and splenic vessel wall IC deposits induced by Hg treatment in WT mice. This is in contrast with the persistence of systemic IC deposits observed in a previous study of Hg-treated IL-4-deficient mice [20]. The obvious difference between these studies is that the daily Hg dose in the previous study was more than threefold higher than in the present study. A relationship exists between the dose of Hg and the induction of IC deposits [30]. Therefore, only the higher dose of Hg used in the previous studies may have provided a sufficiently high autoimmune stimulation to compensate for the lack of IL-4 with regard to induction of IC deposits by Hg.

The crucial role of IFN- γ for induction of HgIA as shown here is in accordance with a previous study [20]. For the first time, we now report this to be also true for induction of ANoA using Ag or Au, indicating that the basic immune mechanisms for induction of ANoA may be similar for all three heavy metals. The actual role played by IFN- γ in HgIA is indicated by a maximum increase of only 25% in lymph node IFN- γ -mRNA during the first 5 days of treatment [18], and the clear dose-response relationship between IFN- γ gene expression and development of ANoA and IC deposits in HgIA observed in heterozygous IFN- $\gamma^{+/-}$ mice [20], which have approximately 55% IFN- γ expression [31]. Therefore, only a certain amount of IFN- γ is crucial for triggering the Hg-induced autoimmune reaction.

The effect of IFN- γ has been studied in other (auto)-immune responses. In experimental myasthenia gravis, the IgG response to the acetylcholine receptor is abrogated in IFN- $\gamma^{+/-}$ mice, while the response to the foreign T cell hapten KLH is intact [32]. In addition, IFN- $\gamma^{+/-}$ mice generally show an intact response to foreign, infectious agents [33]. This difference in antibody induction between self-antigens and foreign, haptenated antigens in IFN- $\gamma^{+/-}$ mice was also observed in HgIA [20]. Taken together, these observations indicate that weakly antigenic molecules, including self-antigens [34], need IFN- $\gamma^{+/-}$ in order to elicit an IgG antibody response. In spontaneous lupus-prone mice the autoimmune manifestations are delayed and survival increases if the IFN- γ gene is deleted or partially reduced [31]. In pristane-induced autoimmunity, anti-ssDNA, anti-chromatin and anti-RNP/Sm autoantibodies, as well as systemic IC deposits, are abrogated in IFN- γ deleted mice, but unchanged in IL-4 $^{-/-}$ mice [35], therefore resembling the findings in the present heavy metal model.

We found that deletion of the IL-6 gene abrogated the induction of ANoA by the heavy metals, and in the case of Hg also the IC deposits. This is at variance with previous studies where IL-6 $^{-/-}$ mice given the more than threefold higher dose of Hg showed reduced hypergammaglobulinaemia but retained ANoA and systemic IC deposits compared with WT mice [36]. Because IL-6 is a co-factor necessary for deviating the T cell development towards induction of Th17 cells instead of T regulatory (T_{reg}) cells,

the lack of ANoA and IC deposits in IL-6 $^{-/-}$ mice might indicate that low doses of Hg causes T_{reg} activity to prevail. However, targeting of the IL-12p40, a cytokine linked tightly to IL-23 and therefore IL-17 (Th17) responses [37], does not alter significantly the manifestations of HgIA [36]. The role of Th17 and associated cytokines (IL-21, IL-22, IL-23) in HgIA therefore needs to be elucidated further. IL-6 is known to be involved in systemic autoimmune diseases, as anti-IL-6 mAbs suppress the production of pathogenic anti-dsDNA antibodies in lupus-prone mice [38]. In the pristane model, IL-6 deficiency caused by blocking the IL-6R has a differential effect on autoantibody induction, with loss of anti-chromatin and anti-ssDNA antibodies, but preserved anti-RNP/Sm antibody response [35].

How do our observations of cytokine requirements in heavy metal (Hg)-induced autoimmunity tally with the emerging picture of three Th cells differentiation/effector pathways – Th1 cells with IFN- γ as the main cytokine, Th2 cells with IL-4, IL-5 and IL-13 as the main cytokines, and finally Th17 cells with IL-17, IL-21 and IL-22 as the main cytokines [37]? The crucial need for (a certain amount of) IFN- γ in order to induce all aspects of heavy metal-induced autoimmunity, coupled with previous observations that single-gene deletions regulating IFN- γ expression [IL-12p35, IL-12p40, STAT4, ICE (IL-18)], do not influence Hg-induced autoimmunity significantly, while deletion of down-stream genes relating to the function of IFN- γ (IFN- γ receptor, IFN response factor 1) strongly reduce manifestations of HgIA [36], indicate IFN- γ as an unrivalled critical factor for HgIA. In contrast, the Th2 pathway with IL-4 as the main cytokine is apparently not needed for induction of ANoA by heavy metals (except the IgG1 isotype). As mentioned above, our findings indicate that the effect of IL-4 on development of IC deposits may depend on the strength of the autoimmune stimulus (in this case the dose of Hg). However, the mechanism behind this observation is not clear.

Finally, we examined the effect of deleting CD28, a crucial factor in delivering an activating, second signal for T cells by interacting with CD80 and CD86 on antigen-presenting cells, also setting in motion expression of CTLA4 on the activated T cells which, after binding to CD80/CD86, suppresses T cell activation. We found that mice lacking the co-stimulatory factor CD28 did not develop ANoA after treatment with any of the heavy metals Hg, Au or Ag. For Hg this tallies with previous studies using a more than threefold higher dose of Hg, where it was also shown that deletion of the T-B cell co-stimulatory molecule CD154 (CD40L) inhibited the induction of ANoA similar to findings after treatment with anti-CD40L mAbs [16]. Biancone *et al.* [39] showed that CTLA-4-Fc administration suppressed induction of ANoA by Hg, illustrating further the effect of blocking CD80/86 and thereby CD28 signalling. Furthermore, Zheng *et al.* [17] showed that blocking of another co-stimulatory receptor, inducible co-stimulatory molecule (ICOS), which is homologous with CD28 and CTLA4 but

interacts mainly with ICOS ligand (ISOSL), abrogated the ANoA response. The mechanism for abrogation of the heavy metal-induced ANoA response after blocking CD28 signaling is likely to be defect priming of Th cells, supporting further the hypothesis that the metal-induced AFA response is analogous with a conventional antigen-driven immune response [40].

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References

- Rhodes B, Vyse TJ. General aspects of the genetics of SLE. *Autoimmunity* 2007; **40**:550–9.
- Rowley B, Monestier M. Mechanisms of heavy metal-induced autoimmunity. *Mol Immunol* 2005; **42**:833–8.
- Hultman P, Enestrom S, Pollard KM, Tan EM. Anti-fibrillar autoantibodies in mercury-treated mice. *Clin Exp Immunol* 1989; **78**:470–7.
- Robinson CJ, White HJ, Rose NR. Murine strain differences in response to mercuric chloride: antinuclear antibodies production does not correlate with renal immune complex deposition. *Clin Immunol Immunopathol* 1997; **83**:127–38.
- Stiller-Winkler R, Radaszkiewicz T, Gleichmann E. Immunopathological signs in mice treated with mercury compounds – I. Identification by the popliteal lymph node assay of responder and nonresponder strains. *Int J Immunopharmacol* 1988; **10**:475–84.
- Johansson U, Hansson-Georgiadis H, Hultman P. The genotype determines the B cell response in mercury-treated mice. *Int Arch Allergy Immunol* 1998; **116**:295–305.
- Hultman P, Hansson-Georgiadis H. Methyl mercury-induced autoimmunity in mice. *Toxicol Appl Pharmacol* 1999; **154**:203–11.
- Reuter R, Tessars G, Vohr HW, Gleichmann E, Luhrmann R. Mercuric chloride induces autoantibodies against U3 small nuclear ribonucleoprotein in susceptible mice. *Proc Natl Acad Sci USA* 1989; **86**:237–41.
- Reichow SL, Hama T, Ferre-D'Amare AR, Varani G. The structure and function of small nucleolar ribonucleoproteins. *Nucleic Acids Res* 2007; **35**:1452–64.
- Kono DH, Park MS, Szydlak A *et al.* Resistance to xenobiotic-induced autoimmunity maps to chromosome 1. *J Immunol* 2001; **167**:2396–403.
- Hultman P, Johansson U, Turley SJ, Lindh U, Enestrom S, Pollard KM. Adverse immunological effects and autoimmunity induced by dental amalgam and alloy in mice. *FASEB J* 1994; **8**:1183–90.
- Havarinasab S, Johansson U, Pollard KM, Hultman P. Gold causes genetically determined autoimmune and immunostimulatory responses in mice. *Clin Exp Immunol* 2007; **150**:179–88.
- Hultman P, Ganowski K, Turley SJ, Pollard KM. Genetic susceptibility to silver-induced anti-fibrillar autoantibodies in mice. *Clin Immunol Immunopathol* 1995; **77**:291–7.
- Johansson U, Hansson-Georgiadis H, Hultman P. Murine silver-induced autoimmunity: silver shares induction of antinuclear antibodies with mercury, but causes less activation of the immune system. *Int Arch Allergy Immunol* 1997; **113**:432–43.
- Hultman P, Johansson U, Dagnaes-Hansen F. Murine mercury-induced autoimmunity: the role of T-helper cells. *J Autoimmun* 1995; **8**:809–23.
- Pollard KM, Arnush M, Hultman P, Kono DH. Costimulation requirements of induced murine systemic autoimmune disease. *J Immunol* 2004; **173**:5880–7.
- Zheng Y, Jost M, Gaughan JP, Class R, Coyle AJ, Monestier M. ICOS-B7 homologous protein interactions are necessary for mercury-induced autoimmunity. *J Immunol* 2005; **174**:3117–21.
- Haggqvist B, Hultman P. Murine metal-induced systemic autoimmunity: baseline and stimulated cytokine mRNA expression in genetically susceptible and resistant strains. *Clin Exp Immunol* 2001; **126**:157–64.
- Bagenstose LM, Salgame P, Monestier M. Mercury-induced autoimmunity in the absence of IL-4. *Clin Exp Immunol* 1998; **114**:9–12.
- Kono DH, Balomenos D, Pearson DL *et al.* The prototypic Th2 autoimmunity induced by mercury is dependent on IFN- γ and not Th1/Th2 imbalance. *J Immunol* 1998; **161**:234–40.
- Ochel M, Vohr HW, Pfeiffer C, Gleichmann E. IL-4 is required for the IgE and IgG1 increase and IgG1 autoantibody formation in mice treated with mercuric chloride. *J Immunol* 1991; **146**:3006–11.
- Havarinasab S, Bjorn E, Ekstrand J, Hultman P. Dose and Hg species determine the T-helper cell activation in murine autoimmunity. *Toxicology* 2007; **229**:23–32.
- Warfvinge K, Hansson H, Hultman P. Systemic autoimmunity due to mercury vapor exposure in genetically susceptible mice: dose-response studies. *Toxicol Appl Pharmacol* 1995; **132**:299–309.
- Chan EKL, Pollard KM. Autoantibodies to ribonucleoprotein particles by immunoblotting. In: Rose N, Conway de Macario E, Fahey JL, Penn H, eds. *Manual of clinical laboratory immunology*. Washington, DC: American Society for Microbiology, 1992:755–61.
- Hultman P, Bell LJ, Enestrom S, Pollard KM. Murine susceptibility to mercury. I. Autoantibody profiles and systemic immune deposits in inbred, congenic, and intra-H-2 recombinant strains. *Clin Immunol Immunopathol* 1992; **65**:98–109.
- Enestrom S, Hultman P. Immune-mediated glomerulonephritis induced by mercuric chloride in mice. *Experientia* 1984; **40**:1234–40.
- Pollard KM, Hultman P. Fibrillar autoantibodies. In: Shoenfeld Y, Gershwin ME, Meroni PL, eds. *Autoantibodies*. Amsterdam: Elsevier, 2006:317–23.
- Kuhn R, Rajewsky K, Muller W. Generation and analysis of interleukin-4 deficient mice. *Science* 1991; **254**:707–10.
- Glimcher LH, Murphy KM. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev* 2000; **14**:1693–711.
- Hultman P, Nielsen JB. The effect of dose, gender, and non-H-2 genes in murine mercury-induced autoimmunity. *J Autoimmun* 2001; **17**:27–37.
- Balomenos D, Rumold R, Theofilopoulos AN. Interferon- γ is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice. *J Clin Invest* 1998; **101**:364–71.
- Balasa B, Deng C, Lee J *et al.* Interferon gamma (IFN- γ) is necessary for the genesis of acetylcholine receptor-induced clinical experimental autoimmune myasthenia gravis in mice. *J Exp Med* 1997; **186**:385–91.

- 33 Graham MB, Dalton DK, Giltinan D, Braciale VL, Stewart TA, Braciale TJ. Response to influenza infection in mice with a targeted disruption in the interferon gamma gene. *J Exp Med* 1993; **178**:1725–32.
- 34 McMahan RH, Slansky JE. Mobilizing the low-avidity T cell repertoire to kill tumors. *Semin Cancer Biol* 2007; **17**:317–29.
- 35 Richards HB, Satoh M, Jennette JC, Croker BP, Yoshida H, Reeves WH. Interferon-gamma is required for lupus nephritis in mice treated with the hydrocarbon oil pristane. *Kidney Int* 2001; **60**:2173–80.
- 36 Pollard KM, Hultman P, Kono DH. Immunology and genetics of induced systemic autoimmunity. *Autoimmun Rev* 2005; **4**:282–8.
- 37 Deenick EK, Tangye SG. Autoimmunity: IL-21: a new player in Th17-cell differentiation. *Immunol Cell Biol* 2007; **85**:503–5.
- 38 Mihara M, Takagi N, Takeda Y, Ohsugi Y. IL-6 receptor blockage inhibits the onset of autoimmune kidney disease in NZB/W F₁ mice. *Clin Exp Immunol* 1998; **112**:397–402.
- 39 Biancone L, Andres G, Ahn H *et al.* Distinct regulatory roles of lymphocyte costimulatory pathways on T helper type-2 mediated autoimmune disease. *J Exp Med* 1996; **183**:1473–81.
- 40 Pollard KM, Lee DK, Casiano CA, Bluthner M, Johnston MM, Tan EM. The autoimmunity-inducing xenobiotic mercury interacts with the autoantigen fibrillarin and modifies its molecular and antigenic properties. *J Immunol* 1997; **158**:3521–8.