

Redirection of allergen-specific T_H2 responses by a modified adenine through Toll-like receptor 7 interaction and IL-12/IFN release

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Background: Natural or synthetic ligands of Toll-like receptors (TLRs), such as CpG-containing oligodeoxynucleotides and imidazoquinolines, affect the functional phenotype of antigen-specific human T lymphocytes by inducing cytokine release by cells of the innate immunity.

Objective: *In vitro* investigation of the ability of substitute adenines (SAs) to affect antigen-presenting cells and shift the functional phenotype of specific human T_H2 cells was performed.

Methods: The functional profile of hapten- and allergen-specific T-cell lines obtained in the absence or presence of modified adenines was assessed by means of quantitative real-time PCR, flow cytometry, and ELISAs. Activation of TLRs was evaluated by means of nucleofection of HEK293 cells.

Results: The synthetic heterocycle, chemically related to adenine with substitution in positions 2-, 8-, and 9- (SA-2), but not its related derivative lacking 2- and 8- substitutions, stimulated the production of high amounts of IL-12, IL-10, TNF- α , and IL-6 by CD14⁺ cells and IFN- α and CXCL10 by blood dendritic cell antigen (BDCA)-4⁺ plasmacytoid dendritic cells. A nuclear factor κ B-dependent signaling pathway mediated by SA-2 ligation of TLR7 was responsible for these effects. SA-2 also redirected the *in vitro* differentiation of either *Dermatophagoides pteronyssinus* group 1 or amoxicillin-specific T_H2 cells toward the T_H1/T_H0 phenotype, with parallel downregulation of GATA-3 and upregulation of T-box expressed in T cells transcription factors.

Conclusion: Critical substitutions of the adenine backbone confer the ability to activate TLR7, inducing the production of modulatory cytokines able to shift human allergen-specific T_H2 cells to a T_H1/T_H0 phenotype.

Clinical implications: Appropriately modified adenines might be used as effective adjuvants for the development of novel immunotherapeutic strategies of allergic disorders. (J Allergy Clin Immunol 2006;118:511-7.)

Key words: Adjuvants, immunotherapy, heterocycles, Toll-like receptors, allergy, T_H1/T_H2 lymphocytes, GATA-3, T-box expressed in T cells, nuclear factor κ B

The 11 members of the Toll-like receptor (TLR) family are type 1 transmembrane receptors broadly distributed on the cells of the immune system, recognizing highly conserved structures of microbial products.^{1,2} The interaction between microbial structures and TLRs activates common signaling pathways, such as nuclear factor (NF) κ B and stress-activated protein kinases, resulting in dendritic cell (DC) maturation and cytokine and chemokine production.^{1,2} Both natural and synthetic TLR ligands have been described.^{1,2} Viral nucleic acid-related sequences, such as double-stranded RNA or unmethylated CpG-containing oligodeoxynucleotides (CpG-ODNs), are detected through TLR3 and TLR9, respectively, whereas ssRNA is a TLR7 and TLR8 activator.^{1,3-6} Heterocycles, such as guanosine derivatives and imidazoquinolines (resiquimod [R-848] and imiquimod) have been identified as synthetic ligands of TLR7 exclusively or of both TLR7 and 8.^{6,7} The interaction of natural or synthetic ligands of TLRs results in the production of proinflammatory and regulatory cytokines and chemokines from the cells of the innate immunity, and most of the TLR ligands usually induce the production of large amounts of IL-12, which plays a crucial role in T_H1 differentiation.

In atopic diseases the role of T_H2 T cells specific for common environmental allergens characterized by the production of IL-4, IL-5, and IL-13 but little or no IFN- γ is well documented.⁸ However, allergen-specific T_H2 responses can be shifted, at least *in vitro*, to T_H0 (production of both T_H2 cytokines and IFN- γ) or even T_H1 (production of IFN- γ in the absence of T_H2 cytokines) responses.⁹ Double-stranded RNA,¹⁰ CpG-ODNs,^{11,12} and imidazoquinolines¹³ can be responsible for the immune deviation from a T_H2 to a T_H0/T_H1 phenotype. On this basis, the administration of the Amb a 1 allergen conjugated to an ODN sequence was recently shown to induce a clinical improvement in allergic subjects, correlating with the shift of the allergen-specific T_H2 response to a less polarized phenotype at either the circulation or nasal level.^{14,15}

In search of new adjuvants suitable for novel, safe, and effective immunotherapeutic strategies, we developed a synthetic heterocycle chemically related to a modified

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Abbreviations used

BDCA:	Blood dendritic cell antigen
CpG-ODN:	CpG-containing oligodeoxynucleotide
DC:	Dendritic cell
NF- κ B:	Nuclear factor κ B
PDC:	Plasmacytoid dendritic cell
PMA:	Phorbol 12-myristate 13-acetate
Poly(I:C):	Polyinosinic-polycytidylic acid
R-848:	Resiquimod
SA:	Substitute adenine
T-bet:	T-box expressed in T cells
TCL:	Short-term T-cell line
TLR:	Toll-like receptor

adenine with substitution in positions 2-, 8-, and 9- (SA-2). SA-2 was able to induce the production of high amounts of IL-6, TNF- α , IL-12, and IL-10 by CD14⁺ cells and high amounts of IFN- α and CXCL10 by circulating plasmacytoid DCs (PDCs) of healthy donors. A signaling pathway dependent on NF- κ B activation was responsible for these effects. Transfection of HEK293 with a TLR7-encoding plasmid, but not TLR3, TLR8, or TLR9, enabled these cells to respond to SA-2. The addition of SA-2 resulted in a dramatic shift of allergen- and hapten-specific T cells from *Dermatophagoides pteronyssinus*- or amoxicillin-sensitive donors to a T_H0/T_H1 phenotype. This immune deviation was also confirmed at the transcriptional level, with downregulation of GATA-3 and upregulation of T-box expressed in T cells (T-bet).

These data suggest that appropriately modified adenines, because of their ability to interact with TLR7 expressed by the cells of innate immunity, resulting in the local production of modulatory cytokines, might represent a novel class of synthetic compounds potentially suitable as adjuvants for the development of immunotherapeutic strategies based on the preferential activation of T_H1-polarized responses.

METHODS**Reagents**

For information on reagents, see this [article's](#) supplementary Methods text in the Online Repository at www.jacionline.org.

Synthesis of modified adenines

See this [article's](#) supplementary Methods text in the Online Repository at www.jacionline.org for the synthesis of modified adenine SA-2 and SA-1.¹⁶ The chemical structures of modified adenines are shown in [Fig 1](#).

Subjects

PBMCs were derived from 11 healthy donors, 9 adult amoxicillin-sensitive donors and 5 *D pteronyssinus*-sensitive atopic volunteers, in accordance with the ethical standards of the responsible regional committee on human experimentation.

Purification of monocytes and PDCs

MACS-selected CD14⁺ or blood dendritic cell antigen (BDCA)-4⁺ cells (1×10^6 /mL) from 11 healthy subjects were cultured in the

absence or presence of R-848, modified adenines (all at 6 μ M), or LPS (1 μ g/mL) for 36 hours, and the amounts of cytokines and chemokines were evaluated by using ELISAs. See this [article's](#) supplementary Methods text in the Online Repository at www.jacionline.org for details.

RNA extraction and quantitative real-time RT-PCR

Refer to this [article's](#) supplementary Methods text in the Online Repository at www.jacionline.org for TaqMan real-time PCR. β -Actin was used as a housekeeping gene for normalization.

Quantitative analysis of NF- κ B translocation

Analysis of NF- κ B translocation in CD14⁺ cells was performed by using the TransAm ELISA kit (Active Motif, Carlsbad, Calif). See this [article's](#) supplementary Methods text in the Online Repository at www.jacionline.org for details.

Stimulation of TLR-transfected HEK293 and reporter assay

See this [article's](#) supplementary Methods text in the Online Repository at www.jacionline.org for details.

Assessment of G-coupled and ion-channel receptor activation

See this [article's](#) supplementary Methods text in the Online Repository at www.jacionline.org for details.

Generation of allergen- and hapten-specific short-term T-cell lines and hapten-specific T-cell clones

Amoxicillin- or Der p 1-specific short-term T-cell lines (TCLs) were derived from 9 β -lactam-sensitive donors and 5 atopic subjects, respectively, and analyzed for specificity and cytokine production as described in detail in this [article's](#) supplementary Methods text in the Online Repository at www.jacionline.org.¹¹ Amoxicillin-specific T-cell clones from one sensitive donor were assessed for their functional phenotype, as previously reported and described in this [article's](#) supplementary Methods text in the Online Repository.¹¹

Flow cytometric analysis of intracellular cytokine production

T blasts, 1×10^6 , from antigen-specific TCLs were analyzed after phorbol 12-myristate 13-acetate (PMA; 10 ng/mL) and ionomycin (1 μ M) stimulation for 6 hours, the last 2 in the presence of brefeldin A (5 μ g/mL), as previously described.¹¹

Measurement of IL-4 and IFN- γ in supernatants of TCLs and T-cell clones

Viable T-cell blasts, 10^6 /mL, were stimulated polyclonally for 36 hours with PMA (20 ng/mL) plus anti-CD3 mAb (UCHT1, BD PharMingen, San Jose, Calif, 50 ng/mL) or specifically with allergen or hapten in the presence of autologous monocyte-derived DCs for 72 hours. IL-4, IL-5 (BD PharMingen), and IFN- γ (Endogen, Woburn, Mass) were measured into supernatants by using homemade ELISAs, as previously described.¹¹ For details, see this [article's](#) supplementary Methods text in the Online Repository at www.jacionline.org.

Statistical analysis

Statistical analysis of the results was performed by using the Student *t* test for paired data.

RESULTS

SA-2 induces the production of cytokines and chemokines by cells of the innate immunity

The ability of modified adenines to stimulate the cells of the innate immunity was first investigated. Highly purified circulating CD14⁺ cells were derived from 11 healthy donors and stimulated for 36 hours with 6 μ M SA-2 or SA-1, and the amounts of IL-12, TNF- α , IL-6, and IL-10 were measured by using ELISAs in the supernatants. As positive controls, LPS (1 μ g/mL) and R-848 (6 μ M) were used. SA-2, but not SA-1, stimulated the production of regulatory (IL-12 and IL-10) and inflammatory (TNF- α and IL-6) cytokines to a similar extent as the TLR4 ligand LPS, whereas R-848 was unable to induce substantial IL-10 production (Fig 2, upper table).

Second, highly purified circulating BDCA-4⁺ cells from 11 healthy donors were cultured for 36 hours in the presence of 6 μ M R-848, SA-2, or SA-1. LPS was used as a negative control because PDCs lack TLR4 expression.¹⁷ Both SA-2 and R-848 were able to induce IFN- α and CXCL10 production (Fig 2, lower table), whereas SA-1 (data not shown) and LPS did not exert any detectable effect. The expression of IFN- α and CXCL10 mRNA was analyzed by using quantitative real-time RT-PCR after 30, 60, and 120 minutes to further confirm these results. Both R-848 and SA-2 (all at 6 μ M) potently upregulated IFN- α and CXCL10 expression (Fig 2, right panel), with mRNA peak at 120 minutes. No expression of mRNA for IFN- α or CXCL10 was observed when 6 μ M SA-1 was used (data not shown).

SA-2 induces the activation of NF- κ B and interacts with TLR7

Because the regulation of cytokine gene transcription signaled by inflammatory receptors, including TLRs, is affected by transcriptional activators, such as NF- κ B, we asked whether SA-2 might target NF- κ B. Thus highly purified CD14⁺ cells from 2 healthy donors were stimulated for 30 and 60 minutes with 6 μ M SA-2, SA-1, or R-848, and p50 and p65 subunits were measured by means of ELISA in nuclear extracts. Rapid and strong activation of both p50 and p65 subunits was observed with either SA-2 or R-848, at higher levels than in the positive control (Raji cells), indicating that both compounds activate the NF- κ B signaling pathway (see Fig E1 in the Online Repository at www.jacionline.org).

TLR-transfected HEK293 cells with or without a reporter gene were used to test whether SA-2 was recognized by any TLR. First, cells were transiently cotransfected by means of nucleofection with purified plasmids encoding for human TLR3, TLR7, TLR8, or TLR9 and endothelial cell-leukocyte adhesion molecule-1 (ELAM-1) promoter NF- κ B luciferase reporter plasmid; incubated for 18 hours; and stimulated for an additional 18 hours in the absence or presence of 1.3 μ M ODN 2006, 50 μ g/mL polyinosinic-polycytidylic acid (poly[I:C]), or 6 μ M SA-2, SA-1, or R-848. As shown in Fig 3, upper panel, SA-2 was able to activate only TLR7-expressing HEK

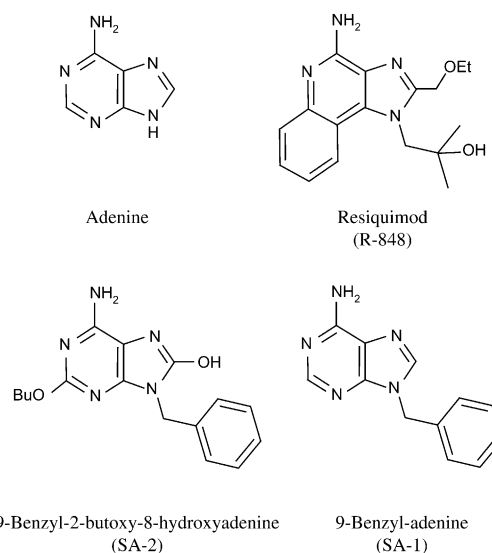


FIG 1. Chemical structure of adenine, imidazoquinoline, R-848, and modified adenines.

cells, whereas R-848 and phosphorothioate ODN 2006 induced NF- κ B expression in TLR7/TLR8- and TLR9-expressing cells, respectively, and poly(I:C) induced NF- κ B expression in TLR3-expressing HEK cells. SA-1 lacked the ability to stimulate any of the TLRs used, and no response was obtained in empty vector-transfected HEK cells (data not shown). Additionally, CXCL8 levels were measured in the supernatants of TLR3-, TLR7-, TLR8-, or TLR9-transfected HEK293 cells after 18 hours of stimulation with the same ligands (Fig 3, lower panel). SA-2 stimulated CXCL8 production in TLR7-transfected, but not in TLR3-, TLR8-, or TLR9-transfected, HEK cells, whereas poly(I:C), R-848, and ODN 2006 activated TLR3-, TLR7/TLR8-, and TLR9-expressing cells, respectively. No cytokine production could be detected (<5 pg/mL) in HEK293 cells cultured in medium alone (data not shown). Moreover, additional evidence that a subcellularly endosome-localized TLR was indeed activated after stimulation with substituted 8-hydroxy adenine was provided by means of preincubation of highly purified CD14⁺ cells with the acidification inhibitor chloroquine, which almost completely abolished TNF- α and IL-12 production induced by 6 μ M SA-2 (see Table E1 in the Online Repository at www.jacionline.org).

Finally, because the structural backbone of SA-2 is chemically related to adenine, the possibility that the active compound was able to activate G protein-coupled receptors, ion-channel nucleotide receptor, or both was also excluded. Actually, a significant increase in cytosolic calcium concentration was only observed in ATP- or ionomycin-stimulated, but not in SA-2- (or R-848-) stimulated, purified CD14⁺ cells (see Videos 1-3, respectively, and Fig E2 in the Online Repository at www.jacionline.org). Moreover, the G_{i/o} protein inhibitor pertussis toxin exerted no effects on SA-2-stimulated CD14⁺ cells (data not shown), whereas forskolin, but not SA-2, upregulated cyclic AMP levels in circulating monocytes (see

Condition	Cytokine production by CD14 ⁺ cells (ng/ml)			
	IL-12	TNF- α	IL-6	IL-10
Medium	0.01 $\pm$ 0.02	0.04 $\pm$ 0.01	2.14 $\pm$ 0.4	0.1 $\pm$ 0.03
LPS (1 μ g/ml)	0.9 $\pm$ 0.1*	0.7 $\pm$ 0.15 [#]	19.0 $\pm$ 1.9*	6.9 $\pm$ 1.0*
R-848 (6 μ M)	1.9 $\pm$ 0.2*	0.96 $\pm$ 0.1*	13.2 $\pm$ 1.3*	0.9 $\pm$ 0.5
SA-2 (6 μ M)	1.5 $\pm$ 0.2*	0.68 $\pm$ 0.16 [#]	17.3 $\pm$ 2.0*	2.3 $\pm$ 0.7 [§]
SA-1 (6 μ M)	0.05 $\pm$ 0.04	0.01 $\pm$ 0.01	3.7 $\pm$ 1.4	0.02 $\pm$ 0.01

Condition	Cytokine production by BDCA-4 ⁺ cells (pg/ml)	
	IFN- α	CXCL10
Medium	17 $\pm$ 5	181 $\pm$ 21
LPS (1 mg/ml)	57 $\pm$ 36	253 $\pm$ 48
R-848 (6 μ M)	2734 $\pm$ 259*	3481 $\pm$ 530*
SA-2 (6 μ M)	2732 $\pm$ 267*	2491 $\pm$ 629 [#]

FIG 2. SA-2 stimulates the production of cytokines and chemokines by the cells of the innate immunity. Mean concentrations ($\pm$ SE) of IL-12, TNF- α , IL-6, and IL-10 (in nanograms per milliliter) or IFN- α and CXCL10 (in picograms per milliliter) produced by purified CD14⁺ (upper table) or BDCA-4⁺ (lower table) cells from 11 healthy donors under the indicated conditions are shown. $P < .01$; $^{\#}P < .005$; $^*P < .0005$. Mean ($\pm$ SE) IFN- α and CXCL10 mRNA expressions, as detected by using real-time RT-PCR in BDCA-4⁺ cells from 7 healthy donors under the indicated conditions, are shown. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

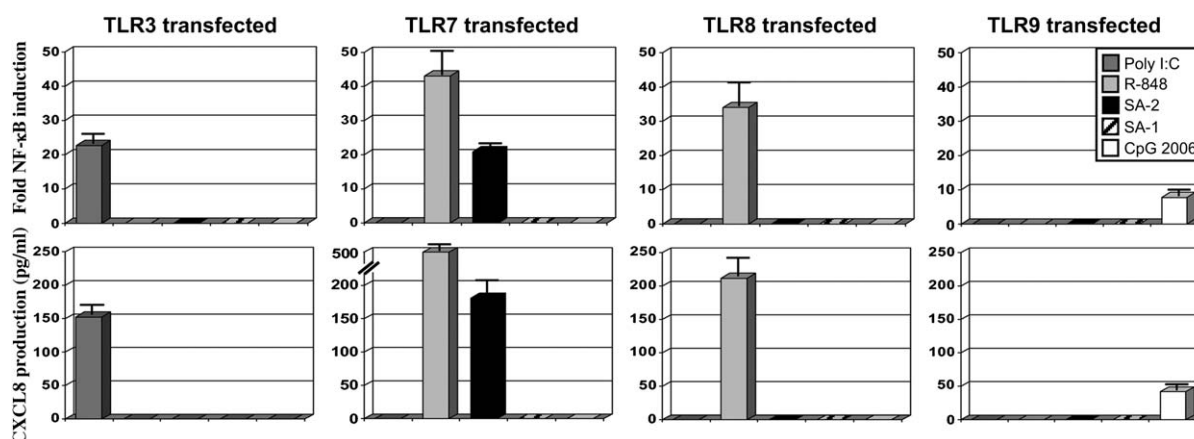
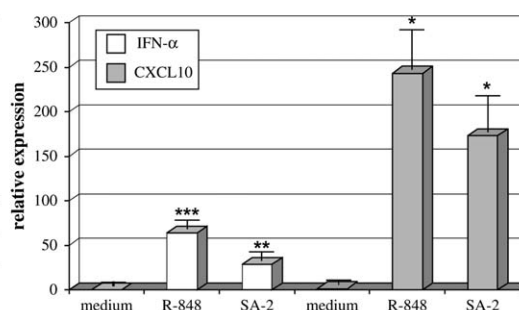


FIG 3. SA-2 ligation of TLR7. Fold increase of NF- κ B activation in HEK293 cells cotransfected by means of nucleofection with human TLR3, TLR7, TLR8, or TLR9 plasmid together with NF- κ B luciferase reporter plasmid and then stimulated under the indicated conditions is shown (upper panel). CXCL8 production (in picograms per milliliter) in human TLR3-, TLR7-, TLR8-, or TLR9-transfected HEK293 cells after stimulation with the indicated compounds is shown (mean $\pm$ SE of 3 different experiments; lower panel).

Table E2 in the Online Repository at www.jacionline.org). These data collectively indicate that active 8-hydroxy-modified adenines uniquely activate the NF- κ B-dependent receptor 7 of the Toll-like family.

SA-2 promotes the *in vitro* shifting of allergen- or hapten-specific T_H2 cells into T_H1/T_H0 effectors

Because SA-2 was able to induce the production of cytokines by innate cells, its ability to redirect the cytokine profile of allergen-specific T_H cells was then investigated. Thus amoxicillin- and Der p 1-specific TCLs were generated from 9 β -lactam- and 5 Der p 1-sensitive donors, respectively, in the presence of medium alone or 6 μ M SA-2, SA-1, or R-848 as a positive control. On day 14,

IL-4 and IFN- γ production by T-cell blasts was evaluated by means of flow cytometry after polyclonal stimulation with PMA and ionomycin. As shown in Fig 4, upper left, either SA-2- or R-848-conditioned Der p 1-specific TCLs showed a significant increase in the proportion of cells expressing IFN- γ ($P < .01$) and a parallel reduction in the proportion of IL-4-expressing T cells ($P < .01$) in comparison with unconditioned cultures. SA-1 did not exhibit any effect (data not shown). Similar and even more profound effects were obtained in amoxicillin-specific TCLs, with the SA-2-induced T_H1 shifting being comparable with that of R-848 (Fig 4, upper right). SA-2 or R-848 did not significantly modify the proportions of cells able to produce both IFN- γ and IL-4 (T_H0 cells) in either allergen- or hapten-stimulated TCLs (data not shown). The addition of a mixture of anti-IL-12 and IFN (α and γ)-neutralizing mAbs reverted the

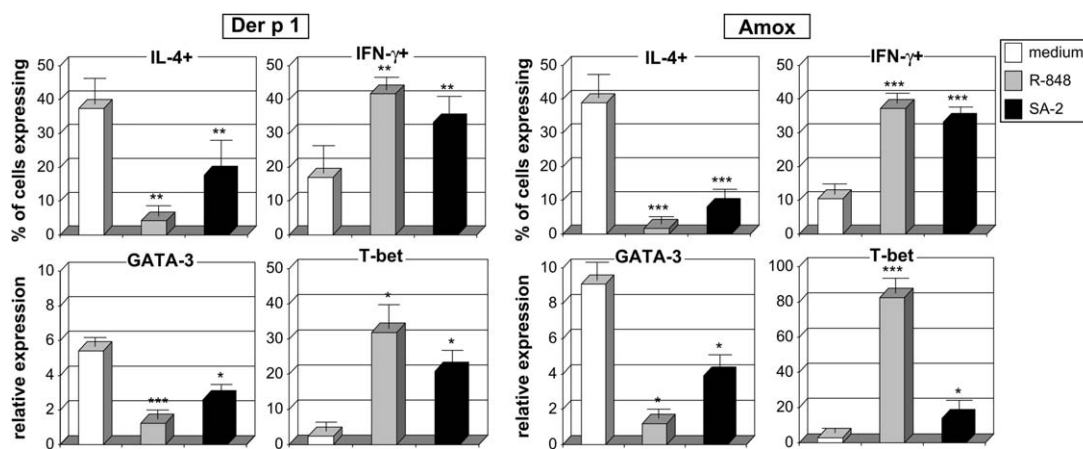


FIG 4. SA-2 downregulates IL-4 production and GATA-3 expression, upregulating IFN- γ and T-bet in allergen- or hapten-specific T cells. Intracellular content (mean percentage $\pm$ SE) of IL-4 and IFN- γ in 5 Der p 1- and 9 amoxicillin- specific T cells derived in the absence (medium) or presence of 6 μ M R-848 or SA-2 after polyclonal stimulation with PMA and ionomycin is shown (* P < .01; *** P < .0001; upper panels). Unstimulated T cells from the same donors were evaluated for GATA-3 and T-bet mRNA expression (mean $\pm$ SE) by using real-time quantitative RT-PCR (lower panels). * P < .05; ** P < .01; *** P < .005.

T_H1-shifting effect of SA-2 (data not shown). Analogous T_H1-modulatory effects were exerted by 8-hydroxy-adenine SA-2 when allergen- and hapten-specific T cells were stimulated by the use of Der p 1 and amoxicillin, respectively, in the presence of autologous monocyte-derived DCs as antigen-presenting cells (see Fig E3 in the Online Repository at www.jacionline.org).

T blasts from Der p 1- and amoxicillin-specific T cells from unconditioned (medium alone) or conditioned (R-848 or SA-2) T cells were also analyzed for the expression of the T_H2- and T_H1-associated transcription factors GATA-3 and T-bet to provide additional evidence for the SA-2-induced T_H1 shift.¹⁸ As expected, T blasts from unconditioned cultures expressed high levels of GATA-3 with low or undetectable levels of T-bet. Conversely, SA-2- (or R-848-) conditioned T cells exhibited a significant downregulation of GATA-3 with a parallel increase in the expression of T-bet (Fig 4, lower panels).

Finally, amoxicillin-specific T-cell clones from one drug-sensitive donor were derived from unconditioned or conditioned (SA-2 or R-848, as control) T cells¹¹ and assessed for their ability to release IL-4 and IFN- γ in the supernatants after PMA plus anti-CD3 mAb polyclonal stimulation (Fig 5) to definitively demonstrate that allergen-specific T cells were the target of the SA-2-induced T_H1 shifting effect. A total of 24 clones were derived from the unconditioned T cells, 36 from the SA-2-conditioned T cells, and 27 from the R-848-conditioned T cells. The cloning efficiency was similar under the 3 experimental conditions (data not shown). Virtually all amoxicillin-specific T-cell clones derived in the absence of any type of conditioning produced IL-4 alone (8 clones, 33%) or IL-4 plus IFN- γ (15 clones, 63%), thus exhibiting a T_H2 or T_H0 phenotype. Conversely, when SA-2 was present from the beginning of culture, only one clone producing IL-4 alone was derived (3%), whereas 13 T_H1 (36%) and 20 T_H0

(55%) clones were obtained. Comparable results were obtained from R-848-conditioned cultures.

DISCUSSION

Although the mechanisms responsible for successful immunotherapy in allergic subjects are still matter of debate, they certainly account for the central role of allergen-specific T_H2 cells. Both inhibition of antigen presentation and, more recently, suppression of T_H responses by regulatory cells have been suggested, but most investigations addressed immune deviation from T_H2 cells toward a less pathogenic T_H1 phenotype.¹⁹ Actually, CD4⁺ T cells from nonatopic subjects produce IFN- γ and little or no IL-4 in response to common environmental allergens or foods.^{20,21} Even if recombinant, modified, or engineered allergens have been proposed for safer treatments, the most promising strategies seem to be based on the use of novel adjuvants potentiating allergen vaccines to induce immune deviation, and several compounds have been tested over the years that can be incorporated with, or conjugated to, allergens to enhance the immune response of allergic subjects.²²⁻²⁴

TLRs are a group of newly discovered receptors regulating pathogen-related responses constitutively expressed in different cell types both extracellularly and intracellularly (TLR3, TLR7, TLR8, and TLR9).²⁵ They are differently coupled to Toll/IL-1 receptor (TIR) domain adapters, mostly MyD88, activating NF- κ B transcription factors and resulting in the production of proinflammatory cytokines, chemokines, type I IFNs, and DC maturation.¹ DCs express a wide spectrum of TLRs on the basis of their myeloid or lymphoid origin.¹⁷ Because DCs play a critical role in IL-12 production that is a key product in innate immunity and drives T_H1 polarization,²⁶ natural^{4,5} and

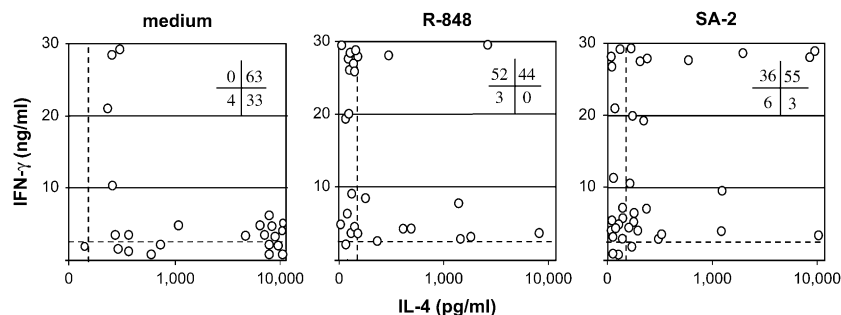


FIG 5. SA-2 shifts the cytokine profile of amoxicillin-specific T cells toward the T_H1 profile. IL-4 and IFN- γ amounts in supernatants from polyclonally stimulated, amoxicillin-specific T-cell clones generated from TCLs under the indicated conditions from one drug-sensitive donor are shown. Dotted lines, Mean cytokine concentrations (± 5 SD) produced by irradiated PMA plus anti-CD3-stimulated feeder cells. Values inside the figures, Percentages of clones exhibiting a T_H1 (upper left), T_H0 (upper right), or T_H2 (lower right) phenotype.

synthetic^{3,7,11} TLR ligands exert powerful immunostimulatory and modulatory effects. Recently, a number of low-molecular-weight compounds belonging to heterocycles, such as adenosine, guanine nucleoside analogues, and imidazoquinolines, have been found to affect both humoral and cellular immune responses.^{7,11} Whereas adenosine and adenine nucleotides are potent NF- κ B inhibitors^{27,28} and induce distorted DC maturation and T_H1 inhibition through P1 and P2 receptors,²⁹ the imidazoquinolines imiquimod and, more strongly, R-848 upregulate IL-12, IL-6, TNF- α , and IFN- α production through TLR7 or TLR8, respectively.^{1,13} Similarly, TLR7 activation is responsible for the immunostimulatory effects exerted by substituted guanines.⁷ Very recently, the synthesis of a series of 8-hydroxyadenines as novel *in vitro* and *in vivo* IFN-inducing agents in experimental animals has been reported.¹⁶ Oral administration in BALB/c mice induced rapid increase of IFN- α levels in plasma with maximal effects in 2- and 9-substituted compounds.³⁰ However, the effects of these compounds on human cells have not been addressed thus far.

The results of this study provide the first evidence that a synthetic heterocycle related to adenine with substitution in position 2- (butoxy), 8- (hydroxy), and 9- (benzyl), termed SA-2, was able to stimulate the production of high amounts of cytokines and chemokines by circulating monocytes (IL-6, TNF- α , IL-10, and IL-12) and PDCs (IFN- α and CXCL10), whereas 9-benzyl SA-1 had no effects, indicating the critical role of substitutions in position 2- and 8-. These effects were mediated by the activation of the NF- κ B signaling pathway, inasmuch as both p50 and p65 subunits were increased at higher levels than Raji cells, where nuclear factors are constitutively active.³¹ Because the regulation of cytokine gene transcription signaled by TLRs involves NF- κ B transcriptional activators,¹ the possibility that SA-2 exerted its effects through the activation of one or more TLRs expressed in monocytes and PDCs was explored. Transfection experiments demonstrated that SA-2 was able to activate TLR7, but not TLR3, TLR8, or TLR9, as shown by NF- κ B-dependent luciferase activity increase and

CXCL8 production. Moreover, no increase in intracellular Ca^{++} levels, cyclic AMP levels, or both were observed. Thus the results of this study identify the modified adenine SA-2 as a new TLR7-specific ligand able to induce the production of both suppressive (IL-10) and T_H1 -polarizing (IL-12 and IFN- α) cytokines in monocytes and PDCs in addition to IL-6 and TNF- α . Moreover, SA-2 induced CXCL10, which promotes IFN- γ production in T cells, contributing to T_H1 polarization of the human adaptive immune response.³² Thus we asked whether SA-2 might be responsible for suppression or redirection of specific T_H2 responses. Independently of the allergen used (Der p 1 or amoxicillin), our results clearly showed that despite its ability to induce the production of IL-10, SA-2 did not suppress the differentiation of allergen- or hapten-specific CD4⁺ cells but rather shifted T_H2 effectors to a T_H0/T_H1 phenotype promoting the IL-12 and IFN production. These findings agree with those of previous *in vitro* studies demonstrating that several TLR ligands mimicking the effects of microbial components (dsRNA, CpG-ODNs, and imidazoquinolines) stimulate the production of T_H1 -polarizing cytokines, therefore modulating the allergen-specific responses by immune deviation rather than immune suppression.^{10,11,13} These findings also support recent *in vivo* results in both mice and human subjects.^{14,15,33,34} First, administration of CpG-ODNs during (or after) the sensitization phase could effectively and lastingly reduce allergic asthma because mice rechallenged with allergens continued to generate strong T_H1 -oriented (instead of T_H2) memory responses protecting against lung inflammation.³³ Second, colonization of germ-free animals with *Bacteroides fragilis* corrects systemic T-cell deficiency and T_H1/T_H2 imbalance through IL-12-induced IFN- γ production by bacterial polysaccharide.³⁴ Finally, specific immunotherapy with a CpG-ODN-Amb a 1 conjugate resulted in T_H1 redirection of the allergen-driven response in rhinitic patients, with rapid and persistent downregulation of T_H2 cytokines and chemokines (IL-5, CCL17, and CCL22) and increase in IFN- γ , CXCL9, and CXCL10.¹⁴ In a second independent trial repeated weekly injections of CpG-ODN-Amb a 1 conjugate reduced

eosinophils and IL-4 mRNA⁺ cells, increased the number of IFN- γ -producing cells in nasal biopsy specimens, and decreased chest and nasal symptoms.¹⁵ These studies demonstrate that the covalent linkage of immunostimulatory ODNs to allergens can represent a safe and well-tolerated strategy to promote immune deviation of allergen-specific T_H2 responses, even if long-term side effects, especially in the case of systemic administration, cannot yet be excluded. CpG-ODNs indeed induce substantial B-cell proliferation and differentiation *in vitro* and are responsible for activation of B-cell compartment, including autoantibody production in experimental animals.³⁵

The results of this study provide the first *in vitro* evidence that a novel synthetic heterocycle chemically related to adenine exhibits similar immunostimulatory activities as CpG-ODNs on human cells. Although the maintenance of the T_H1-skewing effects by substituted adenine conjugated to antigen-allergen molecules still needs to be explored, the limited effects on B cells (data not shown) and the described IFN- α induction after oral administration in mice suggest that SA-2 might represent an efficient and safe adjuvant in vaccination protocols for the treatment of T_H2-mediated diseases or other disorders in which stimulation of IFN- γ -mediated protective responses are hopeful.

REFERENCES

- Kawai T, Akira S. Toll-like receptor downstream signalling. *Arthritis Res Ther* 2005;7:12-9.
- Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of Toll-like receptor-mediated immune responses. *Nat Rev Immunol* 2005;5:446-58.
- Hornung V, Guenther-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, et al. Sequence-specific potent induction of IFN- α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med* 2005;11:263-73.
- Kandimalla ER, Bhagat L, Li Y, Yu D, Wang D, Cong YP, et al. Immunomodulatory oligonucleotides containing a cytosine-phosphate-2'-deoxy-7-deazaguanosine motif as potent toll-like receptor 9 agonists. *Proc Natl Acad Sci U S A* 2005;102:6925-30.
- Sugiyama T, Gursel M, Takeshita F, Coban C, Conover J, Kaisho T, et al. CpG RNA: identification of novel single-stranded RNA that stimulates human CD14⁺CD11c⁺ monocytes. *J Immunol* 2005;174:2273-9.
- Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004;303:1529-31.
- Lee J, Chuang T-H, Redecke V, Liping S, Pitha PM, Carson DA, et al. Molecular basis for the immunostimulatory activity of guanine nucleoside analogues: activation of Toll-Like Receptor 7. *Proc Natl Acad Sci USA* 2003;100:6646-51.
- Romagnani S. The role of lymphocytes in allergic disease. *J Allergy Clin Immunol* 2000;105:399-408.
- Parronchi P, Maggi E, Romagnani S. Redirecting T_H2 responses in allergy. *Curr Top Microbiol Immunol* 1999;238:27-56.
- Manetti R, Annunziato F, Tomasevic L, Giannò V, Parronchi P, Romagnani S, et al. Poly-inosinic acid: poly-cytidylic acid promotes T helper type 1-specific immune responses by stimulating macrophage production of IFN- α and interleukin-12. *Eur J Immunol* 1995;25:2656-60.
- Parronchi P, Brugnolo F, Annunziato F, Manuelli C, Sampognaro S, Mavilia C, et al. Phosphorothioate oligodeoxynucleotides promote the *in vitro* development of human allergen-specific CD4⁺ T cells into T_H1 effectors. *J Immunol* 1999;163:5946-53.
- Annunziato F, Cosmi L, Manetti R, Brugnolo F, Parronchi P, Maggi E, et al. Reversal of human allergen-specific CRTH2 T_H2 cells by IL-12 or the PS-DSP30 oligodeoxynucleotide. *J Allergy Clin Immunol* 2001;108:815-21.
- Brugnolo F, Sampognaro S, Liotta F, Cosmi L, Annunziato F, Manuelli C, et al. The novel synthetic immune response modifier R848 (resiquimod) shifts human allergen-specific CD4⁺ T_H2 lymphocytes into IFN- γ -producing cells. *J Allergy Clin Immunol* 2003;111:380-8.
- Estelle F, Simons R, Shikishima Y, Van Nest G, Eiden JJ, HayGlass KT. Selective immune redirection in humans with ragweed allergy by injecting Amb a 1 linked to immunostimulatory DNA. *J Allergy Clin Immunol* 2004;113:1144-51.
- Tulic MT, Fiset P-O, Christodoulopoulos P, Vaillancourt P, Desrosiers M, Lavigne F, et al. Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy decreases the nasal inflammatory response. *J Allergy Clin Immunol* 2004;113:235-41.
- Hirota K, Kazaoka K, Niimoto I, Kumihara H, Sajiki H, Isobe Y, et al. Discovery of 8-hydroxyadenines as a novel type of interferon inducer. *J Med Chem* 2002;45:5419-22.
- Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kasteleij RA, Bazan E, et al. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J Exp Med* 2001;194:863-9.
- Rangarajan J, Szabo SJ, Glimcher LH. Transcriptional regulation of T_H1/T_H2 polarization. *Immunol Today* 2000;21:479-83.
- Till SJ, Francis JN, Nouri-Aria K, Durham SR. Mechanisms of immunotherapy. *J Allergy Clin Immunol* 2004;113:1025-34.
- Turcanu V, Maleki SJ, Lack G. Characterization of lymphocyte responses to peanuts in normal children, peanut-allergic children, and allergic children who acquired tolerance to peanuts. *J Clin Invest* 2003;111:1065-72.
- Gangur V, Simons FER, HayGlass KT. IP-10 mediated reinforcement of human type 1 cytokine synthesis to environmental allergens among non-atopic subjects. *Int Arch Allergy Immunol* 1999;118:387-90.
- Francis JN, Durham SR. Adjuvants for allergen immunotherapy: experimental results and clinical perspectives. *Curr Opin Allergy Clin Immunol* 2004;4:543-8.
- Tighe H, Takabayashi K, Schwartz D, Van Nest G, Tuck S, Eiden JJ, et al. Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol* 2000;106:124-34.
- Freytag LC, Clements JD. Mucosal adjuvants. *Vaccine* 2005;1804-13.
- Kawai T, Akira S. Pathogen recognition with Toll-like receptors. *Curr Opin Immunol* 2005;17:338-44.
- Manetti R, Gerosa F, Giudizi MG, Biagiotti R, Parronchi P, Piccinini M-P, et al. Interleukin 12 induces stable priming for interferon γ (IFN- γ) production during differentiation of human T helper (T_H) cells and transient IFN- γ production in established T_H2 cell clones. *J Exp Med* 1994;179:1273-83.
- Minguet S, Huber M, Rosenkranz L, Schamel WA, Reth M, Brummer T. Adenosine and cAMP are potent inhibitors of the NF- κ B pathway downstream of immunoreceptors. *Eur J Immunol* 2005;35:31-41.
- Haskò G, Cronstein BN. Adenosine: an endogenous regulator of innate immunity. *Trends Immunol* 2004;25:33-9.
- La Sala A, Ferrari D, Corinti S, Cavani A, Di Virgilio F, Girolomoni G. Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate T_H1 responses. *J Immunol* 2001;166:1611-7.
- Kurimoto A, Ogino T, Ichii S, Isobe Y, Tobe M, Ogita H, et al. Synthesis and structure-activity relationships of 2-amino-8-hydroxyadenines as orally active interferon inducing agents. *Bioorg Med Chem* 2003;11:5501-8.
- Renard P, Ernest P, Houbion A, Art M, Le Calvez H, Raes M, et al. Development of a sensitive multi-well colorimetric assay for active NF- κ B. *Nucleic Acids Res* 2001;29:e21.
- Romagnani P, Maggi L, Mazzinghi B, Cosmi L, Lasagni L, Liotta F, et al. CXCR3-mediated opposite effects of CXCL10 and CXCL4 on type 1 or 2 T helper cytokine production. *J Allergy Clin Immunol* 2005;116:1392-9.
- Banerjee B, Kelly KJ, Fink JN, Henderson JD Jr, Bansal NK, Kurup VP. Modulation of airway inflammation by immunostimulatory CpG oligonucleotides in a murine model of allergic aspergillosis. *Infect Immun* 2004;72:6087-94.
- Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 2005;122:107-18.
- Vigilanti GA, Lau CM, Hanley TM, Miko BA, Schlomchik MJ, Marshak-Rothstein A. Activation of autoreactive B cells by CpG dsDNA. *Immunity* 2003;19:837-47.

METHODS

Reagents

Amoxicillin, LPS (from *Escherichia coli* 0111:B4), and adenine were from Sigma (Sigma Chemical Co, St Louis, Mo). Purified Der p 1 allergen was kindly provided by Lofarma Allergeni (Milan, Italy). Poly(I:C), chloroquine, PMA, ionomycin, brefeldin A, and saponin were from Sigma. Pertussin toxin was from Calbiochem (Darmstadt, Germany). Recombinant IL-2 was from Chiron Corp (Emeryville, Calif). Purified and fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD123, anti-DR, and anti-CD20 mAbs, as well as phycoerythrin-conjugated anti-IL-4 (3010.211, IgG1) and fluorescein isothiocyanate-conjugated anti-IFN- γ (25723.11, IgG2b) mAbs, were purchased from Becton Dickinson (San Jose, Calif). Purified and fluorochrome-conjugated isotype control mAbs were purchased from Southern Biotechnology Associates (Birmingham, Ala). Anti-BDCA2 mAb was from Miltenyi Biotech (Bergisch-Gladbach, Germany). R-848 (S28463) and phosphorothioate ODN 2006 (5'-TCG TCG TTT TGT CGT TTT GTC GTT-3') were purchased from InvivoGen (San Diego, Calif).

Endotoxin levels in R-848, modified adenines, adenine, Der p 1, amoxicillin, and ODN 2006 were less than 0.003 EU/mL, as measured with the Lymulus Amoebocyte Assay (Bio Whittaker, Walkersville, Md).

Synthesis of modified adenines

The synthesis of modified adenines SA-2 and SA-1 was performed as previously described.^{E1} In brief, triphosgene (6.58 mM) was dissolved in 20 mL of dry tetrahydrofuran (THF) and a freshly distilled solution of benzylamine (19.74 mM), and dry N,N-diisopropylethylamine (31.59 mmol) in 40 mL of dry THF was added dropwise at -80°C over 1 hour. A solution of aminomalononitrile *p*-toluenesulfonate (19.74 mM) and N,N-diisopropylethylamine (15.79 mM) in 20 mL of dry THF was added to the mixture dropwise at room temperature over 2 hours. The resulting solution was stirred at room temperature for 24 hours. The solvent was removed under vacuum pressure, and the collected residue was dissolved in a mixture of 200 mL of ethyl acetate (AcOEt) and 100 mL of distilled water. The organic layer was washed with 100 mL of brine and extracted with 2×100 mL of 1N NaOH solution. The basic aqueous phases collected were neutralized with 10% NaHSO₄ solution and extracted with 200 mL of AcOEt. The organic layer was washed with 100 mL of brine and dried over Na₂SO₄. The solvent was removed in a vacuum, and the solid obtained was triturated with 20 mL diethyl ether (Et₂O) and then filtered to produce 5-amino-1-benzyl-4-cyano-2-hydroxyimidazole. The spectroscopic data were in agreement with the reported values. A mixture of 5-amino-1-benzyl-4-cyano-2-hydroxyimidazole (9.34 mM) and urea (93.42 mM) was heated neat at 180°C for 2 hours. The residue was triturated with 30 mL of distilled water, and the resulting precipitate was filtered to produce 9-benzyl-2,8-dihydroxyadenine. The spectroscopic data were in agreement with the reported values. A suspension of lithium hydride (LiH) (2.64 mM) and 9-benzyl-2,8-dihydroxyadenine (1.31 mM) in 1.32 mL of dry dimethyl formamide (DMF) was stirred for 2 hours under argon, and then a solution of butyl bromide (BuBr) (2.64 mM) in 2.64 mL of dry DMF was added, and the mixture was left under stirring under argon. After 2 days, the reaction was quenched with 5 mL of distilled water, and the mixture was neutralized with 10% NaHSO₄ solution and extracted with 3×10 mL AcOEt. The collected organic layer was dried with Na₂SO₄ filtered, and the solvent was removed under vacuum. The remaining oil was triturated in Et₂O and then filtered and crystallized in MeOH/AcOEt 1:1. The solid was purified by

means of flash column chromatography (AcOEt 1% NH₃) to afford SA-2. The spectroscopic data were in agreement with the reported values.

Purification of monocytes and PDCs

Highly purified monocytes were positively selected with anti-CD14 microbeads and the MACS technique (Miltenyi) from PBMCs of 11 healthy subjects. Purity of cell populations was assessed by means of FACS analysis and was always greater than 98%. CD14⁺ cells, $1 \times 10^6/\text{mL}$, were cultured in 24-well flat-bottomed plates (Corning) in complete medium supplemented with 10% heat-inactivated FCS (HyClone, Logan, Utah) in the absence or presence of R-848, modified adenines (all at 6 μM), or LPS (1 $\mu\text{g}/\text{mL}$) for 36 hours. At the end of the culture, supernatants were collected, centrifuged, and stored at -20°C until tested. The amounts of IL-12 (Cytoscreen; Biosource Int, Camarillo, Calif), IFN- α (Cytoscreen), TNF- α (Cytoscreen), IL-10 (BD PharMingen, San Diego, Calif), and IL-6 (R&D System, Minneapolis, Minn) were then evaluated by using commercial kits or homemade ELISAs.

Circulating PDCs were purified from PBMCs of 11 healthy donors by means of MACS (Miltenyi) with the anti-BDCA-4 isolation kit (Miltenyi), according to the manufacturer's recommendations. Purity of the cell populations was assessed by means of FACS analysis and was always greater than 70%. BDCA-4⁺ cells, $1 \times 10^6/\text{mL}$, were cultured for 36 hours in the absence or presence of R-848, modified adenines (all at 6 μM), or LPS (1 $\mu\text{g}/\text{mL}$), and supernatants were collected, centrifuged, and stored at -20°C . IFN- α (Cytoscreen) and CXCL10 (R&D) concentrations were measured by using commercial ELISAs. In the same experiments $2 \times 10^6/\text{mL}$ purified BDCA-4⁺ cells were also stimulated for 30, 60, and 120 minutes in the absence or presence of R-848, modified adenines (all at 6 μM), or LPS (1 $\mu\text{g}/\text{mL}$), and total mRNA was extracted and assessed in quantitative real-time RT-PCR for IFN- α and CXCL10 expression, as described below.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted by using the RNeasy kit (Qiagen, Hilden, Germany) and treated with DNase I (Qiagen) to eliminate possible genomic DNA contamination. cDNA from each sample was synthesized from 1 μg of total RNA with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, Calif), according to the manufacturer's protocol. Real-time PCR was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. All PCR amplifications were performed on MicroAmp optical 96-well reaction plate with Taqman Universal Master Mix and with Assay on Demand (Applied Biosystems). Each assay was carried out in duplicate and included a no-template sample as a negative control. RT-negative samples were used to demonstrate that the signals obtained were RT dependent. Relative expression of mRNA levels was determined, comparing experimental levels with a standard curve generated with serial dilution of cDNA obtained from human PBMCs. β -Actin was used as a housekeeping gene for normalization.

Quantitative analysis of NF- κ B translocation

Nuclear translocation of transcription factors was quantified in nuclear extracts from highly purified CD14⁺ cells by using TransAM ELISA-based kits specific for human-activated NF- κ B (Active Motif, Carlsbad, Calif), according to the manufacturer's protocols. Briefly, $5 \times 10^6/\text{mL}$ CD14⁺ cells from 2 healthy donors were cultured in 15-mL polypropylene tubes in 2 mL of complete medium supplemented with 10% heat-inactivated FCS (HyClone) in the absence

or presence of R-848 or the modified adenines SA-2 or SA-1 (all at 6 μ M) for 30, 60, or 120 minutes. At the end of the culture, cells were extensively washed with PBS solution, placed in pellets, and stored at -80°C until used. Nuclear extracts were obtained by using the Nuclear Extract kit (Active Motif), and 0.5 μ g (p50) or 2 μ g (p65) was plated on 96-well plates coated with the immobilized oligonucleotide containing the activated NF- κ B consensus site (5'-GGGACTTTCC-3')^{E2} followed by the incubation with an antibody recognizing an epitope on p50 or p65 subunits. A horseradish peroxidase-conjugated secondary antibody provided colorimetric read out that was quantified by means of spectrophotometry. Raji nuclear extract was used as a positive control, as suggested by the manufacturer. To monitor the specificity of the assay, a wild-type consensus oligonucleotide was used as a competitor for NF- κ B binding.

Stimulation of TLR-transfected HEK293 cells and reporter assay

HEK293 cells (from ATCC collection) were transiently transfected by means of nucleofection (Amaxa GmbH, Köln, Germany) with TLR3, TLR7, TLR8, or TLR9 plasmids (InvivoGen; 5 μ g of plasmid/ 1×10^6 cells/transfection) purified with EndoFree Plasmid Maxi Kit (Qiagen). pmaxGFP (Amaxa) was used as the transfection efficiency control. After transfection, 1×10^5 /mL cells were plated in 48-well flat-bottomed plates in E-MEM medium (Gibco) supplemented with 5% FCS for 18 hours and then stimulated with or without poly(I:C) (50 μ g/mL), R-848, modified adenines SA-2 and SA-1 (6 μ M), or ODN 2006 (1.3 μ M) for a further 18 hours. At the end of the culture, supernatants were collected, stored at -20° until used, and assessed for their CXCL8 content by using a homemade ELISA with commercial pairs of mAbs (BD PharMingen).

In the same experiments HEK293 cells were transiently cotransfected by means of nucleofection with TLR3, TLR7, TLR8, or TLR9 plasmids (InvivoGen) and the ELAM-1 promoter NF- κ B luciferase reporter plasmid (pNiFty, InvivoGen; 10:1 for TLR7 and 1:1 for TLR3, TLR8, and TLR9) and plated in 48-well flat-bottomed plates at 1×10^5 /mL in E-MEM (Gibco) supplemented with 5% FCS (HyClone). Eighteen hours after transfection, cells were stimulated with or without poly(I:C) (50 μ g/mL), R-848, modified adenines SA-2 and SA-1 (6 μ M), or CpG-ODN 2006 (1.3 μ M) for a further 18 hours. At the end of the culture, luciferase activity was determined in cell lysates by using a luciferase Assay System (Promega, Madison, Wis).

Determination of Ca^{++} influx

Highly purified CD14⁺ cells, 5×10^5 /mL, obtained from the peripheral blood of 2 healthy donors by using the MACS technique were resuspended in complete medium, seeded on L-polylysine-treated chamber slides, and then placed in a 5% CO_2 humidified atmosphere at 37°C for 40 minutes to favor cell adhesion. Adherent cells were washed twice in complete medium, incubated in the presence of 2.5 μ M fluo-4 AM (Molecular Probes, Eugene, Ore) for 30 minutes in the dark at room temperature, washed twice in complete medium, and finally incubated at 37°C in a 5% CO_2 humidified atmosphere for an additional 30 minutes.

The experiment was performed at 37°C in serum-free medium, and the cells were analyzed by using a LSM 510 META Zeiss Confocal Microscope system (Carl Zeiss Inc, Jena, Germany) with a $\times 40$ oil immersion lens. For image analysis, a Confocor 2 (Zeiss) software was used. A krypton-argon laser (488 nm wave length) was used to excite fluo-4; the emission light was selected by using a band pass filter (500–550 nm). For each experiment, at least 70 consecutive pictures were acquired (one picture/2.3 seconds). The size of every picture was 512×512 pixels, and each field dimension was 325.8×352.8 μ m.

The basal fluorescence intensity was assessed on the average of at least 10 images acquired before stimulation and at least 15 images acquired after each stimulation. The stimulation was obtained in the presence of medium alone (negative control) or in the presence of 6 μ M SA-2 or R-848 and 1 μ M ionomycin or 250 μ M ATP (as positive controls).

Inhibition of $\text{G}_{i/o}$ -coupled receptor signaling and cyclic AMP measurement

MACS-purified CD14⁺ cells, 1×10^6 /mL, from the peripheral blood of 2 healthy donors were cultured in the absence or presence of 6 μ M SA-2 or R-848 with or without 1 μ g/mL pertussis toxin for 36 hours. At the end of the culture, supernatants were collected, and IL-12 and TNF- α levels were determined by using ELISAs, as already described.

For cyclic AMP determination, 1×10^6 CD14⁺ cells from 2 healthy donors were stimulated for 30, 60, and 120 minutes in the absence or presence of 6 μ M SA-2 or R-848 and 10 μ M forskolin as positive control. At the end of the culture, the cells were extensively washed and lysed by the use of appropriate buffer. Cyclic AMP levels (in picomoles per milliliter) were measured in supernatants collected after centrifugation by using a commercial ELISA (R&D Systems).

Generation of allergen- and hapten-specific TCLs and hapten-specific T-cell clones

PBMCs from 9 β -lactam-sensitive donors and 5 atopic subjects sensitized to *D. pteronyssinus* were separated by means of Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden) and stimulated with amoxicillin (500 μ g/mL) or Der p 1 (10 μ g/mL) in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 1 mM sodium pyruvate, 1 mM L-glutamine, and nonessential amino acids (all from Euroclone Ltd; complete medium) and 5% autologous serum for 6 days in the absence or presence of R-848 or modified adenines SA-2 or SA-1 (all at 6 μ M). Cells were then expanded with recombinant IL-2 (20 U/ml), and after 14 days, the specificity of TCLs was assessed.

T-cell blasts from amoxicillin-specific TCLs obtained from one sensitive donor were cloned under limiting dilution (0.3 cells per well), and the antigen specificity of T-cell clones was assessed as previously reported.^{E3}

Flow cytometric analysis of intracellular cytokine production

Surface molecules in T-cell blasts from TCLs or T-cell clones were detected by means of incubation for 20 minutes with anti-CD3, anti-CD4, and anti-CD8 or isotype control mAb. The cells were washed and analyzed by using a FACScalibur cytofluorimeter (Becton Dickinson), as previously described.^{E3} Intracytofluorometric analysis of IFN- γ and IL-4 synthesis at the single-cell level was performed as already described.^{E3} Briefly, 1×10^6 T-cell blasts from allergen- and hapten-specific T-cell lines were stimulated with PMA (10 ng/mL) plus ionomycin (1 μ M) for 6 hours, the last 2 in the presence of brefeldin A (5 μ g/mL). For antigen-specific stimulation, 1×10^6 T-cell blasts from TCLs were stimulated with autologous CD14-derived DCs in the absence or presence of 10 μ g/mL Der p 1 or 0.5 mg/mL amoxicillin for 8 hours, the last 6 in the presence of 5 μ g/mL brefeldin A. After incubation, cells were fixed with formaldehyde, permeabilized with 0.5% saponin, incubated with the specific mAb, and analyzed on a FACScalibur cytofluorimeter using the CellQuest software (Becton Dickinson). The area of positivity was determined by using an isotype-matched mAb. In all cytofluorometric analyses, a total of 10^4 events, gated as CD3⁺CD4⁺ cells, were acquired.

Expression of transcription factors by allergen- and hapten-specific T-cell lines

T-cell blasts, 2×10^6 , from 5 Der p 1- and 9 amoxicillin-specific TCLs were collected after the 15-day culture period, total mRNA was extracted, and GATA-3 and T-bet expression was evaluated by means of real-time PCR, as described above.

Measurement of IL-4, IL-5, and IFN- γ levels in T-cell line supernatants

Autologous monocyte-derived DCs were used as antigen-presenting cells to specifically stimulate allergen- and hapten-specific TCLs. Briefly, $3 \times 10^6/3$ mL of MACS-purified CD14⁺ cells obtained from the peripheral blood of one β -lactam-sensitive and one atopic subject were cultured in the presence of 20 ng/mL IL-4 and 50 ng/mL GM-CSF for 5 days. Floating immature DCs were cultured together with 10 μ g/mL Der p 1 or 0.5 mg/mL amoxicillin and 1×10^6 /mL T-cell blasts (ratio, 1:10) from unconditioned or SA-2 (or R-848)-

conditioned T-cell lines. Culture supernatants were collected after 72 hours and stored at -20°C until tested, and IL-4, IL-5, and IFN- γ levels were assessed by using homemade ELISAs with commercial pairs of mAbs (IL-4 and IL-5: BD PharMingen; IFN- γ : Endogen, Woburn, Mass), as previously described.^{E3}

REFERENCES

- E1. Hirota K, Kazaoka K, Niimoto I, Kumihara H, Sajiki H, Isobe Y, et al. Discovery of 8-hydroxyadenines as a novel type of interferon inducer. *J Med Chem* 2002;45:5419-22.
- E2. Renard P, Ernest P, Houbion A, Art M, Le Calvez H, Raes M, et al. Development a sensitive multi-well colorimetric assay for active NF- κ B. *Nucleic Acids Res* 2001;29:e21.
- E3. Brugnolo F, Sampognaro S, Liotta F, Cosmi L, Annunziato F, Manuelli C, et al. The novel synthetic immune response modifier R-848 (resiquimod) shifts human allergen-specific CD4⁺ T_H2 lymphocytes into IFN- γ -producing cells. *J Allergy Clin Immunol* 2003;111:380-8.

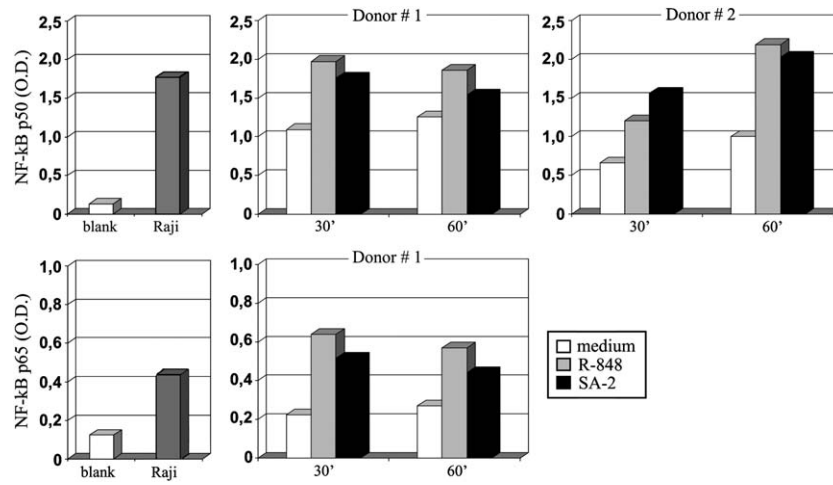


FIG E1. The effects of SA-2 are mediated by the activation of NF-κB. p50 and p65 concentrations were evaluated by using a specific ELISA in nuclear cell extracts from $2.5 \times 10^6/\text{mL}$ highly purified CD14⁺ cells obtained from 2 healthy donors and cultured under the indicated conditions for 30 and 60 minutes. As a positive control, the nuclear extract from Raji cells was used. Values are expressed as optical density (O.D.).

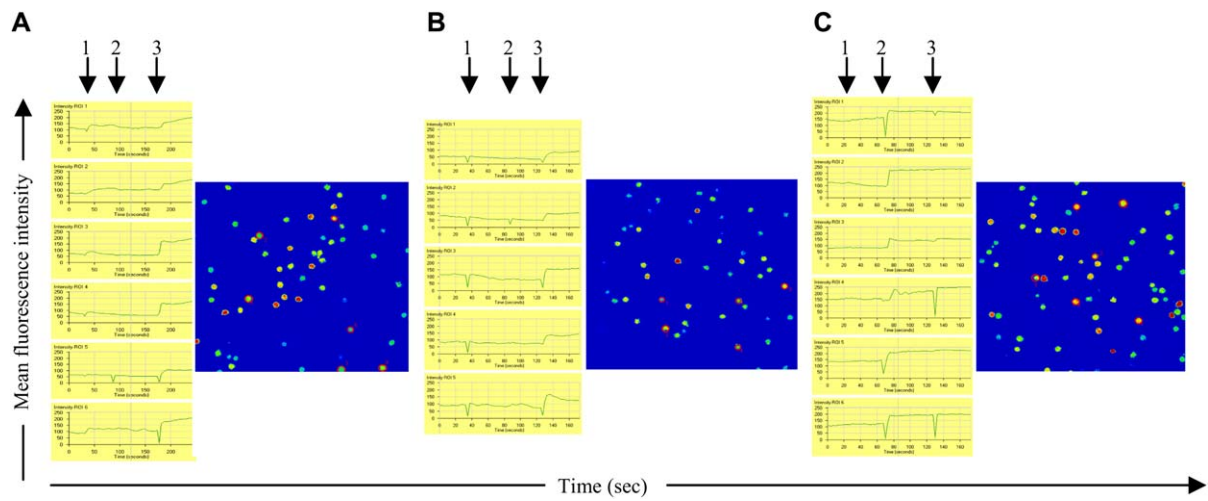


FIG E2. SA-2 does not modify intracellular Ca^{++} levels. Mean fluorescence intensity of fluo-4 AM-loaded, L-polylysine-treated, chamber slide-adherent CD14^{+} cells calculated after treatment with medium (negative control, *arrow 1*) followed by 6 μM R-848 (**A**, *arrow 2*), SA-2 (**B**, *arrow 2*), or 250 μM ATP (**C**, *arrow 2*) and on stimulation with 1 μM ionomycin (positive control, *arrow 3*) is shown. Each single graphic is relative to the mean fluorescence of one single cell randomly selected among those responsive to the positive control. One of 2 experiments is shown.

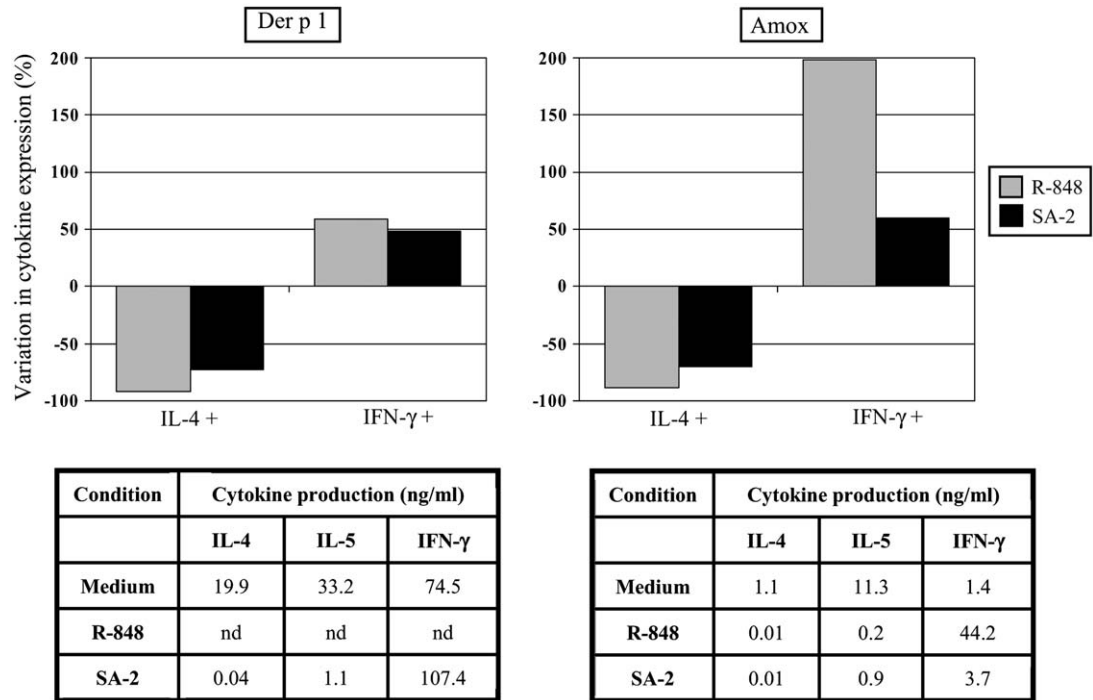


FIG E3. Modified adenine SA-2 downregulates IL-4 and upregulates IFN- γ production in allergen- or hapten-specific T-cell blasts. Intracellular content of IL-4 and IFN- γ in one allergen- and one hapten-specific T-cell line derived in the absence (*medium*) or presence of 6 μ M R-848 or SA-2 after specific stimulation with Der p 1 (10 μ g/mL) or amoxicillin (*Amox*; 0.5 mg/mL) in the presence of autologous monocyte-derived DCs as antigen-presenting cells is shown. Data are represented as percentage variation in cytokine expression (*upper panels*). IL-4, IL-5 and IFN- γ levels (in nanograms per milliliter) were also determined by using specific ELISAs in 72-hour culture supernatants derived from the same conditioned or unconditioned allergen- and hapten-specific T-cell lines after stimulation with Der p 1 or amoxicillin, respectively, in the presence of autologous monocyte-derived DCs, as described above (*lower tables*).

TABLE E1. Endosome acidification inhibitor chloroquine abolishes cytokine production in SA-2-stimulated CD14⁺ cells

Condition	Cytokine production (mean $\pm$ SE) (ng/mL)			
	TNF- α		IL-12	
	Medium	+ chloroquine	Medium	+ chloroquine
Unstimulated	0.2 $\pm$ 0.01	0.1 $\pm$ 0.01	0.01 $\pm$ 0.0	0.01 $\pm$ 0.0
SA-2 (6 μ M)	2.6 $\pm$ 0.3	0.1 $\pm$ 0.01	1.9 $\pm$ 0.6	0.01 $\pm$ 0.0
R-848 (6 μ M)	3.0 $\pm$ 0.15	1.3 $\pm$ 0.4	4.9 $\pm$ 1.1	0.9 $\pm$ 0.2

Highly purified CD14⁺ cells, 1×10^6 , from the peripheral blood of 2 healthy donors were preincubated with medium alone or 5 μ g of chloroquine for 2 hours at 37°C and then further cultured in the absence or presence of 6 μ M SA-2 or R-848 used as a control. After 24 hours, supernatants were collected, centrifuged, and stored at -20°C until tested by using commercial ELISAs. Viability of chloroquine-preincubated cells was always greater than 99%, as indicated by propidium iodide exclusion. The mean concentrations of the different cytokines ($\pm$ SE) are reported.

TABLE E2. SA-2 does not activate cyclic AMP–dependent signaling pathways

Condition	Cyclic AMP levels (pmol/mL)	
	Donor 1	Donor 2
Medium	3.5	13
SA-2 (6 μ M)	4.4	5.6
R-848 (6 μ M)	5.4	9.3
Forskolin (10 μ M)	10.3	21.9

Highly purified CD14⁺ cells, 1×10^6 , from the peripheral blood of 2 healthy donors were cultured in the absence or presence of 6 μ M SA-2 or R-848 and 10 μ M forskolin used as a positive control. After 60 minutes, cells were extensively washed and lysed with the appropriate buffer. Supernatants collected after centrifugation were tested by using a commercial ELISA.