Published May 5, 2014

Immunity induced by a broad class of inorganic crystalline materials is directly controlled by their chemistry

Gareth R. Williams,[1,2] Kaat Fierens,[4,5] Stephen G. Preston,[2] Daniel Lunn,[3] Oliwia Rysnik,[2] Sofie De Prijck,[4] Mirjam Kool,[4,5,6] Hannah C. Buckley,[1,2] Bart N. Lambrecht,[4,5,6] Dermot O'Hare,[1] and Jonathan M. Austyn[2]

[1]Chemistry Research Laboratory, Department of Chemistry; [2]Nuffield Department of Surgical Sciences, John Radcliffe Hospital; and [3]Department of Statistics; University of Oxford, Oxford OX1 2JD, England, UK
[4]Laboratory of Immunoregulation and Mucosal Immunology, Department of Pulmonary Medicine, University Hospital and [5]Flanders Institute for Biotechnology (VIB) Inflammation Research Center, University of Ghent, 9000 Ghent, Belgium
[6]Department of Pulmonary Medicine, Erasmus University Medical Center, 3015 GE Rotterdam, Netherlands

There is currently no paradigm in immunology that enables an accurate prediction of how the immune system will respond to any given agent. Here we show that the immunological responses induced by members of a broad class of inorganic crystalline materials are controlled purely by their physicochemical properties in a highly predictable manner. We show that structurally and chemically homogeneous layered double hydroxides (LDHs) can elicit diverse human dendritic cell responses in vitro. Using a systems vaccinology approach, we find that every measured response can be modeled using a subset of just three physical and chemical properties for all compounds tested. This correlation can be reduced to a simple linear equation that enables the immunological responses stimulated by newly synthesized LDHs to be predicted in advance from these three parameters alone. We also show that mouse antigen–specific antibody responses in vivo and human macrophage responses in vitro are controlled by the same properties, suggesting they may control diverse responses at both individual component and global levels of immunity. This study demonstrates that immunity can be determined purely by chemistry and opens the possibility of rational manipulation of immunity for therapeutic purposes.

CORRESPONDENCE
Bart N. Lambrecht:
Bart.Lambrecht@ugent.be

Abbreviations used: CI, confidence interval; DAMP, damage-associated molecular pattern; DC, dendritic cell; LDH, layered double hydroxide.

The innate immune system senses and responds to danger posed by different types of infection through recognition (by pattern recognition receptors) of conserved components of infectious agents (pathogen-associated molecular patterns [PAMPs]; Medzhitov, 2009). It can also sense and respond to other forms of danger, such as signs of cell stress or damage (damage-associated molecular patterns [DAMPs]), which may or may not be pathogen induced (Bianchi, 2007). PAMPs and DAMPs can trigger dendritic cell (DC) responses that help provide a context for activation of specific adaptive immune responses appropriate to the type of threat, such as different types of antibodies or cytotoxic T lymphocyte responses (Pulendran et al., 2010b). There is increasing evidence that certain inorganic and organic crystalline materials can also be perceived as dangerous, but how these are sensed is little understood. However, it has been shown that alum crystals bind with extraordinary strength to the plasma membrane of DCs (Flach et al., 2011) and are sensed independently of pattern recognition receptors, suggesting that physicochemical principles may be involved; the same is true for uric acid (DeFranco, 2008; Ng et al., 2008) but not its analogue allopurinol, indicating a remarkable selectivity in this process.

G.R. Williams, K. Fierens, and S.G. Preston contributed equally to the experimental work.
B.N. Lambrecht, D. O'Hare, and J.M. Austyn contributed equally to the strategic design of the project.
G.R. Williams' present address is UCL School of Pharmacy, University College London, London WC1N 1AX, England, UK.
S.G. Preston's present address is Dept. of Zoology, University of Oxford, Oxford OX1 3PS, England, UK.

The Rockefeller University Press $30.00
J. Exp. Med. 2014 Vol. 211 No. 6 1019-1025
www.jem.org/cgi/doi/10.1084/jem.20131768

Supplemental Material can be found at:
http://jem.rupress.org/content/suppl/2014/05/01/jem.20131768.DC1.html

Published May 5, 2014

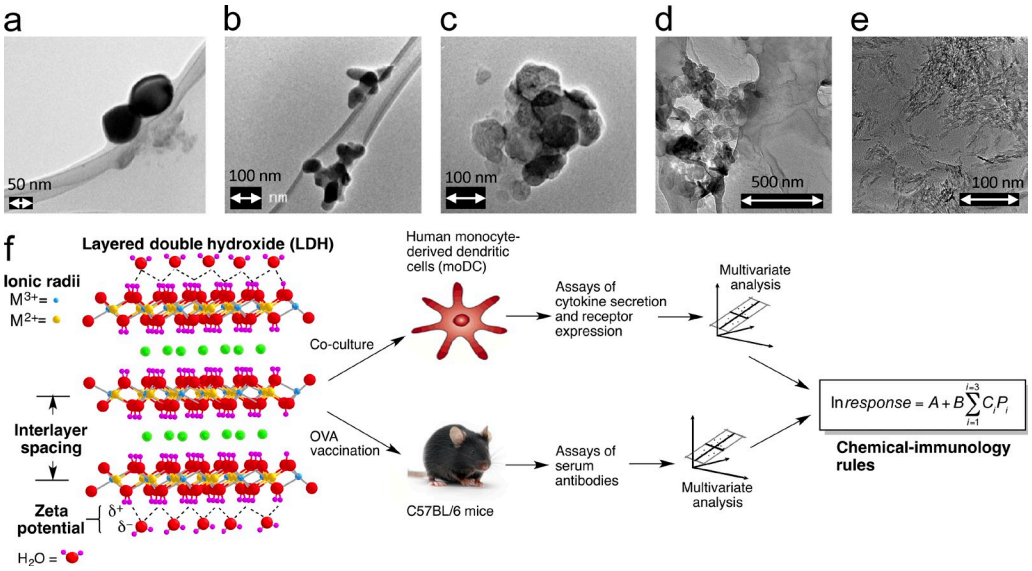

Figure 1. Illustrations of typical LDHs and the systems vaccinology approach used in this study. (a–e) Transmission electron micrographs of $LiAl_2$-CO_3 (a), Mg_2Al-NO_3 (b), Mg_2Fe-Cl (c), Imject alum (d), and Alhydrogel (e). Size data on the LDHs are in Table S1. (f) A schematic showing the systems vaccinology approach. To the left, the general LDH structure is depicted, showing the positively charged layers (yellow/blue/red circles) and interlayer anions (green circles), with a surrounding layer of water (top and bottom). The in vitro DCs and in vivo antibody responses stimulated by a series of LDHs were evaluated, and the datasets were then independently subjected to multivariate analysis, with the physicochemical properties of LDHs detailed in Table S4. All observed responses were highly correlated with the three key physicochemical properties indicated on the left side and conformed to the equation (Eq. 1) illustrated on the right side. This equation was then used to predict, de novo, the immunological (DC) responses stimulated by newly synthesized LDHs from their respective physicochemical properties.

Through their capacity to elicit danger signals, alums have for many decades been incorporated into vaccines to stimulate high levels of protective antibodies against the antigens they contain (Marrack et al., 2009; Coffman et al., 2010). The alum used as adjuvants usually comprises aluminum oxyhydroxide (AlOOH) or aluminum hydroxyphosphate ($Al(OH)_x(PO_4)_y$), but the materials are heterogeneous and poorly characterized. In contrast, layered double hydroxides (LDHs) are structurally and chemically homogeneous crystalline materials represented by the general chemical formula $[M^{z+}{}_{1-x}M^{3+}{}_x(OH)_2]^{p+}(X^{n-})_{p/n}\cdot\gamma H_2O$ (see Fig. 1). In essence, the structure is a sandwich of positively charged mixed-metal hydroxide layers (containing both a trivalent [M^{3+}] and either a monovalent [M^+] or divalent [M^{2+}] cation) with interlayers of negatively charged anions. LDHs can be synthesized in thousands of different chemical compositions with a large range of possible metal cations, varied ratios of $(M^+/M^{2+}):M^{3+}$, and many different anions. Because alums are solid, ionic hydroxyl salts, we reasoned that LDHs may also elicit immunological responses; if so, the versatility of these materials would enable us to explore the impact of systematic chemical substitutions on the types of immunity induced.

RESULTS AND DISCUSSION

Chemically different LDHs stimulate diverse human DC responses in vitro

Because alum adjuvants act on DCs, at least in vivo (Kool et al., 2008a,b; Marrack et al., 2009; McKee et al., 2009), we initially evaluated the immunological properties of LDHs in culture by assessing their ability to stimulate responses of human monocyte-derived DCs (Sallusto and Lanzavecchia, 1994). We measured the capacity of DCs to secrete proinflammatory cytokines (IL-1β, IL-6, and TNF), as well as cytokines that polarize Th1 responses (IL-12p70), maintain memory T cell responses (IL-15), and promote antiviral responses (IFN-α2). We also investigated DC production of chemokines (IL-8, MIP-1β, and MCP-1) and of membrane molecules involved in T cell activation (CD86), DC–T cell cross talk (CD40), and suppression of CD8 T cell proliferation (CD274). To study this systematically, we designed, synthesized, and characterized a series of endotoxin-free LDHs, with systematic substitutions of metal cations and anions (Table S1). The LDHs were observed to comprise crystalline hexagonal platelets (Fig. 1). DCs were co-cultured with LDHs, and the cellular responses stimulated by each compound were evaluated and compared with those elicited by the commercially available alums Imject and Alhydrogel. We found that individual LDHs stimulated distinct DC responses across many immunological outputs but with donor to donor variation. We therefore undertook a statistical repeated measures analysis, with a random effect to account for donor variability, and fitted a multilevel model to obtain estimates of the overall responses. Several compounds were found to stimulate robust DC responses, frequently much greater than the commercially used Alhydrogel adjuvant (Fig. 2 and Tables S2 and S3). In Fig. 2 and subsequently, LDHs are denoted M_xM_y'-X, where M and M′ are the metal cations in the LDH layers, x/y is the ratio of these ions, and X is the interlayer anion.

Published May 5, 2014

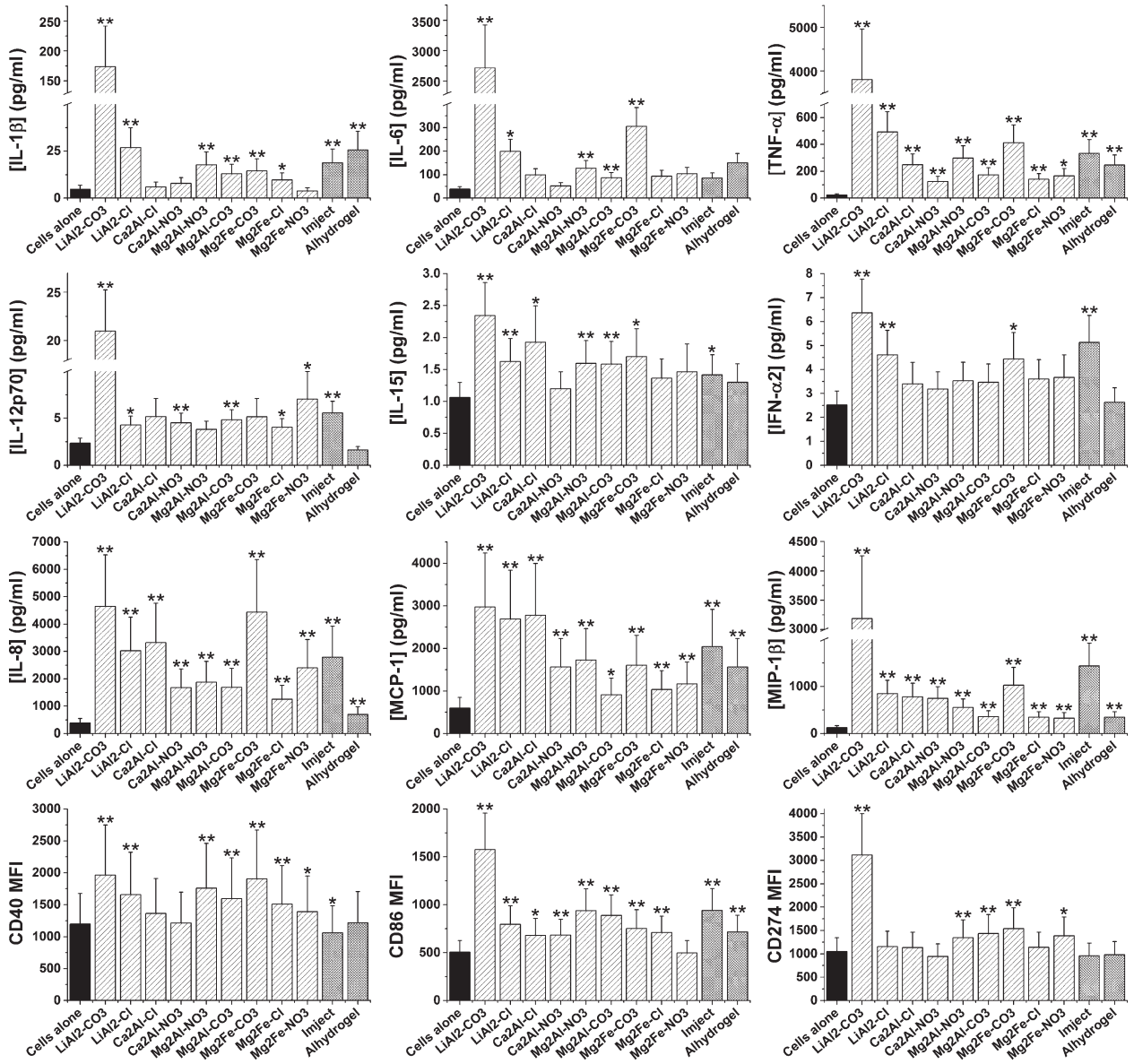

Figure 2. Chemically different LDHs drive diverse DC responses in vitro. Human monocyte-derived DCs were cultured without or with the indicated LDH or commercial adjuvant preparation for a period of 24 h. The concentration of cytokines and chemokines in the supernatant was then determined using ELISA (IL-6 and TNF) or Luminex (others). Cell surface expression of co-stimulatory and co-inhibitory molecules was assessed by flow cytometry. MFI, mean fluorescence intensity. Error bars show one standard error. *, $P < 0.05$; and **, $P < 0.01$ versus cells alone. The number of individual experiments performed for each response ranges from $n = 6$ to $n = 22$ and is given in Table S3. Each experiment contained at least three biological replicates.

DC responses are controlled purely by discrete physicochemical properties of LDHs

We then hypothesized that the varying immunological activities of different LDHs may be determined by their respective physicochemical characteristics. We therefore applied a regression model between all of the DC response datasets and the physicochemical properties of these LDHs obtained from published studies or measured directly (Table S4 a). Essentially, this approach considered that the physicochemical properties were causative of the immune responses and sought to find the closest fit between any given subset of properties and all the immunological responses. Surprisingly, this revealed that all in vitro human DC responses were very highly correlated with a linear combination of three LDH properties: the radius of the spherical M^+ or M^{2+} metal cations; the distance between the LDH layers (interlayer spacing); and the zeta potential, which defines the magnitude of the electrical charge at the interfacial double layer around the LDH particle (Fig. 1; Attwood, 2007). Correlation coefficients between this combination and the observed responses ranged from 0.78 to 1.0, with one exception (IL-8) at 0.65. These correlations can be expressed by a simple linear equation:

Published May 5, 2014

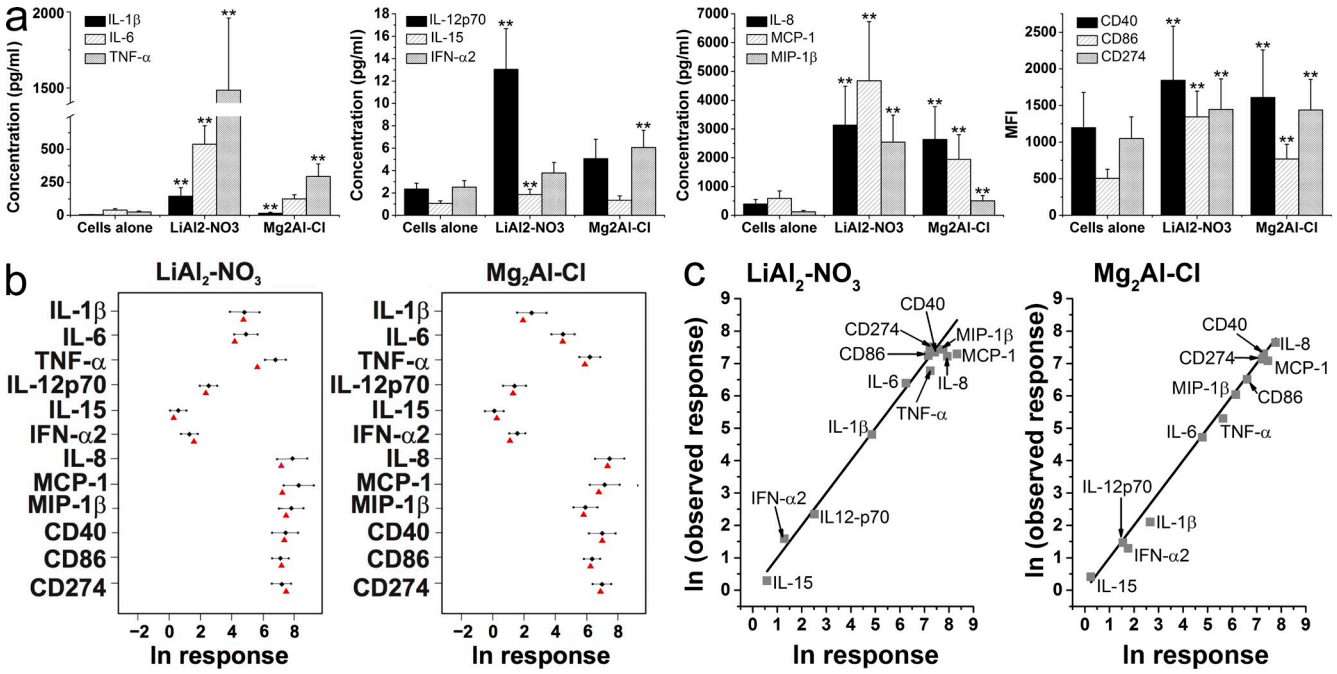

Figure 3. Multiple DC responses induced by newly synthesized LDHs can be predicted with a high degree of accuracy. (a) DC responses to $LiAl_2$-NO_3 and Mg_2Al-Cl were assessed as in Fig. 2. Error bars show one standard error. **, $P < 0.01$ versus cells alone. Four independent experiments were performed, each with three or four biological replicates. (b and c) DC responses to $LiAl_2$-NO_3 and Mg_2Al-Cl were predicted with Eq. 1 following calibration of the model using data from Fig. 2. In b, the mean and 95% CIs for the measured responses are indicated (diamonds and short horizontal lines) with the predicted value (triangles) immediately below. In c, observed ln responses are shown along a straight line of gradient 1, and the predicted ln responses as squares on the same plot.

$$\ln response = A + B\sum_{i=1}^{i=3} C_i P_i. \qquad (1)$$

In this equation, A, B, and C_i are coefficients for any given immunological response, and P_i is the value of each respective physicochemical property. (Values for LDH properties, which were standardized to ensure equal variance, and the coefficients are shown in Tables S4 b and S5.)

DC responses to newly synthesized LDHs can be accurately predicted simply from knowledge of their physicochemical properties

We next investigated the model's ability to predict a priori the immunological properties of newly synthesized LDHs, using the robust and relatively high-throughput DC assays we had previously used. We synthesized two new LDH compounds, $LiAl_2$-NO_3 and Mg_2Al-Cl (Table S1), and made blind predictions of the multiple ($n = 12$) DC responses they might induce based purely on their physicochemical properties. We found the correlation between predicted and actual observed responses to be remarkably high, with a median coefficient of variation of 5.14% (Fig. 3 and Table S6). We observed that 22 of the 24 predicted values fell within the 95% confidence intervals (CIs); the probability of this occurring by chance is $P < 0.0002$. Conversely, just 14 out of 24 predicted values for responses elicited by one compound fall within the CIs for the responses elicited by the other, showing that the model's predictions are composition specific. Because the predictive power of our model is proportional to the size of the datasets, its accuracy should be increased by further calibration across a wider series of LDHs with a broader range of properties.

The same physicochemical properties of LDHs control mouse antibody responses in vivo and may apply to other immunological responses

To explore the potential in vivo adjuvant activities of LDHs, we next evaluated the capacity of some of our compounds to elicit antibody responses in C57BL/6 mice, based on a vaccination protocol to study adjuvant-driven responses against the model antigen OVA. Mice were primed i.p. with OVA admixed with each LDH, or alum, and boosted i.p. with soluble OVA 1 wk later (Eisenbarth et al., 2008; Kool et al., 2008b). Because this leads to primary antigen presentation in the mediastinal lymph nodes, which also drain the lungs, we then challenged the mice with a final, intranasal boost of soluble OVA 1 wk later. The resultant data confirmed that chemically different LDHs can elicit in vivo antibody responses that are at least as potent as those of the alums tested (Fig. 4 and Tables S7 and S8). Notably, vaccination with alum adjuvants resulted in OVA-specific IgG1 and IgE antibody production alone, reflective of a Th2-dominated response as expected, whereas some LDH compounds (e.g., Mg_2Fe-CO_3)

Published May 5, 2014

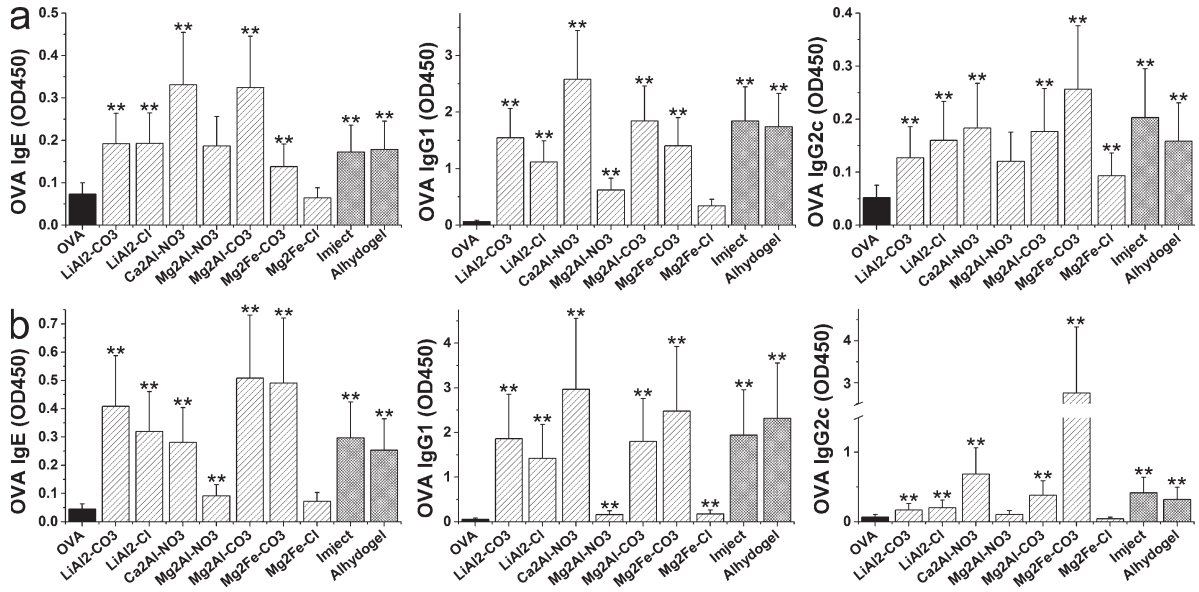

Figure 4. Chemically different LDHs have distinct adjuvant activities in vivo. Mice were immunized i.p. with 10 µg OVA admixed with 1 mg LDH, Alhydrogel, or Imject alum and boosted i.p. 10 d later with 10 µg OVA alone. Control mice were given the booster treatment only. (a) 7 d after boost, mice were bled via the tail vein, and serum levels of OVA-specific antibody isotypes were measured by ELISA. (b) Starting 1 wk later, the prime-boosted mice were challenged on three consecutive days with a 1% OVA aerosol. 1 d after the final challenge, ELISA was used to measure serum levels of OVA-specific antibody isotypes. Antibody titers are shown as OD450, as assessed by ELISA. Error bars depict one standard error. **, $P < 0.01$ via pairwise comparisons versus OVA alone. Data are from two independent experiments, each with at least five mice per group.

stimulated production of OVA-specific IgG2c antibodies, indicative of a Th1-polarized response (Gavin et al., 2006; Eisenbarth et al., 2008). We then applied our regression model (above) to all of these in vivo antibody datasets and found that these, too, were very highly correlated with the same subset of LDH properties as above, conforming to Eq. 1. (Values for the properties and coefficients are shown in Tables S4 and S9.)

Finally, we have investigated the capacity of LDHs to induce human macrophage responses in vitro. Our initial data indicate that proinflammatory responses of human monocyte-derived macrophages and dermal macrophages are highly correlated with the same LDH properties as above, also conforming to Eq. 1 (Table S10). Together, our findings suggest that the same "chemical–immunology rule" for these materials may apply at other levels of immunity, such as T cell responses. In the latter context, we also have preliminary data suggesting that certain LDHs, notably Mg_2Al-NO_3 and Mg_2Al-CO_3, can elicit expansion of mouse OVA-specific effector $CD8^+$ T cells in vivo (not depicted), and the former compound has been reported to elicit cytotoxic T lymphocytes in vivo (Li et al., 2011).

This study suggests a highly discriminatory mode for the sensing of crystalline materials by the immune system. Whether this is caused by the capacity of the plasma membrane of DCs to act as a general sensor for solid structures (Flach et al., 2011), the regulated production of DAMPs, or other mechanisms (Dostert et al., 2008; Hornung et al., 2008) is under investigation; so too is the nature of the interaction between LDHs and DCs (e.g., phagocytosis) and the impact of systematically changing particle dose, size, and/or morphology (Hu and O'Hare, 2005) on immunological responses. The regression model we developed (Fig. 1) derives from a systems vaccinology approach (Plotkin, 2008; Pulendran et al., 2010a; Pulendran and Ahmed, 2011; Nakaya and Pulendran, 2012), one aspiration of which has been to predict which materials will act as potent adjuvants to the immune system. Until now, lack of systematically variable adjuvants has made this impossible to achieve. In contrast, our chemical–immunology rule enables accurate prediction of the multiple DC, and potentially other, responses that can be induced by any given LDH, the first time such an approach has been possible for any material. Ultimately, advances in computational chemistry should permit pure in silico screening of vast numbers of LDHs to select those with the most desirable properties. Hence our findings may comprise a paradigm shift in vaccinology by enabling the selection and synthesis of designer adjuvants that can be incorporated into bespoke vaccines, tailored for use in different disease settings.

MATERIALS AND METHODS

Synthesis and characterization of LDHs. LDHs were synthesized according to previously reported protocols (Khan et al., 2009). $LiAl_2$-Cl and $LiAl_2$-NO_3 were prepared by the direct reaction of LiCl or $LiNO_3$ with gibbsite. Mg_2Al-NO_3, Mg_2Al-Cl, Mg_2Fe-Cl, Mg_2Fe-NO_3, Ca_2Al-Cl, and Ca_2Al-NO_3 were generated via a coprecipitation synthesis. $LiAl_2$-CO_3, Mg_2Al-CO_3, and Mg_2Fe-CO_3 were produced by reaction of the nitrate- or chloride-containing LDHs with an excess of sodium carbonate. Endotoxin was not detectable in any compound by Limulus amebocyte assay. Characterization was performed by x-ray diffraction, infrared spectroscopy, elemental microanalysis, and dynamic light-scattering measurements. The positions of the *00l* reflections in diffraction patterns were used to calculate the interlayer

Published May 5, 2014

spacing (the distance between the LDH layers). The zeta potential was measured for 10 mg/ml solutions of the LDHs in phosphate buffered saline, initial pH 7.2. The M^+ or M^{2+} ionic radius was taken from the literature (Lide, 2000).

Immunological assays in vitro. Human monocyte-derived DCs were generated according to standard procedures (Sallusto and Lanzavecchia, 1994). Monocytes were isolated from human blood products (Buffy coats or leukocyte cones) using density gradient centrifugation (Lymphoprep; Axis-Shield) followed by negative selection (EasySep Human Monocyte Enrichment kit; STEMCELL Technologies) and cultured for 6 d in complete RPMI supplemented with recombinant human (rh) GM-CSF and rhIL-4 (DC-RPMI). Cytokines were supplied by Gentaur Belgium BVBA or PeproTech. On day 6, DCs were suspended in DC-RPMI at 5×10^5 cells/ml and transferred to flat-bottomed, 96-well plates with 200 µl of cells per well. DCs were then co-cultured for ∼24 h with the LDHs or commercial alums (Alhydrogel [Sigma-Aldrich] and Imject alum [Thermo Fisher Scientific]) at 476 µg/ml final concentration. The expression of surface proteins by the DCs was measured by flow cytometry (FACSCanto [BD], with analysis using FlowJo software). Cytokines were measured either by ELISA (Insight Bioscience Ltd.) or by Luminex technology (EMD Millipore) according to the manufacturers' instructions. Macrophages were generated from monocytes isolated in an identical process to that used for generating DCs. Subsequently, monocytes at 5×10^5 cells/ml were seeded in 96-well plates (200 µl/well) and cultured for 6 d with 10 ng/ml rhM-CSF alone to generate macrophages (Becker et al., 1987) or with 10 ng/ml rhM-CSF, 10 ng/ml rhIL-10, and 10 U/ml rhGM-CSF to prepare dermal macrophages (Kwan et al., 2008). Cells were co-cultured with LDHs or commercial alums as above, and cytokine production was measured by ELISA in accordance with the manufacturer's instructions.

Immunological assays in vivo. For antibody assays, C57BL/6 mice (Harlan Laboratories) were immunized with the model antigen OVA (Worthington Biochemical Corporation) with or without each LDH compound or commercial Imject alum (Thermo Fisher Scientific) as a potential adjuvant ($n \geq 5$ per experimental group). Mice were sensitized i.p. with 500 µl OVA at 20 µg/ml admixed with 2 mg/ml LDH (a dose of 10 µg OVA and 1 mg LDH/mouse). 7 d later they were boosted i.p. with 10 µg OVA alone. 1 wk later (day 14) mice were bled via the tail vein, and the serum levels of OVA-specific IgG1, IgE, and IgG2c were measured by ELISA (eBioscience). The prime-boosted mice were then challenged with a 1% OVA aerosol for 30 min on each of days 17, 18, and 19. On day 20 the mice were sacrificed with an overdose of Avertin. Blood was collected to determine the serum levels of OVA-specific IgE, IgG1, and IgG2c antibodies as above. All experiments were approved by the Ethical Committee for Animal Experimentation, Faculty of Science, University of Ghent and VIV site Ardoyen; site license number LA1400091.

Statistical analysis. The observational data comprised repeated measures of immunological responses from several experiments. Therefore, to obtain estimates of the mean response for each compound, it was necessary to fit a series of two-level linear mixed-effects models. This was done using the *lme* function from the *nlme* library in the *R* statistical package with experiment ID as the second level. To obtain normally distributed residuals and stability of variance, the responses were log-transformed, after which all of the usual diagnostic tests proved to be entirely satisfactory. This analysis provided the estimates of the mean responses given in Tables S2 (shown in Fig. 2), S7 (shown in Fig. 4), and S10 a and the p-values detailed in Tables S3, S8, and S10 b.

Investigation of possible relationships between the immune responses and the physicochemical properties of the LDHs given in Table S4 was performed by computing the canonical correlation coefficients between subsets of the properties and the set of mean responses for each adjuvant. Because the magnitude of the different "as-measured" properties varied considerably (Table S4 a), the properties were first standardized to have mean 0 and unit variance (Table S4 b). This was performed to ensure that each property had equal weighting at the start of analysis. All possible subsets of three or four properties were investigated, and the correlation coefficients were calculated, along with their associated p-values. The subset [ionic radius of M^+ or M^{2+}, interlayer spacing, and zeta potential] consistently produced the smallest p-values and very high correlations across all response types (median = 0.863), all of which were statistically significant; note that all p-values for this subset were <0.025 (with a correlation of >0.6075). It should be noted that the physicochemical properties are often internally correlated with one another, so the immune responses were correlated with several subsets of properties. The selected subset was chosen because it gave the highest correlations and lowest p-values. This resulted in the formula

$$\ln response = A + B\sum_{i=1}^{i=3} C_i P_i, \quad (1)$$

where the C_i values were calculated to maximize the correlation between the properties and the mean responses. The constants A and B were then determined by treating the relationship as one of cause and effect, in other words to regard the LDH properties as responsible for the responses; thus, A and B were obtained as regression coefficients. Values for the coefficients A, B, and C_i for the respective immunological datasets are shown in Tables S5, S9, and S10 c.

A priori predictions of the responses to newly synthesized LDHs were calculated using the formula in Eq. 1 with the values for A, B, and C_i determined from experiments previously undertaken with other LDHs. To obtain a conservative estimate for the likelihood of the prediction accuracy being attributable to chance, we first calculated the full range of estimated mean ln response across all compounds, then determined the mean 95% CI width and divided this by the range to give the largest plausible upper bound to the proportion of the range covered by the CI. The mean of this proportion across all DC responses was then taken as an over-conservative estimate of the probability of a single prediction falling within the 95% CI by chance. This was 0.6272, and therefore the overall probability of the accuracy of prediction observed being caused by chance (22 of 24 responses within the 95% CI) is 0.00023.

Online supplemental material. Table S1 shows chemical details and size data for the LDHs used in this study. Tables S2 and S3, included in a separate PDF file, respectively, show actual and ln values and significance data for DC responses to LDHs and alums. Table S4, included in a separate PDF file, shows the actual (a) and standardized (b) physicochemical properties of the LDHs used in this study. Table S5, included in a separate PDF file, shows coefficients for DC responses according to Eq. 1. Table S6, included in a separate PDF file, shows the actual DC response to newly synthesized LDHs and that predicted from Eq. 1. Tables S7, S8, and S9, included in a separate PDF file, respectively, show the actual and ln values, significance data, and coefficients according to Eq. 1 for mouse OVA-specific antibody responses to LDHs and alums. Table S10, included in a separate PDF file, shows the actual and ln values (a), significance data (b), and coefficients (c) according to Eq. 1 for macrophage responses to LDHs and alums. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131768/DC1.

We thank Dr. Jan Digby (Department of Cardiovascular Medicine, University of Oxford, Oxford, England, UK) for help with Luminex measurements; Dr. Kerstin Jurkschat and Dr. Alison Crossley (Department of Materials, University of Oxford) for assistance with zeta potential measurements; and Dr. Mark Suter (University of Zurich, Zurich, Switzerland), Dr. Gordon McPherson (Oriel College, Oxford, England, UK), and Dr. Richard Dillon (King's College London, London, England, UK) for helpful discussions.

D. O'Hare was supported by a Medical Research Council Discipline Hopping Grant (with J.M. Austyn as sponsor), and J.M. Austyn was supported by the EU FP6 Programme (via the DC-THERA Network of Excellence, Project No. LSHB-CT-2004-512074). B.N. Lambrecht is supported by a European Research Council grant and by a University of Ghent MRP-GROUP-ID grant.

The authors declare no competing financial interests.

Published May 5, 2014

Author contributions: G.R. Williams designed and conducted in vitro experiments, synthesized LDHs, performed statistical analysis, and wrote the manuscript; K. Fierens conducted in vivo experiments and wrote the manuscript; S.G. Preston designed and conducted in vitro experiments and wrote the manuscript; D. Lunn designed and performed the systems vaccinology modelling and undertook statistical analysis; O. Rysnik performed in vitro experiments; S. De Prijck performed in vivo experiments and in vitro analysis of immunoglobulin levels; M. Kool designed and performed in vivo experiments; H.C. Buckley prepared and characterized LDH and alum samples; B.N. Lambrecht designed in vivo experiments and wrote the manuscript; D. O'Hare synthesized the LDH compounds, designed experiments, and wrote the manuscript; J.M. Austyn designed in vitro experiments and wrote the manuscript. D. O'Hare, J.M. Austyn, and B.N. Lambrecht jointly conceived of the use of LDHs as adjuvants, and B.N. Lambrecht proposed the systems vaccinology approach.

Submitted: 23 August 2013
Accepted: 14 April 2014

REFERENCES

Attwood, D. 2007. Disperse systems. *In* Aulton's Pharmaceutics: The Design And Manufacture of Medicines. Third edition. M.E. Aulton, editor. Elsevier Ltd, Philadelphia, PA. 76–78.

Becker, S., M.K. Warren, and S. Haskill. 1987. Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. *J. Immunol.* 139:3703–3709.

Bianchi, M.E. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukoc. Biol.* 81:1–5. http://dx.doi.org/10.1189/jlb.0306164

Coffman, R.L., A. Sher, and R.A. Seder. 2010. Vaccine adjuvants: putting innate immunity to work. *Immunity*. 33:492–503. http://dx.doi.org/10.1016/j.immuni.2010.10.002

DeFranco, A.L. 2008. "Dangerous crystals". *Immunity*. 29:670–671. http://dx.doi.org/10.1016/j.immuni.2008.10.005

Dostert, C., V. Pétrilli, R. Van Bruggen, C. Steele, B.T. Mossman, and J. Tschopp. 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science*. 320:674–677. http://dx.doi.org/10.1126/science.1156995

Eisenbarth, S.C., O.R. Colegio, W. O'Connor, F.S. Sutterwala, and R.A. Flavell. 2008. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature*. 453:1122–1126. http://dx.doi.org/10.1038/nature06939

Flach, T.L., G. Ng, A. Hari, M.D. Desrosiers, P. Zhang, S.M. Ward, M.E. Seamone, A. Vilaysane, A.D. Mucsi, Y. Fong, et al. 2011. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. *Nat. Med.* 17:479–487. http://dx.doi.org/10.1038/nm.2306

Gavin, A.L., K. Hoebe, B. Duong, T. Ota, C. Martin, B. Beutler, and D. Nemazee. 2006. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science*. 314:1936–1938. http://dx.doi.org/10.1126/science.1135299

Hornung, V., F. Bauernfeind, A. Halle, E.O. Samstad, H. Kono, K.L. Rock, K.A. Fitzgerald, and E. Latz. 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat. Immunol.* 9:847–856. http://dx.doi.org/10.1038/ni.1631

Hu, G., and D. O'Hare. 2005. Unique layered double hydroxide morphologies using reverse microemulsion synthesis. *J. Am. Chem. Soc.* 127:17808–17813. http://dx.doi.org/10.1021/ja0549392

Khan, A.I., A. Ragavan, B. Fong, C. Markland, M. O'Brien, T.G. Dunbar, G.R. Williams, and D. O'Hare. 2009. Recent Developments in the use of layered double hydroxides as host materials for the storage and triggered release of functional anions. *Ind. Eng. Chem. Res.* 48:10196–10205. http://dx.doi.org/10.1021/ie9012612

Kool, M., V. Pétrilli, T. De Smedt, A. Rolaz, H. Hammad, M. van Nimwegen, I.M. Bergen, R. Castillo, B.N. Lambrecht, and J. Tschopp. 2008a. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. *J. Immunol.* 181:3755–3759.

Kool, M., T. Soullié, M. van Nimwegen, M.A. Willart, F. Muskens, S. Jung, H.C. Hoogsteden, H. Hammad, and B.N. Lambrecht. 2008b. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J. Exp. Med.* 205:869–882. http://dx.doi.org/10.1084/jem.20071087

Kwan, W.-H., E. Navarro-Sanchez, H. Dumortier, M. Decossas, H. Vachon, F.B. dos Santos, H.W. Fridman, F.A. Rey, E. Harris, P. Despres, and C.G. Mueller. 2008. Dermal-type macrophages expressing CD209/DC-SIGN show inherent resistance to dengue virus growth. *PLoS Negl. Trop. Dis.* 2:e311. http://dx.doi.org/10.1371/journal.pntd.0000311

Li, A., L. Qin, W. Wang, R. Zhu, Y. Yu, H. Liu, and S. Wang. 2011. The use of layered double hydroxides as DNA vaccine delivery vector for enhancement of anti-melanoma immune response. *Biomaterials*. 32:469–477. http://dx.doi.org/10.1016/j.biomaterials.2010.08.107

Lide, D.R., editor. 2000. CRC Handbook of Chemistry and Physics. 81st edition. CRC Press, Boca Raton, FL. 2556 pp.

Marrack, P., A.S. McKee, and M.W. Munks. 2009. Towards an understanding of the adjuvant action of aluminium. *Nat. Rev. Immunol.* 9:287–293. http://dx.doi.org/10.1038/nri2510

McKee, A.S., M.W. Munks, M.K. MacLeod, C.J. Fleenor, N.Van Rooijen, J.W. Kappler, and P. Marrack. 2009. Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J. Immunol.* 183:4403–4414. http://dx.doi.org/10.4049/jimmunol.0900164

Medzhitov, R. 2009. Approaching the asymptote: 20 years later. *Immunity*. 30:766–775. http://dx.doi.org/10.1016/j.immuni.2009.06.004

Nakaya, H.I., and B. Pulendran. 2012. Systems vaccinology: its promise and challenge for HIV vaccine development. *Curr. Opin. HIV AIDS*. 7:24–31. http://dx.doi.org/10.1097/COH.0b013e32834dc37b

Ng, G., K. Sharma, S.M. Ward, M.D. Desrosiers, L.A. Stephens, W.M. Schoel, T. Li, C.A. Lowell, C.-C. Ling, M.W. Amrein, and Y. Shi. 2008. Receptor-independent, direct membrane binding leads to cell-surface lipid sorting and Syk kinase activation in dendritic cells. *Immunity*. 29:807–818. http://dx.doi.org/10.1016/j.immuni.2008.09.013

Plotkin, S.A. 2008. Vaccines: correlates of vaccine-induced immunity. *Clin. Infect. Dis.* 47:401–409. http://dx.doi.org/10.1086/589862

Pulendran, B., and R. Ahmed. 2011. Immunological mechanisms of vaccination. *Nat. Immunol.* 12:509–517. http://dx.doi.org/10.1038/ni.2039

Pulendran, B., S. Li, and H.I. Nakaya. 2010a. Systems vaccinology. *Immunity*. 33:516–529. http://dx.doi.org/10.1016/j.immuni.2010.10.006

Pulendran, B., H. Tang, and S. Manicassamy. 2010b. Programming dendritic cells to induce T_H2 and tolerogenic responses. *Nat. Immunol.* 11:647–655. http://dx.doi.org/10.1038/ni.1894

Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and down-regulated by tumor necrosis factor alpha. *J. Exp. Med.* 179:1109–1118. http://dx.doi.org/10.1084/jem.179.4.1109