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Cutting Edge: New Chimeric NOD2/TLR2 Adjuvant Drastically Increases Vaccine Immunogenicity

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TLR ligands are critical activators of innate immunity and are being developed as vaccine adjuvants. However, their usefulness in conjunction with NOD-like receptor agonists remains poorly studied. In this study, we evaluated a new ligand that targets both TLR2 and NOD2 receptors. We assessed its ability to enhance dendritic cell maturation in vitro in addition to improving systemic and mucosal immune responses in mice. The chimeric NOD2/TLR2 ligand induced synergistic upregulation of dendritic cell maturation markers, costimulatory molecules, and secretion of proinflammatory cytokines compared with combinations of separate ligands. Furthermore, when coadministered with biodegradable nanoparticles carrying a model Ag, the ligand was able to induce high Ag-specific IgA and IgG titers at both systemic and mucosal sites after parenteral immunizations. These findings point out the potential utility of chimeric molecules TLR/NOD as adjuvants for vaccines to induce systemic and mucosal immune responses. The Journal of Immunology, 2014, 193:000–000.

New directions in vaccine research concern the improvement of Ag immunogenicity and the design of new adjuvants. Recombinant protein subunits or synthetic peptides are being tested in new vaccine strategies and offer many advantages, such as a reduced toxicity compared with live-attenuated vaccines. However, they often remain poorly immunogenic when administered alone, particularly with regard to their capacity to induce mucosal immune responses. The enhancement of Ag-specific immune responses through the development of improved vaccine adjuvants remains an important challenge (1).

The discovery of the role of TLRs and NOD-like receptors (NLRs) in the action of certain adjuvants has provided new insights for vaccine development (2, 3). These observations are particularly important with regard to the cross-talk between NLRs and TLRs, which has been the subject of numerous investigations in recent years (4).

Although TLR ligands are pyrogenic and crucial for the induction of inflammatory cytokines, NLR ligands have a poor capacity to trigger these pathways without a synergistic boost from TLR ligands in vitro (5–7). Despite these apparently weak stimulatory properties, the presence of NLR subfamily NOD1 and NOD2 ligands positively triggers the potential of some immune adjuvants, including TLR ligands (8–10). Therefore, the interaction between NLRs and TLRs seems to be more crucial for adaptive immunity than previously appreciated. An interesting new area in the field of adjuvants could be the combination of immunostimulatory agents that target different pattern recognition receptor pathways (11).

Our purpose was to target both TLR2 and NOD2, aiming at inducing systemic and mucosal immune responses after parenteral immunization. Indeed, TLR2 appears to have a special role in T cell polarization and differentiation (especially Th17) (12), mucosal homing receptor expression, and IgA production by human B cells (13), whereas NOD2 has a role in the production of proinflammatory cytokines and autophagy (14, 15) and is highly expressed at the mucosal level (3, 10).

In this study, we evaluated the potential synergistic effects of NOD2 and TLR2 stimulation by a chimeric ligand on the induction of dendritic cell (DC) maturation and proinflammatory cytokine secretion in vitro, as well as the induction of systemic and mucosal immune responses after parenteral immunization in mice in the context of a nanoparticle (NP)-based vaccine carrying the Gag p24 (HIV-1) Ag.

Materials and Methods

NOD2, TLR2, chimeric NOD2/TLR2 ligands, and p24-coated polymeric NPs

CL429 is a novel chimeric compound that was designed to stimulate both TLR2 and NOD2. This compound is composed of Murabutide (NOD2 ligand) covalently linked to Pam2C (TLR2 ligand) via a spacer (Supplemental Fig. 1A). Murabutide, Pam2C, and CL429 were developed and manufactured by InvivoGen.

The ligands’ specific activity was determined using the HEK-Blue-hTLR2, HEK-Blue-hNOD2, and RAW-Blue reporter cell lines (InvivoGen) to monitor the activation of the NF-κB and AP-1 pathways using a secreted embryonic alkaline phosphatase (SEAP). Stimulation with ligands activates NF-κB and...

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Abbreviations used in this article: DC, dendritic cell; MHC-II, MHC class II; MoDC, monocyte-derived DC; NLR, NOD-like receptor; NP, nanoparticle; PLA, poly(lactic acid); PLA-p24, p24 adsorbed on PLA NP; SEAP, secreted embryonic alkaline phosphatase.

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AP-1, which induce the production of SEAP. The level of SEAP can be easily quantified with a detection medium that turns purple/blue in the presence of alkaline phosphatase (HEK-Blue Detection; InvivoGen).

HIV-1 p24 Ag was produced and purified by PXTerapeutics (Protein’expert, Grenoble, France).

Poly(lactic acid) (PLA) NPs (180 nm) were prepared by nanoprecipitation, as previously described (16), and provided by Adjuvatis (Lyon, France). P24 protein and PLA particles were diluted in PBS at 200 μg/ml and 5 mg/ml, respectively. The two solutions were mixed volume to volume. The adsorption reaction occurred within 2 h at room temperature, with moderate overhead stirring. Endotoxin contents were assessed using an Endosafe test (Charles River) and were <5 EU/ml.

In vitro maturation of human monocyte-derived DCs and cytokine secretion

Monocytes were purified from peripheral human blood and cultured in the presence of IL-4 and GM-CSF to differentiate into DCs (monocyte-derived DCs (MoDCs)) (17). After 6 d, NOD2, TLR2, or chimeric NOD2/TLR2 ligands (10 nmol/ml) were added to the MoDCs for 24 or 48 h. LPS (2.5 μg/ml) was used as a positive control. Cells were then stained with FITC-labeled anti-CD1a (DC marker), and DC maturation was assessed by cell immunostaining using PE-labeled mAbs against the maturation marker CD83, costimulatory molecules (CD80, CD86), MHC class II (MHC-II) (BD Pharmingen), and the intracellular DC-LAMP maturation marker (Dendritics). Ten thousand events were acquired by FACS with a FACSCanto II (BD), and the data were analyzed using FlowJo software. MoDC culture supernatants were collected after 24 or 48 h, and expression of IL-12p70, TNF-α, IL-6, IL-1β, and IFN-α was analyzed by Luminex using the Bio-Plex Pro assay (Bio-Rad). Autophagy was analyzed by immunofluorescence analysis of LC3. After stimulation (8 h), cells were fixed, permeabilized, incubated with LC3-specific Ab (NanoTools; clone 5F10), and incubated with secondary Ab goat anti-mouse FITC. An agent containing DAPI was added; cells were observed using fluorescence microscopy, and quantification of cells containing LC3+ autophagosomes was performed.

Mouse study design

Eight-to-twelve week-old female BALB/c mice were purchased from Charles River Laboratories. Mice were divided into five groups of five animals and immunized s.c. with 50 μl PBS on days 0, 14, and 28. A total of 20 nmol ligands (NOD2, TLR2, both or chimeric) was coadministered; one group was immunized with PLA-p24 alone. Sera, vaginal lavage fluid, and fecal pellets were collected at days 0, 14, 28, and 34 and tested for the presence of p24-specific IgA, IgG, IgG1, and IgG2a by ELISA.

Statistical analysis

Analyses were performed using SigmaPlot software, version 11.0. Statistical data analyses were performed using one-way ANOVA with Bonferroni post hoc test for comparison of pairs. Statistical significance is indicated in the figures.

Results and Discussion

The chimeric ligand CL429 activates both TLR2 and NOD2

The specificity of each ligand at different concentrations was monitored in vitro using cell lines expressing only TLR2, NOD2, or both. As shown in Supplemental Fig. 1B, HEK cell line activation was dose dependent, and the TLR2 ligand (Pam2C) was able to stimulate only HEK cells expressing TLR2.

In vitro maturation of human monocyte-derived DCs and cytokine secretion

Monocytes were purified from peripheral human blood and cultured in the presence of IL-4 and GM-CSF to differentiate into DCs (monocyte-derived DCs (MoDCs)) (17). After 6 d, NOD2, TLR2, or chimeric NOD2/TLR2 ligands (10 nmol/ml) were added to the MoDCs for 24 or 48 h. LPS (2.5 μg/ml) was used as a positive control. Cells were then stained with FITC-labeled anti-CD1a (DC marker), and DC maturation was assessed by cell immunostaining using PE-labeled mAbs against the maturation marker CD83, costimulatory molecules (CD80, CD86), MHC class II (MHC-II) (BD Pharmingen), and the intracellular DC-LAMP maturation marker (Dendritics). Ten thousand events were acquired by FACS with a FACSCanto II (BD), and the data were analyzed using FlowJo software. MoDC culture supernatants were collected after 24 or 48 h, and expression of IL-12p70, TNF-α, IL-6, IL-1β, and IFN-α was analyzed by Luminex using the Bio-Plex Pro assay (Bio-Rad). Autophagy was analyzed by immunofluorescence analysis of LC3. After stimulation (8 h), cells were fixed, permeabilized, incubated with LC3-specific Ab (NanoTools; clone 5F10), and incubated with secondary Ab goat anti-mouse FITC. An agent containing DAPI was added; cells were observed using fluorescence microscopy, and quantification of cells containing LC3+ autophagosomes was performed.

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Serum samples were obtained from whole blood recovered by performing a small incision at the retro-orbital vein. Vaginal secretions were collected from mice with 50 μl PBS (two times) placed in the vagina of the animal using an automatic pipette (Pipette M100) and tips (CP100; both from Gilson). A total of five microliters of 25 mM PBS on days 0, 14, and 28. A total of 20 nmol ligands (NOD2, TLR2, both or chimeric) was coadministered; one group was immunized with PLA-p24 alone. Sera, vaginal lavage fluid, and fecal pellets were collected at days 0, 14, 28, and 34 and tested for the presence of p24-specific IgA, IgG, IgG1, and IgG2a by ELISA.

To study acute cytokine production, new groups of mice were immunized twice, one week apart. Sera and vaginal and intestinal tissues were collected 24 h after the boost. The vaginal cell and intestine cell lysates were prepared with the Bio-Plex Cell Lysis Kit (Bio-Rad), as specified by the manufacturer. Evaluation of multiple cytokines/chemokines was performed with a Luminex 100 instrument, in combination with the Bio-Plex mouse cytokine panels (Bio-Rad). Cytokine concentrations were determined as the mean of three replicates/time point.

Statistical analysis

Analyses were performed using SigmaPlot software, version 11.0. Statistical data analyses were performed using one-way ANOVA with Bonferroni post hoc test for comparison of pairs. Statistical significance is indicated in the figures.

Results and Discussion

The chimeric ligand CL429 activates both TLR2 and NOD2

The specific activity of each ligand at different concentrations was monitored in vitro using cell lines expressing only TLR2, NOD2, or both. As shown in Supplemental Fig. 1B, HEK cell line activation was dose dependent, and the TLR2 ligand (Pam2C) was able to stimulate only HEK cells expressing

FIGURE 1. Maturation-related patterns expressed by human MoDCs upon exposure to NOD2, TLR2, or chimeric NOD2/TLR2 ligands. (A) Phenotypes of MoDCs were determined after 24 h of stimulation, and mean expression of maturation markers (CD83 and DC-LAMP), costimulatory molecules (CD80 and CD86), and MHC-II were analyzed. (B) Quantification of cytokine-secretion pattern of MoDCs by a Bio-Plex Pro assay. The supernatants were collected, and the concentrations of IL-6, IL-12p70, TNF-α, IL-1β, and IFN-α were analyzed. Data are means ± SD (n = 3). (C) Chimeric molecule NOD2/TLR2 improves autophagy induction in DCs. DCs were incubated in the presence of the ligands for 8 h. Cells were stained with Ab to LC3, and cells containing LC3+ autophagosomes were quantified. Data are mean ± SD from eight independent counts. Data are representative of more than three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
TLR2, whereas the NOD2 ligand (Murabutide) was able to stimulate only HEK cells expressing NOD2. Both cell lines were efficiently stimulated with the chimeric ligand CL429, meaning that the chimeric ligand can activate both TLR2 and NOD2 and induce NF-κB and/or AP-1 signaling. Moreover, stimulation of the RAW Blue cell line expressing both receptors showed that the chimeric ligand was significantly more efficient than separated ligands.

The chimeric ligand NOD2/TLR2 induces synergistic human DC maturation in vitro

Adjuvants and vaccine-delivery systems address the interface between innate and adaptive immune responses, where the extent and direction of innate immune activation determine the quality and magnitude of the resulting adaptive response. DCs play a major role at this interface; their maturation is correlated with the presentation of Ag fragments by MHC molecules, as well as with the expression of specific cell markers. To determine the adjuvant potential of NOD2 and/or TLR2 ligands to stimulate human DCs in vitro, expression of the cell markers of maturation was assessed by flow cytometry 24 h after equimolar ligand addition. As shown in Fig. 1A, a statistically significant increase in the expression of CD80, CD86, and DC-LAMP was observed in MoDCs incubated with NOD2 or TLR2 ligand alone (p < 0.05). CD83 expression was enhanced to a statistically significant extent by the NOD2 ligand (p < 0.001), whereas the amount of MHC-II was not modified by the NOD2 or TLR2 ligand. The combination of the two ligands significantly increased the expression of CD86, CD80, and DC-LAMP markers, but not CD83, compared with NOD2 alone, suggesting a cumulative effect of these ligands (p < 0.01). With the notable exception of DC-LAMP, the expression of DC maturation markers was synergistically and significantly upregulated by the use of the chimeric ligand containing both NOD2 and TLR2 in comparison with monospecific (TLR2 or NOD2) and combination (TLR2 + NOD2) of molecules (p < 0.001). Moreover, this chimeric ligand was the only molecule able to increase MHC-II expression.

DC maturation triggered by external signals is accompanied by the expression of different cytokines that modulate the immune responses. For this reason, we compared the impact of different combinations of the NOD2 and TLR2 ligands on the production of different cytokines. We obtained data after 24 and 48 h of ligand exposure for IL-1β, TNF-α, IFN-α, IL-6, and IL-12p70 (Fig. 1B). In comparison with untreated cells, NOD2 or TLR2 ligands significantly increased the amount of IL-6 in MoDC culture medium (p < 0.001), as well as TNF-α and IFN-α, respectively (p < 0.05); their impact was more disparate for other cytokines. With both ligands, an additive effect was significantly detectable for IL-6, TNF-α, and IL-1β (p < 0.001). Contrary to monospecific ligands, the chimeric NOD2/TLR2 molecule synergistically increased (2- to 30-fold) the secretion of these cytokines (p < 0.001).

As already shown, NOD2 stimulation is able to induce autophagy in DCs (15). In this study, we showed that stimulation with the chimeric ligand induced high autophagosome formation in MoDCs (Fig. 1C). This observation is in accordance with the high cytokine production observed, as autophagy was shown to promote innate cytokine expression in DCs (18).

This strong adjuvant effect could be explained by improved NOD2 agonist uptake by DCs. Although TLR2 receptors are expressed on the cell surface and so widely exposed to ligands, NOD2 receptors are within the cytoplasm and are exposed to few diffusing NOD2 agonists. We hypothesized that the chimera is taken up through its TLR2 agonist domain in lipid rafts and internalized within endosomes (19, 20), enabling more NOD2 ligands to enter the cells. How the NOD2 agonist domain of the chimera interacts with the NOD2 receptor after endosomal internalization is still beyond our comprehension and requires further study, but involvement of...
the SLC15A3 and SLC15A4 transporters seems to be crucial (21, 22).

Although it is well known that stimulation of NOD2 and TLR2 activates NF-κB and AP1 signaling pathways, it is still not fully understood where the synergy takes place. Recent discoveries point out that the ser/thr kinase TAK1 is involved in the TLR2 signaling pathways (23) and Hasegawa et al. (24) postulated that NOD2 activation recruits RIP2 allowing recruitment and activation of the TAK1 complex, leading to proinflammatory cytokine secretion. This double activation could be part of the synergistic mechanisms between these two receptors.

**NOD2/TLR2 chimeric ligand enhances systemic and mucosal immune responses against HIV-1 p24 Ag**

Numerous studies validated the vaccine potential of PLA NPs as an Ag-carrier system (25). PLA NPs coated with Gag p24 (HIV-1) were evaluated for their immunostimulatory potential after s.c. coadministration with the different NOD2/TLR2 ligands in mice. At days 0, 14, 28, and 35, sera, vaginal lavage fluids, and feces were collected, and p24-specific Abs were quantified.

As shown in Fig. 2A, the chimeric NOD2/TLR2 ligand significantly increased anti-p24 IgG and IgA titers in sera, up to 2 logs in comparison with other formulations \( (p < 0.001) \). No significant differences were observed in terms of systemic anti-p24 responses between mouse groups immunized with PLA-p24 alone or coadministered with free NOD2, TLR2, or TLR2 plus NOD2 ligands. The predominant IgG subclass was IgG1, a Th2-associated isotype, for all groups of mice. IgG1 titers in the group of mice treated with the chimeric ligand were significantly higher than in other assays \( (p < 0.001) \). IgG2a (a Th1-associated isotype) also was significantly increased with the chimeric ligand or the combination of NOD2 and TLR2 ligands \( (p < 0.05) \), which resulted in a significant decrease of the IgG1/IgG2a ratio with these formulations.

Mucosal immune responses also were monitored (Fig. 2B). Compared with other assays, the group of mice injected with the chimeric ligand showed a significant increase in Ab responses (IgG and IgA) in feces and vaginal lavage fluids at day 34 postimmunization. This effect is probably due to the induction of high IgG titers in sera, which lead to the transudation and/or FcRn transport to mucosal surfaces (26). The specific IgA responses at mucosal surfaces might be due to an efficient stimulation of B cells in mucosal tissues or transport of blood IgA by the polymeric IgR (27).

Consistent with the knowledge that TLR2 and NOD2 pathways act synergistically on human B lymphocytes (28) and DCs (29) to induce immune responses, as well as our in vitro data, the strong adjuvant effect observed in vivo is probably due to the induction of inflammation at the injection site, which leads to the recruitment of APCs such as macrophages and DCs. The latter are able to uptake Ag carried by PLA NPs and then migrate to draining lymph nodes to present Ag to naïve T cells. Moreover, upregulation of MHC-II, as well as the costimulatory molecules CD80 and CD86, can improve the presentation to naïve T cells (signal 1) and induce costimulatory signals (signal 2) necessary for the activation and survival of the lymphocytes. The production of IL-12 by the DCs stimulated by the chimeric ligand orchestrates the differentiation of T cells into Th1 cells, leading to a decrease in the IgG1/IgG2a ratio.

To determine the ability of the vaccine to promote chemotaxis of immune effector cells, the chemokinome and cytokine levels in different compartments were analyzed. The formulation PLA-p24-chimeric ligand was a potent inducer of chemokines, including G-CSF and MCP-1, as well as proinflammatory cytokines TNF-α and IL-1β in sera (Supplemental Fig. 2A). These observations are consistent with the in vitro data on MoDCs showing that these cytokines were synergistically increased with the chimeric ligand. Increase of the pro-Th2 cytokines IL-5 and IL-9 in sera has also been observed in mice immunized with the chimeric ligand in comparison to other formulations. These cytokines are known to be important for mouse B cell differentiation, particularly for fate determination of terminal B cell differentiation to Ab-secreting plasma cells (30, 31), supporting the high humoral responses observed with the chimeric ligand. IL-9 also was shown to promote Th17 development (32). Despite the fact that IL-17 expression was not increased in sera, a slight, but significant increase, in the level of IL-17F was observed in vaginal tissue but not in intestinal tissue (Supplemental Fig. 2B, 2C), meaning that there was a proinflammatory reaction in the mouse vagina (33).

The NOD2 ligand induced a slight increase in IL-33, IL-23p19, MIP-3α, IL-31, CD40L, and IL-21 in vaginal tissue compared with PLA-p24 alone. The expression of these cytokines was improved by coadministration of the TLR2 ligand, whereas the chimeric ligand improved the production of MIP-3α and CD40L, which are known to interact with B cells via its cognate receptor to trigger Ig isotype class switching and promote their secretion and the differentiation of memory B cells (34). Pro-Th1 cytokines were not markedly different between the experimental groups.

In this article, we have described a new way to improve adjuvant activity by combining multi–pattern recognition receptor stimulation simultaneously using a chimeric molecule. The chimeric TLR2/NOD2 molecule we described possesses a very potent adjuvant activity, with no apparent toxicity, and it allows strong induction of both systemic and mucosal immunity against vaccine Ag. This activity seems to be mediated by an optimal DC maturation process, which induces strong B and T cell stimulation and autophagy.

**Disclosures**

G.T., E.P., T.L., and F.V. are employees of Cayla InvivoGen. They provide molecules and test their ability to stimulate transfectected cells. The other authors have no financial conflicts of interest.

**References**


