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Innate Imprinting by the Modified Heat-Labile Toxin of Escherichia coli (LTK63) Provides Generic Protection against Lung Infectious Disease

Andrew Evan Williams,2* Lorna Edwards,† Ian Robert Humphreys,* Robert Snelgrove,* Aaron Rae,* Rino Rappuoli,1 and Tracy Hussel*2

In a healthy individual, the lung contains few lymphoid cells. However, amplified immune responses, as exemplified during lung infection, can cause extensive tissue damage. We have previously demonstrated that one lung infection modulates the immunopathological outcome to a subsequent unrelated pathogen. Mimicking heterologous immunity may provide a means of enhancing both innate and acquired immunity. We now show that prior lung administration of a modified heat-labile toxin from Escherichia coli (LTK63) enhances immunity to respiratory syncytial virus, influenza virus, and the fungus Cryptococcus neoformans. Treatment with LTK63 decreased lung inflammation and tissue damage and improved the ability to resolve the infection. APCs expressing the activation markers MHC class II, CD80, and CD40 increased in number in the lung. LTK63 treatment increased the pathogen-specific IgA response in the nasal mucosa and simultaneously decreased inflammatory cytokine production (IFN-γ and TNF-α) after infection. The number of activated CD8+ CD44+ T cells and the respiratory syncytial virus- or influenza-specific CD8+ proliferative responses increased, although the total inflammatory infiltrate was reduced. LTK63 treatment matured lung APCs (LTK63 prevented efficient presentation of whole OVA to DO11.10 cells, whereas OVA peptide presentation was unaffected), enhanced immunity in both a Th1 and Th2 environment, was long lasting, and was not pathogen or host strain specific; the protective effects were partially independent of T and B cells. Innate imprinting by toxin-based immunotherapeutics may provide generic protection against infectious disease in the lung, without the need for coadministered pathogen-specific Ag. The Journal of Immunology, 2004, 173: 7435–7443.

Most human pathogens enter and infect their host across a mucosal surface, in particular the gastrointestinal tract or respiratory tract. However, most vaccines are poorly immunogenic when delivered mucosally. This is due to a combination of factors including Ag dilution or denaturation and tight immune regulation at mucosal surfaces. Effective mucosal vaccination against pathogens therefore often requires the codelivery of an adjuvant, although a suitable mucosal adjuvant that is both immunogenic and safe for human use has yet to be developed. Potential adjuvant candidates have been proposed including cholera toxin (CT),3 heat-labile enterotoxin (LT) from Escherichia coli, DNA that contains immunostimulatory CpG motifs, and the codelivery of cytokines, although they are either too toxic or insufficiently immunostimulatory for approval status.

Due to the toxicity of native CT, and specifically wild-type LT (1), several LT mutants have been produced with single-point mutations that inhibit or completely prevent the ADP-ribosylating enzyme activity of the A subunit. These LT mutants are nontoxic but still retain their ability to bind cellular membranes through the B subunit and maintain their immunogenicity (1, 2). The nontoxic LT mutant LTK63 has a serine to arginine substitution at aa 63 of the A subunit. Coadministration with candidate vaccines enhances, e.g., the cytotoxic T cell response to HIV gag-p55 (3) and respiratory syncytial virus matrix proteins (2), increases the protective efficacy of the VacA protein of Helicobacter pylori lyse (4) and induces Ab to tetanus toxin when delivered transcutaneously (5). Delivery via the respiratory tract improves the efficacy of the group C meningococcal conjugate vaccine (6) and enhances immunity to the SAG1 Ag of Toxoplasma gondii (7). The response induced by LT and related LT mutants depends on the degree of retained enzymatic activity. Intact LT synergizes with CpG to improve immunity to hepatitis B surface Ag, whereas nonenzymatic LTK63 does not (8). Coadministration of LTK63 with pertussis Ag enhances both Th1 immunity to Bordetella pertussis, whereas the partially detoxified mutant LTR72 promotes a Th2 environment (9, 10).

The mechanism of how nontoxic LT adjuvants function in the context of enhancing protective immunity to an invading pathogen is poorly understood. In particular, the influence of LT mutants alone on subsequent immune responses has received little attention. We, and others, have previously shown that prior lung infection modulates subsequent immunity and pathology to subsequent unrelated pathogens (11, 12). We now show that administration of LTK63 prevents the immunopathology associated with a number of respiratory pathogens. We use several mouse models of lung

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3 Abbreviations used in this paper: CT, cholera toxin; LT, heat-labile enterotoxin; RSV, respiratory syncytial virus; rVV-G recombinant vaccinia virus expressing the RSV G protein; HA, hemagglutinin; BAL, bronchoalveolar lavage; MDCK, Madin-Darby canine kidney cells.
infection, including the BALB/c model of respiratory syncytial virus (RSV) infection as it provides a useful tool for the study of virus-induced lung immunopathology and mimics the pathology observed in human infants after formalin-inactivated RSV vaccination. Mice primed with the attachment protein G of RSV (expressed by a recombinant vaccinia virus construct rVV-G) and then challenged with live RSV develop Th2-driven pulmonary eosinophilia, weight loss, and cachexia (13). Influenza virus infection of BALB/c mice results in acute pulmonary inflammation and weight loss, providing a model for Th1-induced immunopathology. Furthermore, a model of nonviral lung infection is provided by infection of susceptible C57BL/6 mice with the fungus Cryptococcus neoformans, which induces severe Th2-driven eosinophilia.

Intrasal treatment of mice with LTK63 reduced the cellular inflammation in the lung, abolished pulmonary eosinophilia and weight loss, but did not affect RSV clearance. The immunomodulatory effect of LTK63 was dose dependent, reliant on administration into the lung, was associated with a Th1 cytokine profile and APC maturation. Similarly, prior treatment with LTK63 greatly reduced pathology, inflammation, and pathogen burden after severe influenza virus and C. neoformans lung infection. Alteration of the lung microenvironment by nontoxic LTK63 may therefore provide generic protection against a number of lung infectious diseases by a phenomenon of immune modulation we now term “intra imprinting.” This immunotherapeutic approach provides an alternative prophylactic treatment to contemporary vaccination against respiratory pathogens.

Materials and Methods
Mice and virus stocks
Female BALB/c, C57BL/6, or RAG1 knockout mice (Harlan Olac, Bicester, U.K.), 8–10 wk old, were kept in specified-pathogen-free conditions, according to institutional and Home Office (U.K.) guidelines. RSV (A2 strain) and rVV-G were grown in HEp-2 cells and assayed for infectivity (14). All stocks were free of Mycoplasma as determined by DNA hybridization (Gen-Probe, San Diego, CA). Influenza A strain X31 (hemagglutinin (HA) titer 1024) was a gift from Dr. A. Douglas (National Institute for Medical Research, London, U.K.). C. neoformans strain 52 was obtained from the American Type Culture Collection (ATCC, Manassas, VA (ATCC 24067). Yeast were cultured in Sabouraud dextrose broth (1% yeast extract, 2% dextrose; Difco, Detroit, MI) for 48–72 h until the mycelial growth was at least 10 mm. The mycelia were harvested, washed with 10% BSA-PBS, and suspended in R10F medium at 2 × 10^6 cells/ml. To each well of 10-mm diameter filters to obtain single-cell suspensions, the RBC were lysed, and the cell pellet was resuspended to 1 × 10^6 cells/ml in RPMI 1640 containing 10% FCS, 2 mM l-glutamine, 50 µg/ml penicillin, and 50 µg/ml streptomycin (R10F). Lung tissue was disrupted using 0.8-µm pore size filters to obtain single-cell suspensions, the RBC were lysed, and the cell pellet was resuspended at 1 × 10^6 cells/ml in R10F. Cell number was quantified using a hemocytometer and trypan blue exclusion. A single lobe of lung tissue was fixed in 2% formaldehyde and embedded in paraffin. Sections were stained with H&E.

Enumeration of eosinophils
Eosinophils were enumerated by flow cytometry based on their size (forward scatter) and granularity (side scatter). From cytospin preparations, the proportion of eosinophils was confirmed by their distinctive nuclear morphology and presence of acidophilic red granules. Neutrophils were identified by the presence of a multilobed nucleus and the absence of acidophilic granules.

Flow cytometry
Cells (1 × 10^6) obtained from the airways or the lung were stained with the following Ab combinations: 1) anti-CD44-PE, anti-CD45RB-FITC, anti-CD-APC, and anti-CD8-PerCP; 2) anti-B220-PerCP, anti-4-48E8-FITC, anti-CD80-PE, and anti-CD40-APC; 3) to detect intracellular cytokines, 1 × 10^6 cells were incubated with 50 ng/ml PMMA (Sigma-Aldrich), 500 ng of 3,3′-diaminobenzidine (DakoCytomation, Carpinteria, CA), and 10 µg/ml brefeldin A (Sigma-Aldrich) for 4 h at 37°C. Cells were stained with anti-CD4-APC and anti-CD8-PerCP on ice for 30 min, and then fixed in 2% formaldehyde for 20 min at room temperature. Cells were permeabilized with 0.5% saponin in PBS containing 0.1% BSA and 0.01% azide for 10 min. A combination of anti-TNFα-FTTC or anti-IFNγ-FTTC and anti-IL-4-PE or anti-IL-5-PE Abs, diluted in saponin buffer, was then added to the cells. After 30 min, cells were washed once in saponin buffer and twice in PBS containing 0.1% azide and 1% BSA; and 4) TCR Vβ expression was determined on cells stained with anti-CD4-PerCP and anti-CD8-APC using the screening panel kit from BD Biosciences (Oxford, U.K.), which contains FITC-conjugated Abs to 15 different murine Vβ gene products. Samples were analyzed on a FACS Calibur (BD Biosciences), collecting data on at least 30,000 lymphocytes.

RSV- and influenza-specific proliferation assays
Spleens were removed from individual mice under sterile conditions and passed through a 0.8-µm pore size filter to produce a single-cell suspension. By RBC lysis in 0.13 M ammonium chloride, 1 M potassium hydrogen carbonate, and 0.01 mM EDTA, pH 7.2, spleen cells were resuspended in R10F medium at 2 × 10^6 cells/ml. To each well of 10-mm bottom 96-well plate, 4 × 10^6 cells were added together with 25 µg of purified G protein peptide (AICKRPICKPKPGKKT) or 25 µg of nonstimulatory G protein peptide (AICKRPKNPKPGKKT) in which the anchor lysine residue at position 193 had been changed to asparagine. All samples were analyzed in triplicate. After 48 h at 37°C, 50 µCi/ml [3H]thymidine (Amersham, Little Chalfont, U.K.) were added to each well for a further 24 h. Cells were harvested, the incorporated [3H]thymidine was transferred to a filter, and the β emissions were read using a scintillation counter. The proliferation index was calculated by dividing the value obtained for thestimulatory peptide by the equivalent well containing control peptide. Following an identical method, influenza-specific proliferation assays were conducted on Ficol/lives (Sigma-Aldrich) purified lymphocytes from the lung. Mouse MHC class II Abs (clone TYO1) or TRAVL as the stimulus and the RSV peptide SYIGSINNI as control peptide. The incorporation of [3H]thymidine was counted in the same way.

IgA-specific Ab ELISA
Immuno Maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated with either sonicated RSV Ag or UV-inactivated influenza virus (100 HA/ well) in PBS and incubated at 4°C overnight. Plates were washed with PBS-Tween (0.05%) and between each subsequent step. To each well, 200 µl of 10% BSA-PBS were added and incubated at room temperature for 90 min. Bovine serum albumin fluid was diluted 1/2 in 10% BSA-PBS, and each sample was incubated in duplicate for 2 h at room temperature. Then 100 µl of bioneutralized anti-IgA (Serotec, Oxford, U.K.) diluted 1/500 were added to each well and incubated for 1 h at room temperature. To each well, 100 µl of streptavidin-HRP (DakoCytomation, Carpenteria, CA) diluted 1/500 were added and incubated for 1 h at room temperature. Finally 100 µl of substrate o-phenylenediamine (Sigma-Aldrich) solution were added to each well and incubated for 30 min; the reaction was stopped with 2 M H2SO4. Plates were read at 490 nm, and optical density was compared between sample groups (no standard was available for quantification).
Results

LTK63 modifies the lung microenvironment in naive mice

Prior lung immune stimulation by pathogens alters the immune response to subsequent heterologous infection (17–19). This reduces overexuberant inflammation, which can cause morbidity and mortality. Modulation of the lung microenvironment by whole pathogens is not therapeutically viable. Therefore, we investigated the effect of a nontoxic E. coli-derived labile toxin (LTK63) on the lung microenvironment. Naive BALB/c mice were treated i.n. with 5 μg of LTK63 and lung compartments periodically sampled over 28 days. Lymphocytic (T and B cell) expansion within the lung tissue primarily accounted for the mild increase in total cellularity (Fig. 1A). Both CD4+ and CD8+ T cells (Fig. 1B) increased in number; the proportion of these subsets was, however, constant throughout the analysis (data not shown). The most striking effect exerted by LTK63 was on the APC compartment. The proportion and total number of B cells increased, as did their expression of markers of activation (MHC class II, CD40, and CD80; Fig. 1C). Although the proportion of granulocytes and macrophages remained constant, the macrophage population expressing CD80 and MHC class II increased (Fig. 1D). LTK63 induced a Th1-type cytokine profile in the lung, with increased IFN-γ and TNF-α (Fig. 1E) but not IL-4 or IL-5 (data not shown). The enhanced state of activation of T cells and APCs persisted for 14 days. By day 28, the lung had essentially returned to a pretreatment condition. Furthermore, changes in CD4+ and CD8+ T cell clonality after LTK63 treatment were analyzed based on the proportion of cells expressing different Vβ TCRs. The CD4+ Vβ1.8,2 population and the CD8+ Vβ2- population increased in the lung (Fig. 1, F and G). These populations, however, receded with time and were back at preinfection levels by day 14.

LTK63 prevents weight loss during respiratory viral infection

Respiratory virus infection in mice is associated with profound weight loss due to excessive cellular infiltration and the production of inflammatory cytokines. In BALB/c mice, prior vaccination with rVV-G induces up to 25% weight loss after intranasal whole virus challenge (G/RSV) (20). Human infection with influenza A virus typically causes tracheobronchitis and occasional fatal pneumonia (21, 22). In mice, influenza A virus infection induces similar pulmonary inflammation with an infiltration of neutrophils and Th1-driven CD8+ T cells. To therapeutically manipulate the lung immune cell compartments, mice were treated with 5 μg of LTK63 before G/RSV or influenza virus infection. LTK63 prevented weight loss in G/RSV mice (Fig. 2A) when given i.n. but not i.p., and prevented weight loss in mice infected with influenza virus (Fig. 2B). In both infections, the reduced weight loss was associated with reduced cellular infiltration into the lung (Fig. 2, C and D) and the higher the dose of LTK63, the greater was the reduction in inflammation (Fig. 2C) as shown in the G/RSV model.

LTK63 prevents eosinophilic inflammation during respiratory infection

Vaccination with rVV-G followed by intranasal RSV challenge induces Th2-driven pulmonary eosinophilia in BALB/c mice (23). Similarly, a primary infection of C57BL/6 mice with C. neoformans induces extensive lung eosinophilia again driven by induction of Th2 cells (24). We determined the effect of LTK63 treatment on eosinophilia in these two model systems. Intranasal but not intraperitoneal LTK63 treatment reduced airway eosinophilia in G/RSV mice (Fig. 3A). This effect was dose dependent (Fig. 3B)
given that 0.2 μg of LTK63 had minimal effect, 1 μg reduced pulmonary eosinophilia in 3 of 4 mice, whereas a 5-μg dose completely ameliorated eosinophilia (neutrophils predominated). To determine the long term effects of LTK63, mice were scarified with rVV-G and challenged with RSV 12 wk after LTK63 treatment. Mice treated with LTK63 had less weight loss (Fig. 3C), reduced pulmonary eosinophilia (Fig. 3D), a reduction in activated CD4+CD44+ T cells, and a reduction in TNF-α production (data not shown) compared with PBS-treated controls. Although the effects of LTK63 treatment were shown to be long lasting, the extent of these effects was less significant than administration with a shorter interim before infection. A similar reduction in airway eosinophilia was observed in LTK63-treated C57BL/6 mice after challenge, whereas those given 5 μg of LTK63 had only 7,083 ± 87 (p < 0.05). IFN-γ production dominated over IL-5 in both CD4+ (Fig. 5A) and CD8+ (data not shown) T cell subsets, which was dose dependent. In addition, this effect was only evident in mice given LTK63 i.n. and not i.p. (Fig. 5B). Treatment of mice with 5 μg of LTK63 14 days before influenza infection also reduced the proportion and total number of CD4+ T cells producing IFN-γ (Fig. 5C) and IL-5 (Fig. 5D). Despite reduced cytokine production, significant RSV- or influenza-specific IgA was detected in nasal washes taken from mice infected with G/RSV (Fig. 5E) or influenza virus (Fig. 5F). In both cases, prior intranasal LTK63 treatment enhanced recovered Ag-specific IgA.

LTK63 improves lung histology and either does not affect or improves pathogen clearance

Histological (H&E) analysis of lung sections confirmed that prior LTK63 treatment significantly reduced the inflammatory infiltrate following G/RSV (Fig. 6A), influenza virus (Fig. 6B) and C. neoformans (Fig. 6C) infections. Analysis of pathogen titer in lung homogenates showed that LTK63 had no effect on RSV titers 7 days after infection (Fig. 6D) but markedly reduced both influenza
LTK63 modifies Ag processing and exerts its influence independent of T and B cells

The data presented thus far imply that LTK63 stimulates a mild Th1 environment in the lung and matures APCs by increasing MHC class II, B7 costimulatory molecules, and CD40. The outcome and immune phenotype of any subsequent lung infection tested to date are altered beneficially. Although total T cells are reduced during pathogen challenge, of those remaining the proportion of activated cells is increased. This may imply an alteration in pathogen processing and presentation. We therefore tested the ability of peritoneal lavage cells (>95% macrophages by flow cytometry) to process and present whole OVA to OVA-specific T cells (DO11.10 T cells) and compared the proliferative responses with those of OVA-specific peptide. Untreated peritoneal macrophages efficiently induced proliferation of DO11.10 T cells in the presence of OVA or OVA peptide. However, macrophages removed from LTK63-treated mice were effective at eliciting T cell proliferation in response to peptide but not whole OVA (Fig. 7A).

This suggests that presentation of peptides to Ag-specific T cells is not affected by LTK63 but that processing of whole Ags is reduced.

The alteration of lung APCs by LTK63 may explain the difference in subsequent immune pathology to unrelated infections. To prove the impact of LTK63 modulated APCs on subsequent infection we removed alveolar cells by lavage (>95% macrophages) from RAG<sup>−/−</sup> mice that had been pretreated or not with 5 μg LTK63 3 wk earlier. Cells (1 × 10⁵) were transferred i.n. into immunocompetent littersmates. Mice were then infected with influenza virus 1 day later, and weight loss was evaluated. Transfer of LTK63 but not PBS-treated lung cells reduced weight loss during subsequent influenza virus infection (Fig. 7B). These results imply that the protective influence of LTK63 is partially independent of T and B cells.
FIGURE 4. LTK63 enhances the proportion of activated T cells and proliferative responses to specific Ag. LTK63 (5, 1, or 0.2 µg) or PBS was administered i.n. to 8–10 wk old BALB/c mice 2 wk before scarification with rVV-G and challenged i.n. with whole RSV 2–3 wk later. The proportion of cells expressing both CD44 and CD4 (A) or CD44 and CD8 (B) was determined by flow cytometry 7 days after RSV infection. A similar analysis was performed on CD4 (C) and CD8 (D) T cells from influenza-infected mice previously given 5 µg of LTK63 i.n. The proliferative response of CD8+ T cells from mice pretreated with LTK63 and challenged with G/RSV (E) or influenza virus (F) was tested in a [3H]thymidine incorporation assay. The stimulation index was calculated by dividing the cpm to the immunodominant T cell peptide (AICKRIPNKKPGKKT) from the G-protein of RSV by the control peptide (AICKRIPNKPGKKT) in which the major MHC class II E6 anchor region had been substituted (E). Cells were incubated with the immunodominant CD8 epitope for RSV (SYIGSINNI) or influenza (TYQRTRALV), LTK63 itself, or Con A (F). F, □, influenza-infected mice pretreated with PBS; ■, those pretreated with LTK63. Average values are represented as horizontal bars ± SEM. **, p < 0.05 or *, p < 0.1 compared with the PBS-treated sample group.

Discussion
We have shown that prior administration of a modified bacterial toxin (LTK63) that lacks toxicity but retains immunogenicity prevents immunopathology to a number of respiratory infections. This is the first time that the protective capabilities of a bacterial toxin have been tested in isolation without admixed Ag. Treated mice still develop powerful Ab responses to each virus but lack the exuberant cellular inflammation characteristic of these infections. Moreover, LTK63 prevents eosinophilic disease in mice previously sensitized with the attachment G protein of RSV or infected with C. neoformans. We believe that the major influence in protection is the maturation of APCs, because we were able to transfer the LTK63-modified environment from mice lacking B and T cells (RAG−/− mice).

There have been several major obstacles that have prevented the development of effective vaccines against respiratory infections: 1) natural infection does not always confer immunity to the recurrence of disease; 2) the induction of powerful cellular immune responses has been associated with an increase in disease severity during subsequent infection (25, 26). This effect was seen when children were given formalin-inactivated RSV virus vaccines in clinical trials during the 1960s (27). Subsequent infection led to greatly increased hospitalization rates and a small number of vaccine-related deaths; 3) there is a lack of appropriate adjuvants suitable for the induction of long term immunity, particularly for the generation of effective anti-viral CTL responses; 4) stimulating immunity at the site of infection, e.g., the mucosa of the respiratory tract, is still problematic. Therefore, alternative approaches have been sought that exploit the host immune response by means of immune modulation or immune intervention, rather than contemporary vaccination.

The use of the nontoxic LTK63 fulfills many of the criteria required for an effective protection strategy against respiratory infection. It induces appropriate immune responses to both viral and nonviral pathogens, and to both Th1- and Th2-driven disease or immunopathology. LTK63 treatment effectively matures the APC compartment of the respiratory tract and induces potent CD8+ T cells and local IgA production. Excessive inflammation is eliminated without sacrificing effective pathogen clearance, and it is most effective at the site of pathogen entry and replication. In addition, the i.n. route of administration benefits from being noninvasive, and the beneficial effects are long lasting. Furthermore, treatment with LTK63 also bypasses the problem of antigenic shift or drift in surface exposed pathogen proteins because the mechanism of protection is not restricted to one Ag. This strategy may also negate the "unnatural immunity" described by Casadevall and Pirofski (28), who highlight that vaccines often induce immune responses that differ from the one elicited by natural infection (29). LTK63 primes the lung to cope with infection more efficiently but not in a way that one would anticipate a vaccine working.

Treatment with LTK63 significantly alters the APC compartment by increasing MHC II-, CD80-, and CD40-expressing cells. The ability of LT and mutant derivatives to modify dendritic cells, macrophages, and B lymphoma cells in vitro has been described.
FIGURE 6. LTK63 improves lung histology and enhances pathogen clearance from the lung. Mice were given PBS (top row) or 5 μg of LTK63 (bottom row) 2 wk before G/RSV (A), influenza virus (B), or C. neoformans (C). Lungs were formalin fixed and embedded in paraffin at the peak of the inflammatory response (day 7 in A and B and day 12 in C), and sections were stained with H&E. Pathogen titers were enumerated at multiple time points in G/RSV (D), influenza virus (E), or C. neoformans (F)-infected mice at days 7, 4, and 12, respectively. The results represent the mean ± SD for 5 mice (D and E) or individual mice (F) and are representative of two to three independent experiments. Average values are represented as horizontal bars ± SEM; **, p < 0.05 compared with the PBS-treated sample group.

FIGURE 7. LTK63 treatment enhances non-T and B cells. BALB/c mice were treated with either PBS or 5 μg of LTK63 1 wk before cell recovery. Peritoneal cells were removed by lavage and washed before addition of OVA or OVA-specific peptide. 24 h later 5 × 10⁶ DO11.10 T cells were added and cultured for 72 h, with the inclusion of 1 μCi of [³H]thymidine for the last 16 h. Results show the mean and SD from four separate experiments containing triplicate samples (A). In B, RAG⁻/⁻ mice were given 5 μg of LTK63 or PBS i.n., and the lungs were removed after 14 days. Single lung homogenate cells (10⁷) were transferred i.n. into four immunocompetent congenic mice. Weight loss during intranasal influenza virus infection was monitored for 7 days. The results show the mean ± SD of five separate mice. **, p < 0.05 compared with the PBS-treated sample group.

before (10, 30, 31), the overall requirement for any effect being at least partial ADP ribosyltransferase activity. Our data clearly show that the mutant LTK63, devoid of enzymatic activity, can induce profound effects on APCs in vivo. The discrepancy can be explained in many ways, not least that in vivo studies may highlight indirect influences of LT mutants on APCs, that the toxins may function differently in vivo, and/or that the effects observed require the concerted action of several cell types not present in in vitro cultures.

Similar to wild-type LT, nasally administered LTK63 promotes a mild Th1-type cytokine environment (32) and may therefore be useful in imprinting this phenotype on the lung, a phenomenon we term innate imprinting. This would be particularly useful in infants at risk from the development of asthma or viral-induced atopic disorders. Recently, studies indicate that immune responses in children are predominantly skewed to a Th2 phenotype at birth and become more Th1 biased as they experience subsequent infection (33). In support of this, infants generally produce lower levels of IFN-γ, but this gradually increases with age (34). LTK63 could therefore provide the necessary Th1 trigger without the need for infection. The ability of LTK63 to induce a Th1 cytokine environment is also due to the production of TNF-α and likely the production of IL-12 by APCs (10). The absence of enzymatic activity is critical in this regard because wild-type CT inhibits IL-12 production by dendritic cells and impedes IL-12 receptor expression on activated T cells (35).

The effect of LTK63 treatment on the T cell population is reminiscent of studies modulating the lung microenvironment using whole pathogens (11). Although wild-type CT and LT are thought to cause lymphocyte apoptosis, mutants with reduced or absent toxicity do not (36). Although T cell numbers were reduced, the proportion of activated cells was enhanced in LTK63-treated mice. We suggest that reduced T cell numbers may arise under the following conditions.

Reduced Ag processing
As did Petrovska et al. (30), we noted that LTK63-stimulated peritoneal macrophages were less efficient at stimulating Ag-specific T cells. Reduced pathogen Ag processing would therefore result in a reduced level of inflammation. Presentation of peptide Ag, however, was unaffected.

Increased efficiency of T cell activation
APCs must undergo maturation to efficiently prime T lymphocytes (for a review, see Ref. 37). LTK63 therefore enhances APC maturation, thus increasing the efficiency of T cell priming (38, 39). The T cell pool therefore does not have to expand excessively to combat the infection. Indeed, influenza virus and C. neoformans were cleared more rapidly by LTK63 pretreatment.
Refractory microenvironment

Prior LTK63 treatment may reduce the amplitude of subsequent cellular responses. This may involve the induction of IFN-α/β and MHC class I expression by respiratory epithelial cells, the activation of a resident NK cell population, and the production of IL-12 by dendritic cells. In addition, dendritic cells become refractory to subsequent stimulation (40). Furthermore, alveolar macrophages are essentially nondividing cells (41) and display a relatively long turnover time (42, 43) and thus become refractory relatively easily. This results in a diminished cellular recruitment, prevents disease-induced weight loss and lung pathology, but still allows pathogen clearance. These processes are likely to act in concert with the direct stimulation of cell surface receptors, such as ganglioside receptors, which activate immune cells in a way similar to that of pathogen-associated molecular patterns binding to pattern recognition receptors. Alternatively, although not exclusively, LTK63 treatment may enhance T cell bystander activity analogous to viral-induced heterologous immunity (11), which may partially explain the longevity of LTK63 treatment.

The increase in pathogen-specific, nasal IgA is an important effect of LTK63 treatment. Naturally acquired Abs occur spontaneously after Ig gene rearrangement, are of low specificity, and are predominantly IgM; but IgG and IgA isotypes are known to exist and are often cross-reactive (44, 45). Sera from naive mice contain Abs capable of reacting with purified preparations of lymphocytic choriomeningitis virus, vaccinia virus, Listeria, and vascular stromatosis virus (45, 46). So-called natural Abs are an evolutionary advantage because they enhance pathogen phagocytosis and presentation of Ags in organized lymphoid tissue. Enhanced IgA production to influenza hemagglutinin and pneumococcal polysaccharides is evident in mice given either of these proteins admixed with LTK63 (47, 48). However, in both studies, the production of IgA after LTK63 treatment alone was not described. Controlled or reduced pathogen titers after LTK63 treatment may therefore reflect a combination of efficient T cell priming by activated APCs and binding of pathogens by additional nasal IgA.

Many vaccination strategies increase the frequency of Ag-specific T cells but fail to increase the availability of mature APCs in the appropriate compartment. We show that LTK63 matures APCs so that Ag presentation is more efficient but Ag processing is curtailed. Smaller numbers of T cells will therefore be activated but in a globally more efficient manner. The transfer of LTK63-treated airway cells from RAG knockout mice highlights the influence of non-T and -B cells. We therefore propose that prior stimulation of the lung microenvironment produces long-lived, or indeed irreversible, changes, which may include alterations in APCs and chemokine receptors or adhesion molecule expression profiles. The first signal to the developing lung will therefore influence all subsequent immune responses, a phenomenon we call innate imprinting.

The use of live, even attenuated, vaccines is contraindicated in hosts with impaired immune function, first because they may not mount an efficient immune response and, secondly because the vaccine pathogen itself may disseminate or persist. LTK63 would not necessarily suffer from these problems and may afford generic protection against a number of unrelated pathogens.

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