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The Acquired Immune Response to the Mucosal Adjuvant LTK63 Imprints the Mouse Lung with a Protective Signature

Elaine Tritto,* Alessandro Muzzi,* Isabella Pesce,* Elisabetta Monaci,* Sandra Nuti,* Grazia Galli,* Andreas Wack,* Rino Rappuoli,* Tracy Hussell,† and Ennio De Gregorio†*†

LTK63, a nontoxic mutant of Escherichia coli heat labile enterotoxin (LT), is a potent and safe mucosal adjuvant that has also been shown to confer generic protection to several respiratory pathogens. To understand the mechanisms of action underlying the LTK63 protective effect, we analyzed the molecular and cellular events triggered by its administration in vivo. We show here that LTK63 intrapulmonary administration induced in the mouse lung a specific gene expression signature characterized by the up-regulation of cell cycle genes, several host defense genes, chemokines, chemokine receptors, and immune cell-associated genes. Such a transcriptional profile reflected the activation of alveolar macrophages and the recruitment to the lung of T and B cells and innate immune cells such as granulocytes, NK, and dendritic cells. All of these events were T cell dependent and specific for LTK63 because they were absent in SCID and nude mice. Additionally, we showed that LTK63 induces a potent adaptive immune response against itself directed to the lung. We propose that acquired response to LTK63 is the driving force for the local recruitment of both adaptive and innate immune cells. Our data suggest that LTK63 acts as an airway infection mimic that establishes a generic protective environment limiting respiratory infection by innate immune mechanisms and by improving adaptive responses to invading pathogens. The Journal of Immunology, 2007, 179: 5346–5357.

The mucosal surface of the airways is a large area of contact with many inhaled Ags and is the first point of entry for respiratory pathogens. Therefore, mucosal immunity is believed to have great potential as a first line of defense against invading microorganisms in the airways (1). Several reports have shown that protective immune responses against respiratory infections can be achieved by delivering the Ag directly to the mucosal surface, provided that the Ag is delivered together with a potent mucosal adjuvant (2). Unfortunately, the number of known mucosal adjuvants is limited, the best characterized being the heat labile enterotoxin from Escherichia coli (LT),2 Vibrio cholerae secreted enterotoxins, CpG-containing oligodeoxynucleotides, and an LPS mimic, monophosphoryl lipid A (2–4). LT is an ADP-ribosylating enterotoxin belonging to the A/B family of toxins that are made up by two structurally distinct components, an A subunit containing the enzymatic catalytic site and the pentameric B subunit that binds to GM1 (Gal(β1–3)GalNAc(β1–4)NeuAc(α2–3)Gal(β1–4)Glc(β1–1)ceramide), a ganglioside ubiquitously expressed on the surface of most mammalian cells, and to a lesser extent to other gangliosides, GM2 and asialo-GM1 (5). The binding of the B pentamer promotes the internalization of LT into target cells followed by processing of the complex and release of the A subunit into the cytosol, where it catalyzes the ADP-ribosylation of trimeric G proteins (6). When used as vaccine adjuvant, LT has been shown to enhance Ag presentation, stimulate T cell proliferation and cytokine production, and promote strong mucosal IgG and IgA Ab responses (7, 8). Besides stimulating the immune response directed against a coadministered Ag, LT is also highly immunogenic, eliciting strong humoral and cellular responses against itself (9). To circumvent the harmful drawbacks of the native LT toxin, several LT mutants with significantly reduced toxicity have been generated (10). LTK63 is an LT mutant with a serine-to-lysine substitution in position 63 of the A subunit and has a ∼0.6% residual ADP-ribosyltransferase enzymatic activity (9). Finally, LTB consists of the B pentamer alone. These LT variants have been tested in mice with a large number of Ags by different routes of administration (12–17). Similarly to LT, both LTK63 and LTR72 are highly immunogenic and are able to enhance the immune response against coadministered Ags, whereas LTB is less immunogenic and displays poor adjuvanticity (6). Recently, LTK63 has been tested as mucosal adjuvant for an intranasal, trivalent, inactivated influenza vaccine in a phase I clinical trial, demonstrating a good safety profile and mucosal adjuvanticity in human (18).

In addition to vaccination, protection of the mucosal surfaces can be elicited by several non-antigenic immune-stimulatory compounds; however, little is known about the molecular and cellular mechanisms associated with such generic protection (4, 19, 20). Pulmonary administration of CpG to the mouse lung protects from Cryptococcus neoformans challenge in an IL12-dependent manner (20). Similarly to LT, both LTK63 and LTR72 are highly immunogenic and are able to enhance the immune response against coadministered Ags, whereas LTB is less immunogenic and displays poor adjuvanticity. In these infection models the net effect of LTK63 pretreatment is characterized by enhanced adaptive responses to the infectious agent,

References

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reduced inflammation and tissue damage, and more efficient clear-
ance of the pathogen. In particular, in the case of influenza infec-
tion mice pretreated with LTK63 display reduced lung viral bur-
pron, negligible weight loss, and improved flu-specific CD4+ and
CD8+ T cell response (21).

Despite the great potential of LTK63 as vaccine adjuvant and
immune therapeutic, little is known about its mechanism of action
in vivo. To identify the genes and cell types involved in LTK63-
mediated generic protection, we monitored the effect of LTK63 by
combining oligonucleotide microarray analysis of the lung tran-
scription profile and FACS analysis of lung infiltrate. We show
here that intrapulmonary administration of LTK63 up-regulates
several classes of host defense genes in the lung and triggers T
cell-dependent leukocyte recruitment and the activation of alveolar
macrophages in the airways. On the basis of these results we dis-

cuss the possibility that LTK63 protects against respiratory patho-
gens by innate immune mechanisms and by improving adaptive
immune responses.

Materials and Methods

LTK63 formulation and immunization protocol

We found that the addition of 0.25% CHAPS and 200 mM arginine to the
LTK63 preparation in PBS increased LTK63 stability at 4°C by preventing
precipitation of the complex and dissociation of the A subunit from the B5
pentamer (data not shown). Therefore, LTK63 was formulated in buffer L
(0.2 M arginine, 0.25% CHAPS, 200 mM NaHPO4, in PBS (final pH 7.4)).
All preparations of LTK63 used in this study were tested for GM1 binding.

Buffer L or 5 μg of LTK63 in buffer L were delivered intrapulmonarily
by administering a total volume of 50 μl (25 μL/rostri) to anesthetized
mice (22). Anesthesia was performed by injecting i.p. 100 μL of solution
containing 50 mg/kg ketamine plus 2.6 mg/kg xylazine.

Wild type and nude BALB/c mice, as well as wild-type and SCID Fox
Chains-17 mice, were purchased from the National Cancer Institute/River Laboratories. All animals were housed and treated according to internal animal ethical com-
mittee and institutional guidelines.

Lung RNA extraction and purification

Whole lungs were homogenized in 5 ml of TRIzol (Invitrogen Life Tech-
nologies) with an Ultra-Turrax T25 and the total RNA was extracted from
the tissue following the manufacturer’s protocol. One hundred micrograms of
RNA from each lung was purified using RNAeasy RNA purification col-
umns following the producer’s protocol (Qiagen). The residual DNA was
removed by an additional on-column DNase digestion step using the Qui-
gen RNase-free DNase set. RNA quality was assessed by using the auto-
mated Experion electrophoresis system (Bio-Rad) coupled with the RNA
StdSens analysis kit (Bio-Rad) following the producer’s protocol. Lungs
for microarray analysis were taken from three mice per group 3, 6, and
12 h and 1, 2, 4, 6, 14, and 28 days after treatment. BAL was performed
by injecting 1.5 ml/mouse PBS and 0.1% BSA intratracheally. Cells were
washed with PBS plus 5% FCS and plated into a U-bottom 96-well plate
and incubated for 5 min with 20% rabbit serum before the addition of either
Ab mix 1 or Ab mix 2. Ab mix 1 was used to define the lymphocyte subsets
and was composed of the following mAbs: anti-CD3-PerCP Cy5.5, anti-CD4-
PE, anti-CD8-biotin, anti-DX5-FITC, and anti-B220-allophycocyanin (all BD
Pharmingen). Ab mix 2 was used to define the polynuclear cells (PMN), monocytes, macrophages, and dendritic cells (DCs), and was
composed of anti-CCR3-PE (R&D Systems), anti-CCR5-biotin, anti MHC-II-FTTC, anti-CD11b-PE Cy7, anti-CD11c-allophycocyanin, anti CD3-
PerCP Cy5.5, and anti-B200-PerCP Cy5.5 (all from BD Pharmingen). Anti-
CD8 and anti-CCR5 biotinylated Abs were revealed by a second incubation
step with streptavidin-Pacific Blue. All incubations were conducted for 20 min
on ice in the dark. At the time of reading, samples were diluted in equal
volumes of PBS and the same amount of BD Pharmingen counting beads
was added to normalize the data. Data acquisition was stopped after counting
10,000 beads for each sample.

Three different cell populations were morphologically identified on the
basis of their forward (FSC) vs side (SSC) scatter profile. Lymphocytes were
identified as FSC<15SSC<75, granulocytes as FSCmedium SSCmedium,
and monocytes/DCs as FSChighSSC<75. Within the lympho-
cyte gate, T lymphocytes were identified as CD4+CD3+ or CD8+CD3-
NK cells were identified as CD3 DX5+, and B lymphocytes as CD3DX5
B20+. Within the granulocyte population, eosinophils were identi-
fied as MHC class II CCR3+ and neutrophils as MHC class II CCR3
(25). Within the monocyte/DC gate, alveolar macrophages were identified as
MHC class II CD11b+ whereas DCs were identified as MHC class II
CD11b+CD11c+. For flow cytometric analysis of the lung single cell suspension, lungs
were taken from four mice per group 12 h, 1, 2, 4, 6, 8, and 14 days after

treatment with LTK63 or buffer L and were cut into small pieces in cold
HBSS medium (Invitrogen Life Technologies). The dissected tissue was
then incubated in HBSS containing 5% FBS (HyClone), 20 μg/ml DNAse I
(Roche), and 200 U/ml type I collagenase (Invitrogen Life Technologies) for
45 min at 37°C under gentle swirling. The digested samples were then
centrifuged and further incubated in Ca2+ Mg2+-free HBSS containing 10
mM EDTA for 5 min at room temperature. Finally, samples were filtered
through a 70-μm cell strainer (BD Biosciences). RBCs were removed by a

RNA microarray data analysis

Raw images were initially analyzed using GenePix 6.0 software (Molecular
Devices). The data were then transferred to the BASE 1.2 database/analysis
software (23). For each spot, local background was subtracted from the
mean fluorescence intensity of the Cy3 and Cy5 dyes. Spot intensities were
then normalized by the global median. Spots with a signal-to-noise ratio ≤
3 in both channels or manually flagged for bad quality were filtered. The
average intensity ratio of repeated spots from experimental repetitions was
estimated by geometric mean and the accuracy and statistical significance of
the observed ratios were determined using the Student’s t test. Only genes having a t test p values <0.05 and average intensity ratios >4 (log2
ratio >2) in one time point were selected. Hierarchical clustering was per-
formed with The Institute for Genomic Research (TIGR; Rockville,
MD) Multiexperiment Viewer (MeV) 3.1 software (24) on the log2-trans-
formed data set applying the Euclidean distance matrix and the average
linkage clustering method. The complete set of microarray data has been
submitted to the ArrayExpress database EMBL-EBI (http://www.ebi.ac.uk/
arayexpress/) with accession number E-TABM-303.

Real-time PCR analysis of CXCL10 expression

For each sample 5 μg of RNA extracted from the lungs was retrotrans-
cribed into cDNA with the SuperScript reverse transcriptase transcriptase (200
U/ml; Invitrogen Life Technologies) using the manufacturer’s protocol.
Real-time PCR to detect and measure CXCL10 gene transcripts was con-
ducted with the Brilliant SYBR Green QPCR master mix (Stratagene)
using the oligonucleotides 5′-AGTCCTAATTCCCTGTGTT
TTC-3′ (forward primer) and 5′-CGTCGGACCTCACATAG-3′ (re-
verse primer). As a control, the housekeeping ribosomal protein 10
(RPS10) was amplified in parallel using the oligonucleotides 5′-GG
GACAGGAGACCTAC-3′ (forward primer) and 5′-ATACACATA
AUCTCAACCTGCAC-3′ (reverse primer).

FACS analysis

All samples were analyzed using a FACS LSR-II device and the FACS
Diva software. For flow cytometric analysis of cells recovered in the bron-
chialalveolar lavage (BAL), lungs from eight mice per group were taken 8
days after treatment and lungs from three mice per group were taken 6
and 12 h and 1, 2, 4, 6, 14, and 28 days after treatment. BAL was performed
by injecting 1.5 ml/mouse PBS and 0.1% BSA intratracheally. Cells were
washed with PBS plus 5% FCS and plated into a U-bottom 96-well plate
and incubated for 5 min with 20% rabbit serum before the addition of either
Ab mix 1 or Ab mix 2. Ab mix 1 was used to define the lymphocyte subsets
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10,000 beads for each sample.

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short incubation step in ammonium chloride potassium carbonate (ACK) solution. Cell viability was determined by trypan blue dye exclusion. To reduce nonspecific Ab binding, cells were incubated with the Fc block reagent (anti-CD16/CD32; BD Pharmingen) for 10 min before the addition of the Ab mixture. Cells were morphologically identified on the basis of their FSC vs side SSC profile. Within the lymphocyte gate, CD4+ T lymphocytes were identified as CD4+ CD3+ , CD8+ T cells as CD8+ CD3+ , and NK cells as CD3+ DX5+ using the same mAbs adopted in the BAL staining. In addition, to determine the activation of T and NK cells the mAbs anti-CD44-allophycocyanin (BD Pharmingen) and anti-CD69-PerCP Cy5.5 (BD Pharmingen) were used. Isotype-match-matched mAbs (PE, Pacific Blue, FITC, allophycocyanin, and PerCP-Cy5.5-conjugated) were used to determine background staining.

Measure of LTK63-specific immune responses
To assess LTK63-specific B and T cell responses spleens, lymph nodes, sera, BAL, and nasal washes were taken at day 14 after treatment with buffer L or LTK63 from groups of 3–8 mice. LTK63-specific Abs in BAL fluids, sera, or nasal washes were measured by endpoint ELISA as described (9). Ab titers were expressed as the geometric mean titer of the Ab mixture. Cells were morphologically identified on the basis of the Ab mixture. Cells were incubated with the Fc block reducing nonspecific Ab binding, cells were incubated with the Fc block

Results
Transcriptional changes induced by LTK63 in mouse lung
We examined genomic scale changes in the gene expression profile induced in the whole mouse lung from 3 h to 14 days after the pulmonary administration of 5 μg of LTK63 by using whole genome oligonucleotide microarrays. As a control, we examined the transcriptional changes induced in the lung after the administration of control buffer (buffer L) under the same experimental conditions. For each time point we performed three independent experiments and calculated the mean expression ratios. After data quality check and filtering, we selected 712 genes having an absolute average fold change >4 (log, of ratio ≥ [2]), and a p value <0.05 in at least one time point tested. For details on data filtering and p value calculations see Materials and Methods. The selected genes were hierarchically clustered based on the expression profile using TIGR Multiexperiment Viewer software and examples of subtrees are shown in Fig. 1. We identified an early response cluster of genes up-regulated 3–12 h by both LTK63 and buffer L. In this cluster we found genes, such as coagulation factor III (F3) and metallothionein 2 (M2), induced by tissue damage and cell stress (Fig. 1A). Starting 12 h after administration, TIGR Multiexperiment Viewed Viewer identified a second cluster of genes associated with the cell cycle such as CDC28 subunits, cyclins, cyclin-dependent kinases (Cks2 and Cks1b), and mitotic checkpoint components (Cdc2a, Cdc23) (Fig. 1B). These genes were transiently up-regulated by LTK63 from 12 h to 8 days but not in response to buffer L. The majority of the up-regulated genes (112 genes) were induced by LTK63 at 6–8 days but not by the control buffer. Four representative subtrees of this large cluster are shown in Fig. 1, C–F. This cluster included genes involved in pathogen recognition (peptidoglycan recognition protein PGRP1 (Fig. 1F)), inflammation (IL18bp and prostaglandin E receptor), chemokine receptor genes (CCR5, CXCR6 (Fig. 1C), and CCR2 (Fig. 1E)), chemokines (CCL4, CCL8, CCL13 (Fig. 1E), CCL5 (Fig. 1F), and XCL1), antiviral genes (2′-5′-oligoadenylate synthase 2, Oas2 (Fig. 1D)), and oxidative stress-related genes such as cytochrome b245b subunit (CYBB (Fig. 1F)). In this cluster there were also some cell signature genes expressed on all lymphocytes (CD53 and CD48 (Fig. 1F)) or specifically on neutrophils (neutrophil cytosolic factor, NCF4 (Fig. 1F)) and NK cells (NK cell protein 2A1 and KLRA19, see Table 1). Next, hierarchical clustering allowed us to characterize a cluster of genes specifically up-regulated by LTK63 at 8–14 days (Fig. 1G). Most of these genes were components of the B cell Ig complex and were detected by oligonucleotides specific for different variable regions of the Ig H chain (VH), light λ- and κ-chains (VL), or the joining region of the H chain and for the constant region of the IgG isotype (IgG H chain region). Genes in the subclusters H, I, and L of Fig. 1 were down-regulated in the buffer L control experiments. This inhibitory effect was reversed by LTK63, which up-regulated them transiently between 6 h and 8 days, with a peak at 6–8 days. These subclusters are part of a T cell cluster that included TCR genes (TCRβ, CD8 α-chain (CD8α), T cell-specific transcription factors (TCF7), and T cell signaling genes (lymphocyte protein tyrosine kinase, LCK, and IL2-inducible T cell kinase, ITK). Subtree M in Fig. 1 contained genes induced at early time points (3–12 h) in both LTK63-treated and control lungs but which were specifically up-regulated by LTK63 in a second wave of induction 6 days to 8 days after treatment. In this cluster we found several chemokines (CCL19, CCL12, CCL4, and CCL3) and one chemokine receptor (CCR1), cytokines (IL6 and IL1b) macrophage inflammatory genes (calgranulin A and B), recognition proteins (macrophage scavenger receptor, MSR), proteins involved in tissue remodeling and repair (tissue inhibitor of metalloproteinase 1, TIMP1), iron metabolism genes (lipocalin 2), and acute phase proteins (serum amyloid A1 and A3 and resistin-like α). To assess whether the presence of 0.25% CHAPS and 200 mM Arg in buffer L contributed to the activation of the early response genes found in clusters A and M in Fig. 1, we compared the expression profile of buffer L-treated lungs with lungs treated with the same volume of PBS at 3, 6, and 12 h. We found that there was no significant difference in the gene expression profile between buffer L and PBS-treated lungs, suggesting that the observed early gene activation is triggered by i.p. anesthetic injection or by the administration of a relatively large volume (50 μl) of liquid in the lower respiratory tract.

Chemokine and cell signature gene expression profiles
It has been shown that the pulmonary administration of LTK63 affects the composition of lung cellularity, indicating a recruitment of several blood cell types (21). The B cell and T cell gene clusters identified by hierarchical clustering analysis (Fig. 1, G and H, I, and L, respectively) further support the hypothesis that these cells

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FIGURE 1. Microarray analysis of LTK63-induced genes. Examples of subtrees deriving from the automatic TIGR Multiexperiment Viewer hierarchical clustering of the 712 selected genes having a fold change $> 2 \log_2$ and $p < 0.05$. Each column represents one time point. Each row represents the average kinetic of expression of one gene. Some genes, such as $B2m$, can be represented more than once in the cluster if they are detected by multiple unrelated probes. The expression value is shown in log2 scale and the color scale ranges from $-3$ (green, down-regulation) to $3$ (red, up-regulation).

are recruited into the lung following LTK63 administration. Additionally, we identified other cell-specific genes up-regulated mainly at 6 or 8 days, indicating that LTK63 promotes the recruitment into the lung also of NK cells, macrophages/DCs, and PMNs (Table I).

Because the network of chemokine/chemokine receptors is known to drive cell recruitment from the bloodstream into the tissues, we analyzed in detail the expression profile of C-C- and C-X-C-type chemokines (Fig. 2, A and B) and chemokine receptors in response to LTK63 treatment (Fig. 2C). We found a defined panel of 16 chemokines and a small subset of chemokine receptors (CCR1, CCR2, and CCR5) that were induced by LTK63 and might be involved in LTK63-mediated cell recruitment events in the lung (Fig. 2, A–C). All chemokines selected by microarray analysis were up-regulated at 6–8 days with a subset of them also induced at 12 h. Interestingly, the expression pattern of C-C-type chemokines parallels the expression of the corresponding receptors CCR1, CCR2, and CCR5 (Fig. 2A and B). To validate the microarray data, we performed real-time PCR experiments on one representative up-regulated chemokine, CXCL10. The expression pattern of CXCL10 mRNA measured by microarray and real-time PCR was very similar with a first modest increase at 12 h and a major late response at 8 days (Fig. 2D).

Altogether, these data suggest that the up-regulation of a defined subset of chemokines at 6–8 days promotes the arrival and accumulation in the lung of blood cells in a process mainly mediated by

### Table I. Cell type-specific genes not included in the B and T cell clusters in Fig. 1 having an average fold change >2 log₂ and p < 0.05

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Gene Name</th>
<th>LTK63 Average Fold Change (Log₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK</td>
<td>Cd160</td>
<td>0.00 0.17 −0.19 0.27 −0.09 0.28 0.00 2.83 0.00</td>
</tr>
<tr>
<td>NK</td>
<td>Klra19</td>
<td>1.41 0.94 0.69 1.05 1.13 0.83 2.01 2.24 0.00</td>
</tr>
<tr>
<td>NK</td>
<td>Klrc1</td>
<td>1.06 0.51 0.94 0.96 0.71 0.59 2.75 2.87 0.00</td>
</tr>
<tr>
<td>NK</td>
<td>Klra20</td>
<td>0.14 3.64 2.46 1.45 −0.56 −0.37 0.00 0.42 0.00</td>
</tr>
<tr>
<td>Mature B, Mo/M, DC</td>
<td>Ly86</td>
<td>0.27 0.35 0.81 0.64 0.78 0.54 1.15 2.41 0.93</td>
</tr>
<tr>
<td>T, Mo, M</td>
<td>Cd52</td>
<td>0.04 0.43 0.82 0.61 0.52 1.34 2.16 2.60 0.93</td>
</tr>
<tr>
<td>T, DC</td>
<td>Cd8a</td>
<td>−1.74 0.23 0.15 0.33 0.19 2.04 3.31 1.96 −2.80</td>
</tr>
<tr>
<td>T</td>
<td>CD3z</td>
<td>−0.02 0.11 0.26 0.24 0.25 1.00 1.17 2.89 −0.19</td>
</tr>
<tr>
<td>NK, B, T, Mo</td>
<td>Lair1</td>
<td>0.01 0.33 0.19 0.24 0.24 0.14 2.60 2.12 0.00</td>
</tr>
<tr>
<td>Mo act, DC, M, N, Bas</td>
<td>Cd68</td>
<td>0.55 0.49 1.02 0.72 1.04 0.67 2.04 1.79 0.00</td>
</tr>
<tr>
<td>Mo, B act, T act, DC</td>
<td>Cd86</td>
<td>1.07 0.74 0.62 0.77 0.67 0.87 2.04 1.79 0.00</td>
</tr>
<tr>
<td>T, NK</td>
<td>Cd28</td>
<td>0.00 0.68 0.15 0.86 0.39 2.89 2.58 1.80 0.00</td>
</tr>
<tr>
<td>T act, B act, NK act, G act</td>
<td>Cd69</td>
<td>1.45 1.07 0.55 0.33 0.52 1.96 2.67 2.40 0.00</td>
</tr>
<tr>
<td>NK, G, T, Mo, B</td>
<td>Cd48</td>
<td>0.32 0.53 0.95 0.82 0.69 1.02 1.66 2.12 0.86</td>
</tr>
<tr>
<td>B, M, DC</td>
<td>H2-Ab1</td>
<td>0.27 0.04 0.19 0.23 0.42 0.24 1.22 2.39 0.69</td>
</tr>
<tr>
<td>Mo/M</td>
<td>MSR-1</td>
<td>0.00 1.06 2.75 1.46 1.42 0.37 2.60 2.37 0.00</td>
</tr>
<tr>
<td>B, M, N, E, Mast</td>
<td>Fcgr2b</td>
<td>0.71 0.62 1.23 0.87 0.77 0.31 1.19 2.40 1.00</td>
</tr>
<tr>
<td>Bas, E, Mo, Mast</td>
<td>Fcer1g</td>
<td>0.77 0.70 1.50 1.17 1.17 0.73 1.64 2.39 1.10</td>
</tr>
<tr>
<td>CD8⁺ T and NK</td>
<td>GranzymeB</td>
<td>1.46 0.95 0.74 0.75 0.72 0.32 3.80 2.98 0.34</td>
</tr>
</tbody>
</table>

*a Cell type abbreviations: Mo, monocytes; M, macrophages; G, granulocytes; Bas, basophils; E, eosinophils; N, neutrophils; Mast, mast cells; act, activated. Time symbols: d, day(s), h, hours.

**FIGURE 2.** LTK63-dependent chemokines and chemokine receptors. A–C, Expression profiles measured by microarray of the chemokine ligands of the CC family (A) or CXC family (B) and the chemokine receptors (C) up-regulated by LTK63. D, CXCL10 expression profile measured by quantitative PCR. Each bar represents the log average CXCL10 mRNA expression level from three mice relative to ribosomal protein RPS10. d, Day(s).
CCR1, CCR2, CCR5, and CXCR6. The expression profiles of T, NK, PMN, and macrophage/DC cell signature genes (Fig. 1 and Table I) are predictive of lung recruitment of these cell types 6–8 days after treatment. The expression profile of the B cell cluster (Fig. 1G) suggests that B cell recruitment is delayed compared with the other cell types and peaks at 8 to 14 days after treatment.

**FACS analysis of cell recruitment to the airways following LTK63 administration**

To confirm that the up-regulation of cell-signature genes monitored by microarray is consequent to the recruitment of the corresponding cell types in the lung, mice were sacrificed at different time points following LTK63 or buffer L administration and their lungs were subjected to a BAL. As depicted in Fig. 3, BAL samples taken at 8 days after LTK63, but not after buffer L treatment, contained increased numbers of CD8+ T cells, CD4+ T cells as CD3+CD4+, CD8+ T cells as CD3+CD8+, B cells as CD3+DX5+B220+, and NK cells as DX5+CD3+. Counts were normalized by the use of counting beads (BD Pharmingen) in each sample. B, Flow cytometric analysis of the polymorphonuclear/macrophage/DC population. In the region of granulocytes (SSCmedium/FSCmedium) we identified eosinophils (E) as CCR3+ MHC class II+ and neutrophils (N) as CCR3+ MHC class II+ cells (25). In the region of monocytes/DCs, macrophages (M) were identified as CD11chighCD11b+MHC class II+cells (where “int” is intermediate). DCs were identified as CD11chighCD11bhighMHC class II+. CD11clowCD11bMHC class II+ cells were generally defined as granulocytes (Gr) (26). APC, Allophycocyanin.
between 6 and 8 days (Fig. 4, A–D). A similar kinetic was observed for CD4^+ and CD8^+ T cells (Fig. 4, E and F), whereas B cell recruitment peaked between 8 and 14 days (Fig. 4 G). In contrast to DCs, the number of alveolar macrophages was unchanged at all time points (data not shown). At 14 days the numbers of B cells, eosinophils, DCs and, to a lower extent, CD8^+ T cells and NK cells were higher in the BAL from LTK63-treated mice compared with the BAL from buffer L-treated controls. At the same time point, the numbers of CD4^+ T cells and neutrophils were almost equal in the two groups of mice. The cellular recruitment induced by LTK63 was transient and was reversed completely for all cell types at 28 days. The recruitment of T cells at 6–8 days (Fig. 4, E and F) and B cells at 8–14 days (Fig. 4 G) was in agreement with the gene expression profile for the B and T cell signature genes (Fig. 1, G, H, I, and L). Although we did not observe any LTK63-dependent increase in the number of alveolar macrophages, MHC class II expression was up-regulated on this cell type at 8 days (Fig. 3 B and Fig. 4 H), concomitant with general cell recruitment, and persisted for 14 days. In agreement with this finding, the expression profile of MHC class II genes measured by microarray analysis on whole lung was up-regulated with a major peak at 6–8 days (Fig. 4 I).

**FIGURE 4.** Kinetics of LTK63-dependent cell recruitment in the BAL. BAL from mice immunized intrapulmonarily with LTK63 (filled bars) or buffer L (open bars) were taken between 6 h and 28 days (d) and analyzed by FACS. A, NK cells. B, Neutrophils. C, DCs. D, Eosinophils. E, CD4^+ T cells. F, CD8^+ T cells. G, B cells. H, Mean fluorescence intensity measured by FACS for MHC class II protein expression on BAL-derived alveolar macrophages over time (6 h to 28 days). I, Kinetics of MHC class II gene expression measured by microarray between 3 h and 14 days.

**LTK63 activates CD4^+ and CD8^+ T cells in the lung**

To compare the microarray data with cell recruitment data from the same biological samples, we performed FACS analysis of whole lung cell suspension. In agreement with a previous report and with the data originated from the BAL, we found that LTK63 administration triggered the recruitment at 6–8 days of CD4^+ T cells, CD8^+ T cells, NK cells (Fig. 5, A–C), and B cells (data not shown) (21). We extended the analysis of lung single cell suspension by monitoring the activation status of T and NK cells. Both CD8^+ and CD4^+ T cell subsets found in the lung tissue at 6 to 8 days after treatment showed an activated phenotype as measured by the surface expression of the activation markers CD69 (Fig. 5, D and E) and CD44 (data not shown). In contrast, DX5^+ NK cells appeared to be less activated than T cells (Fig. 5 F). The kinetics of increase in CD69^+ T cell numbers were in agreement with the expression profile of the CD69 gene measured by microarray analysis (Table I).

**Cellular and humoral adaptive response to LTK63**

It has been shown in several systems that LTK63 is highly immunogenic (9, 17, 29–32). Therefore, we asked whether we could measure specific cellular and humoral responses to LTK63 in our experimental conditions. Spleens from mice treated at day 0 with either LTK63 or buffer L were taken at day 14 and analyzed for the presence of LTK63-specific CD4^+ T cells. As shown in Fig. 6, following a short in vitro pulse with LTK63 but not with other Ags (data not shown), LTK63-treated mice displayed increased frequencies of TNF-α-producing CD4^+ T cells in their spleens (Fig. 6 A) as compared with mice treated with buffer L alone.
Increased frequencies of IgG memory B cells were found in the mediastinal lymph nodes and in the spleens of LTK63-treated, but not buffer L-treated, animals at 14 days. Moreover, cells spontaneously secreting LTK63-specific IgG were found in the same organs as well as in the airways (Fig. 6, B and C). These cells displayed typical features of recently activated plasmablasts, because

**FIGURE 5.** FACS analysis of lung single cell suspension after LTK63 (filled bars) and buffer L (open bars) treatment. A–C, Whole lung cellular composition. The absolute number of CD4⁺ T cells (A), CD8⁺ T cells (B), and NK cells (C) was obtained by multiplying the frequency of each cell type by the total viable cell number measured by trypan blue exclusion. D–F, State of activation of T and NK cells. The percentage of activated CD4⁺ T cells (D), CD8⁺ T cells (E), and NK cells (F) was determined by measuring the percentage of CD69⁺ cells. d, Day(s).

**FIGURE 6.** Analysis of B and T cell responses to LTK63. A, Percentage of CD4⁺ T cells in spleen producing TNF-α after treatment with LTK63 or buffer L. Splenocytes were stimulated ex vivo with medium only (open bars), anti-CD3 Ab (gray bars), or 0.5 μg/ml LTK63 (filled bars). The results are expressed as the average of three mice with SD in one representative experiment. B–C, Numbers (×10⁶ CD19⁺ cells) of LTK63-specific plasma cells (B) and memory B cells (C) recovered in the BAL, lymph nodes, and spleens after 14 days of treatment with LTK63 (filled bars) or buffer L (open bars). The results are expressed as the average of three experiments (eight, three, and four mice per group for BAL and three, four, and six mice per group for lymph nodes and spleen) with SE values. Memory B cells have not been identified in the BAL. D–F, Measurement of LTK63-specific IgA (open bars), IgG (gray bars), and total Ig (filled bars) produced in the BAL (D), serum (E), and nasal washes (F) 14 days after LTK63 or buffer L administration. Each bar represents the geometric mean of the Ab titers (GMT) measured by ELISA and the 95% confidence of interval of four mice in one representative experiment. *, Significant difference between LTK63 and buffer L, p < 0.05 calculated by two-tail Student’s t test.
they spontaneously secreted LTK63 Abs while still expressing CD19 (data not shown). Consistently, 14 days after LTK63 administration, detectable levels of LTK63-specific IgG and IgA were found in BAL fluids (Fig. 6D) and nasal washes (Fig. 6E), as well as in sera (Fig. 6F).

**LTK63-mediated cell recruitment and macrophage activation in the BAL requires T cells**

In the previous paragraph, we have shown that under our experimental conditions LTK63 elicits an adaptive response that is measurable in spleens, lymph nodes, and the lung on day 14. Then, we asked whether all of the recruitment events occurring at 8 days after LTK63 administration were linked to the ongoing development of the adaptive response to LTK63. To clarify this issue, we administered LTK63 or buffer L to Fox Chase CB-17 SCID mice, which lack both T and B lymphocytes, and to BALB/c nude mice, which lack only T cells (33), and performed a comparative analysis of the cellular composition of the BAL at day 8. Fox Chase and BALB/c wild type mice were included as controls. As expected, we did not find T or B lymphocytes in the BAL from SCID or nude mice treated with either LTK63 or buffer L (Fig. 7, A and B), whereas increased numbers of both cell types were found in the BAL from control mice treated with LTK63. Interestingly, BAL samples taken from LTK63-treated SCID or nude mice were also devoid of DCs, NK cells, eosinophils, and neutrophils (Fig. 7, C–E). Furthermore, resident macrophages displayed a resting phenotype (Fig. 7, C–E). Taken together, these results demonstrate that the presence of T lymphocytes is required for LTK63-mediated cell recruitment in the BAL at 8 days and strongly suggest that the adaptive response to LTK63 is the main force driving the recruitment of innate and adaptive immune cells in the lung and the activation of resident macrophages.

**Discussion**

Despite the fact that LTK63 has great therapeutic potential as an immune modulator of the lung mucosa, little is known about the molecular and cellular events associated with LTK63 administration in vivo. To characterize in detail LTK63-dependent lung immune modulation in mice, we have adopted a system biology approach combining DNA microarray analysis of the gene expression profile in the whole lung and FACS analysis of the cellular compositions of the BAL and the lung infiltrate. Using microarray analysis, we observed that the very early changes (3–12 h) detected in the transcriptome of the lung were also present in the control group, so that a specific response to LTK63 could not be identified. We cannot exclude that at these time points LTK63 administration is associated with specific transcriptional changes in a subset of lung resident cells; however, in our experimental conditions this readout might be masked by the overall effect of the treatment. Two events could induce early gene activation in our experimental conditions: the i.p. anesthetic injection and the administration of a relatively large volume (50 μl) of liquid in the lower respiratory tract. By contrast, genes up-regulated at later time points were all specifically induced by LTK63. Genes induced at 6–8 days after
LTK63 administration belonged to various host defense mechanisms including pathogen recognition, oxidative burst, inflammation, antiviral activity, and Ag presentation, whereas those up-regulated at 14 days were essentially B cell-specific genes. The analysis of cell recruitment and cell signature genes was predictive of a CCR1-, CCR2-, CCR5-, and CXCR6-mediated recruitment of different population of adaptive and innate immune cells (T, NK, PMN, and macrophage/DC) at 8 days and B cells at 14 days. FACS analysis of the BAL confirmed that eosinophils, neutrophils, NK cells, DCs, and CD4^+ and CD8^+ T cells were recruited at 6 to 8 days, whereas B cells recruitment peaked at 14 days. The number of lung macrophages remained constant over time, even though the expression levels of MHC class II on their surfaces increased greatly with a peak at 8 days. Comparable results were obtained using two different strains of mice, BALB/c and Fox Chase CB-17. The increased cellularity observed in the BAL of LTK63-treated mice reflected a mild lung inflammatory infiltrate and was not a consequence of tissue damage, because a parallel lung histological analysis did not reveal any change in the tissue architecture (data not shown). A previous report showed that LTK63 promotes the recruitment of immune cells in the lung (21). We have confirmed these results by FACS analysis of whole lung cell suspension (data not shown). Furthermore, our FACS analysis of the BAL allowed a better characterization of the cell subsets recruited in the lung in response to LTK63. In particular, we show here for the first time that LTK63 triggers the recruitment of DCs (CD11c^{hi/hi}CD11b^{hi/hi}) and the activation of resident alveolar macrophages (CD11c^{hi}CD11b^{low}).

In line with several reports showing that LTK63 is strongly immunogenic (9), we could measure both cellular and humoral responses to LTK63 in our experimental conditions. Notably, for the first time we could detect specific cellular and Ab responses to LTK63 14 days after a single dose. We could monitor specific LTK63 B cells in the BAL, mediastinal lymph nodes, and spleen and LTK63-specific IgA and IgG Abs in the BAL, nasal wash, and the blood. These data suggest that extensive LTK63-specific T and B cell activation and differentiation were already ongoing at 14 days. Furthermore, the results obtained in SCID and nude mice demonstrate the requirement of T cells for all the LTK63-induced cell recruitment events in the BAL. At this stage we do not know which T cell type (CD4^+, CD8^+ or γδ T cells) is required for this process. We suggest that CD4^+ T cells may play a central role and that the adaptive response to LTK63 itself might be the driving force of LTK63 lung immune modulation.

It is very likely that under our experimental conditions LTK63 exploits the local innate immune response to i.p. treatment that we could measure by microarray at 3 to 12 h, which was characterized by up-regulation of several proinflammatory cytokines such as IL-6 and IL-1β and several chemokine ligands such as CCL3, CCL4, CCL12, CXCL10, and CCL20, one of the main DC chemoattractants in the lung (34) (Fig. 8A). This early immune-stimulating environment, together with the specific ability of LTK63 to enter APCs (DCs) through GM1 binding, may result in an efficient priming of CD4^+ T cell responses to LTK63 that, in turn, may promote cognate and noncognate activation of B cells, as well as the bystander activation of CD8^+ T cells. Eight days after LTK63 administration, LTK63-specific T and B cells migrated to the lung where they might trigger all of the recruitment events and changes in gene expression that we could monitor by FACS and microarray analysis, allowing a sustained modulation of the lung microenvironment (Fig. 8B). Finally, at 14 days we could measure an increase of LTK63-specific B cells and Abs in the BAL.

Combining the microarray and the FACS data, we can hypothesize that LTK63 provides generic protection to pathogen challenge by multiple mechanisms that reduce pathogen load and promote an adaptive immune response. LTK63 induced an up-regulation of several innate immunity genes involved in pathogen sensing (TLR, PGRP, and lectins) (35–37) or killing (complement system, antiviral genes, and oxidative stress genes), which could limit the survival of pathogens in the infected lung. LTK63 also induced the up-regulation of chemokines that attracted cells of the innate immune system such as NK cells, eosinophils, and neutrophils that might be involved in the first line of defense against pathogens in the airways. Additionally, although the alveolar macrophage number remained constant in buffer L- vs LTK63-treated mice, LTK63 induced an increase of MHC class II expression reflecting the activated state of these cells (38). Another important event that might contribute to pathogen killing is the observed presence at 14 days in the BAL of a relatively large number of B cells that might produce cross-reactive Abs. It has been shown, that DCs have an increased Ag processing and presentation capacity.
LUNG IMMUNE MODULATION BY LTK63


