Real-time quantitative RT–PCR after laser-assisted cell picking

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An investigation of cell type-specific gene expression in intact tissues and an understanding of its regulation requires mRNA quantitation in minute numbers of cells. For this purpose, the sensitivity of PCR has to be complemented by the precision achieved with northern blotting or RNase-protection assays. Quantitative competitive-PCR focuses on this target but design of an internal competitor and validation of amplification efficiencies require a dedicated effort. A recently available approach is based on the 5' nuclease activity of *Taq* polymerase[1] for fragmentation of a dual-labeled fluorogenic hybridization probe[2,3]. As long as this sequence-specific probe is intact, emission of a reporter dye at its 5' end is quenched by a second fluorescence dye at the 3' end. During extension of PCR, *Taq* polymerase hydrolyses the probe and releases the reporter dye, the state of which is monitored online by an increase in fluorescence intensity in each tube. As the signal is directly proportional to the amount of amplified copies, valid measurement over a large range of starting quantities is possible and was proven to be a highly reproducible tool with easy handling as compared to conventional PCR cycling[4]. Absolute quantitation using a standard curve of a target with known quantity is possible, as well as relative quantitation using an appropriate housekeeping gene for internal calibration.

The need for quantitative mRNA measurement is one prerequisite when addressing cellular events in intact tissue; another is the accurate access of cells to be examined. However, commonly applied mRNA extraction procedures are performed with homogenized tissue and reflect an average expression, not allowing analysis of a single cell type. The use of microdissection and micromanipulation techniques to obtain pure cell populations have so far been used only for DNA analysis[5–7], while mRNA studies required the use of at least small tissue fragments still containing heterogeneous cell types. Our group recently showed that UV-microbeam-assisted cell picking is an appropriate morphology-based technique for contamination-free isolation of cells from complex tissue sections, and mRNA examination of "picked" cardiac neurons was demonstrated on a qualitative basis[8]. We now describe an approach that allows quantitation of mRNA in a few isotypic cells within intact tissue using a combination of laser-assisted cell picking and real-time PCR.

To validate our technique, we selected alveolar macrophages as target cells, which contain transcriptionally inducible genes and may—apart from cell picking—be alternatively acquired from the same lung by bronchoalveolar lavage. Tumor necrosis factor-α (TNF-α) mRNA served as the target gene and is known to be substantially upregulated in macrophages in response to inflammatory agents like lipopolysaccharide (LPS, Sigma, Deisenhofen, Germany) and interferon-γ (IFN-γ, Laboserv, Giessen, Germany; ref. 9). Use of isolated and perfused rat lung enabled us to stimulate TNF-α expression in alveolar macrophages by nebulization of LPS and IFN-γ for comparison with control lung tissue. High-challenge lung differed from low-challenge lung by an additional stretching maneuver. In each experiment, left lung was lavaged and an aliquot of alveolar macrophages was mRNA-extracted. Another aliquot was cytocentrifuged in a reaction tube, overlaid with TissueTek (Sakura Finetek, California) and frozen in liquid nitrogen. Then, sections of tube with alveolar macrophages were prepared in a cryotome and stained. Ten to fifteen alveolar macrophages each were picked for subsequent analysis. The right lung was instilled with TissueTek, frozen in liquid nitrogen and cryosections were prepared from different regions. After staining, 10–15 alveolar macrophages were picked. Thus, laser-picked alveolar macrophages were compared (1) to lavaged alveolar macrophages of these lungs undergoing mRNA extraction, and (2) to lavaged cells secondarily embedded in a tissue block for *in vitro* mimicry of the laser-assisted, cell-picking procedure.

Real-time PCR

Real-time PCR (TaqMan-PCR, ABI Prism 7700 Sequence Detection System, Perkin Elmer Applied Biosystems, Foster City, California) was recently established as a rapid and sensitive technique for precise quantitation[4,10]. As the validity of this approach is critically dependent on suitable design of primers and probe, some important features are described in Table 1. We used comparative quantitation normalizing target gene to an internal standard gene, as both templates undergo the sectioning and stressing procedure of staining and cell picking in parallel. For internal calibration, mRNA transcribed from the gene encoding porphobilinogen deaminase (PBGD) was used, a ubiquitously—as well as consistently—expressed standard gene that is free of pseudogenes (L.F. *et al.* manuscript submitted).

Relative quantitation is given by the following equation (deduction is described in 'Methods'—Real-time PCR):

$$\frac{T_o}{R_o} = K \cdot (1 + E)^{(C_{T,R} - C_{T,T})}$$

T_o: Initial number of target gene copies; R_o: Initial number of standard gene copies; E: Efficiency of amplification; $C_{T,T}$: Threshold cycle of target gene; $C_{T,R}$: Threshold cycle of standard gene; and K: Constant.

Determination of threshold value C_T and example of calculation is shown in Fig. 1. The relation is based on the precondition that efficiency of target and reference amplification be approximately equal. Comparing serial dilution of target and standard gene simultaneously, it has to be demonstrated that the absolute value of the slope of log input amount versus ΔC_T (C_T target gene - C_T standard gene) is < 0.1. In our case, it varied between 0.014 and 0.04.

As K is dependent on factors like (1) the reporter dye used in the probe (2) sequence context effects on the fluorescence properties of the probe (3) purity of the probe, and (4) efficiency of the probe cleavage, the exact value of K need not be equal to

one[11]. However, affecting factors will vary only negligibly among single samples so that K is assumed to be equal and thus does not influence the comparison of calculated relative ratios.

For comparison of different runs the run efficiencies have to be calculated. The efficiency of PCR provides information about the amplification rate and varies from 0 to 1. One (= 100%) means that in each cycle the amount of copies is doubled. A serial dilution of a PCR product of PBGD in concentrations corresponding to copy numbers of picked cells served for generating a standard curve. The slope of this standard curve is required for calculation of the run efficiency. The relation is given by:

$$E = 10^{-1/s}-1$$

E: Run efficiency; and s: Slope of generated standard curve.

Among all runs the calculated efficiencies varied from 0.86 to 0.96.

C_T values for the target (TNF-α) and reference (PBGD) cDNA were measured in each sample. For calculation of relative copy numbers it was a prerequisite that both values were obtained.

Table 1 Sequences, amplicon sizes and exon localizations of primers and probes that were used for PCR

Primer name	Sequence	Exon
PBGD amplicon size: 135 bp		
PBGD-forward	CAAGGTTTTCAGCATCGCTACCA	e4
PBGD-reverse	ATGTCCCGGTAACGGCGGC	e1
PBGD-hybridization probe	CCAGCTGACTCTTCCGGGTGCCCAC	e4-e3
TNF-α-amplicon size: 173 bp		
TNF-α-forward	GGTGATCGGTCCCAACAAGGA	e1
TNF-α-reverse	CACGCTGGCTCAGCCACTC	e4
TNF-α-hybridization probe	TGGCCCAGACCCTCACACTCAGATCA	e2-e3

Differentiation between cDNA and genomic DNA was achieved by use of intron-spanning primers. The cDNA amplicon was much shorter than genomic DNA amplicon (PBGD: >1,000 bp; TNF: ≈1,000 bp) so that synthesis of the latter was suppressed by suitable selection of cycling conditions. The hybridization probe also spanned an intron to exclude annealing to genomic DNA. Both the target gene and the housekeeping gene were shown to be pseudogene-free to exclude falsification by amplification of genomic DNA with cDNA sequence. By this, mRNA stressing DNase digestion was avoided. Both sets of primers and probes were selected to work under identical cycling conditions.

Efficiency of picking

The data presented here are based on 171 samples (each containing 10–15 picked alveolar macrophages), in 133 of which PBGD mRNA was detected. An additional 126 samples of picked alveolar macrophages were investigated; 110 were PBGD-positive (data not shown). Thus, the overall efficiency of RT– and real-time PCR in detecting the housekeeping gene in a limited number of picked cells was 82% (243/297 samples). Negative samples may be mostly caused by methodological imponderables, including degradation of RNA during staining and preparation, loss during transfer of the needle, and failure of the polymerase during 60 cycles.

Quantitation and time-course of TNF-α mRNA induction *in vitro*

As a basis for the experiments in intact lung tissues, we first evaluated the time-course of TNF-α mRNA induction in alveolar macrophages by stimulation *in vitro*. As depicted in Fig. 2*a*, rapid TNF-α mRNA upregulation during adhesion was noted and further enhanced by LPS/IFN-γ stimulation with a maximum after 6 h (2 h adhesion inclusive).

Quantitation of TNF-α mRNA induction *in vivo*

Control, low-challenge and high-challenge lungs underwent both cell picking and lavage, the latter approach being split into direct mRNA extraction and secondary embedding of lavaged alveolar macrophages in 'tissue' blocks.

TNF-α mRNA quantitation in lavaged alveolar macrophages

Aliquots of lavaged alveolar macrophages served for mRNA extraction. PBGD mRNA, as well as TNF-α mRNA, were detected in all samples (48/48). As evident from Fig. 2*b*, a dramatic upregulation of the number of TNF-α mRNA copies in high-challenge lung and a moderate upregulation in low-challenge lung was noted.

TNF-α mRNA quantitation in picked alveolar macrophages from stained cytospin sections

mRNA analysis in picked alveolar macrophages differs in many respects from processing mRNA extracted directly from lavaged

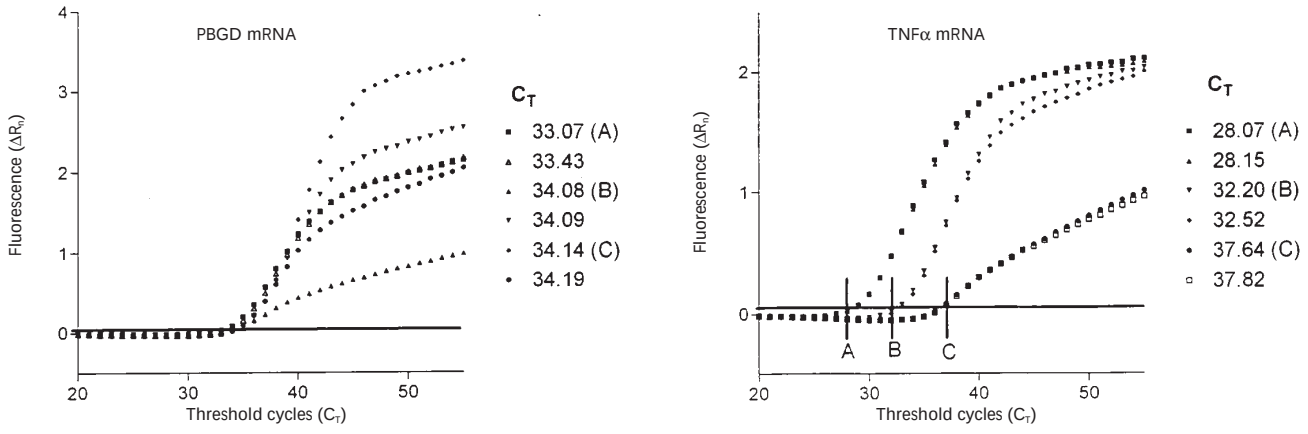

Fig. 1 Original recording of real-time PCR using mRNA extracted from lavaged alveolar macrophages. A: High-challenge lung; B: Low-challenge lung; and C: Control lung. Increasing fluorescence emission during amplification is displayed for both the housekeeping gene (PBGD) and the target gene (TNF-α). Calculated threshold cycles C_T are given. Minimal deviation among duplicate samples indicates high reproducibility of this approach. Among the 133 PBGD-positive samples used for this investigation, the mean C_T of PBGD was 41.13 ± 0.16 (mean ± s.e.m.). To calculate, for example, the relative expression of TNF-α mRNA normalized to PBGD mRNA in alveolar macrophages from high-challenge lung, TNF-α C_T of A (28.07) was subtracted from PBGD C_T of A (33.07) resulting in 5.0. Deduced from the co-amplified PBGD standard curve, a PCR efficiency of 0.9 was calculated. Thus, TNF-α mRNA copies amounted to $K \times 1.9^{5.0} = K \times 24.8$ copies of PBGD mRNA in this example. K = constant.

macrophages. Whereas lavaged cells are intact, with integral cell membranes, and are immediately and without loss transferred into mRNA extraction medium, picked alveolar macrophages are sectioned. This implies that they lose a varying but considerable part of their cytoplasm, including mRNA, in addition to protecting membrane portions. Moreover, they have to pass an mRNA-stressing procedure with staining, storage in alcohol, lasering and picking. To investigate reliability of the current approach under these conditions we first introduced picking of lavaged alveolar macrophages that were treated like tissue sections. This ensured that identical cell populations were examined.

Using this approach with investigation of a few single cells, approximately half of the alveolar macrophages were found to express PBGD mRNA but not TNF-α mRNA under control lung conditions, suggesting remarkable differences in individual TNF-α expression (Table 2*a*). Quantifying TNF-α mRNA in the remaining subset with both detectable PBGD and TNF-α expression revealed a somewhat higher ratio of TNF-α/PBGD mRNA copies as compared to the mRNA extraction from the bulk of lavaged alveolar macrophages, averaging all alveolar macrophage subsets (Fig. 2*b*). In alveolar macrophages picked from cytospin sections of LPS/IFN-γ-challenged lungs, the percentage of alveolar macrophages expressing both PBGD and TNF-α increased significantly to 77% in low- and 92% in high-challenge lung. Moreover, calculating the number of TNF-α copies per PBGD copy in this subset, in which both PBGD C_T and TNF-α C_T values were obtained, again demonstrated a highly significant upregulation of TNF-α message, although the TNF-α/PBGD ratio was lower than that calculated for mRNA directly extracted from lavaged alveolar macrophages.

Table 2 Percentage of samples with detectable TNF-α mRNA among all samples of picked alveolar macrophages, in which PBGD mRNA was found. Picked alveolar macrophages from stained cytospin sections (*a*), and from stained tissue sections (*b*).

a	Control lung (25 samples)	Low-/High-challenge lung (13/24 samples)
PBGD+ TNF-α+	44%	77%/92%
PBGD+ TNF-α–	56%	23%/8%
b	Control lung (35 samples)	Low-/High-challenge lung (18/18 samples)
PBGD+ TNF-α+	31%	61%/89%
PBGD+ TNF-α–	69%	39%/11%

In control lung, significantly fewer samples expressed TNF-α mRNA than those originating from low-challenge lung ($P = 0.05$ for *a*, $P = 0.02$ for *b*) and high-challenge lung ($P < 0.001$ for *a* and *b*, Pearson Chi-square test).

TNF-α mRNA quantitation in picked alveolar macrophages from stained tissue sections

By instillation of TissueTek into the lung airways, alveolar collapse was prevented and alveolar macrophages could easily be distinguished from alveolar epithelial cells. The technique of laser-assisted cell picking in intact lung tissue is demonstrated in Fig. 3. For TNF-α negative control, we picked a comparable number of endothelial cells (10–15 cells) from large vessels of the control lung in parallel with the macrophage picking. Among the 22 PBGD-positive samples investigated, a weak TNF-α message was detected in one sample.

In analogy to alveolar macrophages from stained cytospin sections, only a subset of alveolar macrophages picked from control lung expressed both PBGD mRNA and TNF-α mRNA (Table 2*b*). In lungs undergoing LPS/IFN-γ challenge the percentage of PGBD+/TNF-α+ samples again significantly increased. Moreover, calculated from all picked alveolar macrophages expressing both PBGD and TNF-α, a dose-dependent, highly significant increase in the TNF-α mRNA/PBGD mRNA ratio was again

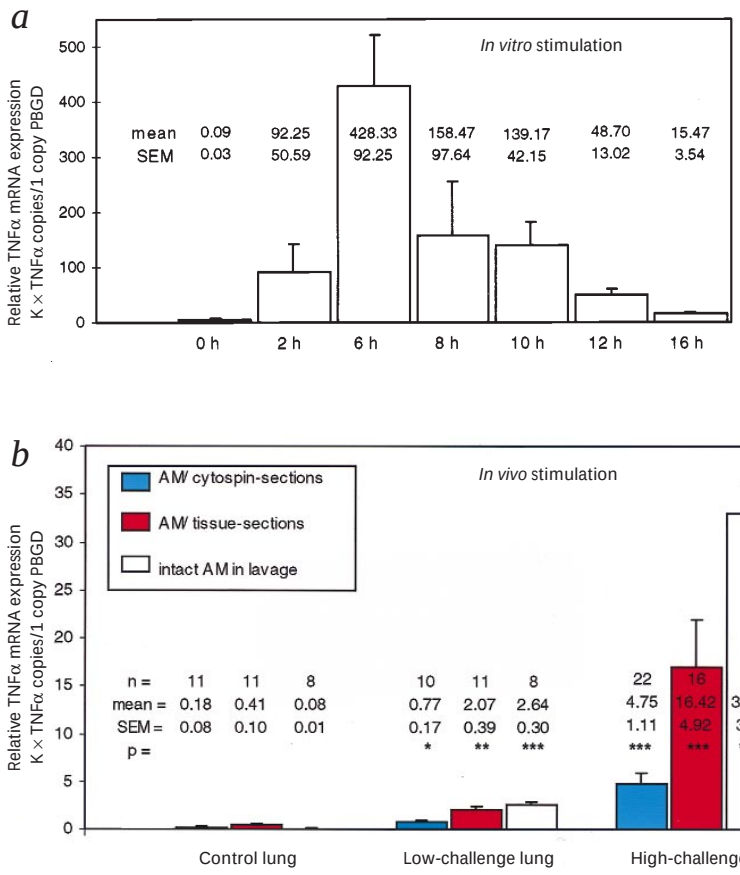

Fig. 2 ***a***, Upregulation of TNF-α mRNA in alveolar macrophages exposed to LPS/IFN-γ *in vitro*. Alveolar macrophages were analyzed post-lavage (= 0 h), after 2 h adhesion to the plastic dishes (= 2 h) and were then stimulated with LPS/IFN-γ for different time periods (time post-lavage is given; $n = 3$ independent experiments each). K = constant. ***b***, Quantitation of TNF-α mRNA in alveolar macrophages of control lung and lungs undergoing low- and high-challenge with LPS/IFN-γ aerosolization. Relative TNF-α mRNA expression was assessed by different techniques: (1) mRNA extraction of intact alveolar macrophages obtained by bronchoalveolar lavage; (2) cell picking of stained cytospin sections; and (3) cell picking of alveolar macrophages from intact lung tissue sections. Mean and s.e.m., as well as numbers of assays, are given in the figure. Statistical comparison was performed for low- and high-challenge lungs as compared to control lung for the respective techniques (***: $P < 0.001$; **: $P = 0.002$; *: $P = 0.008$; Mann-Whitney test). K = constant.

Materials and methods

Lung isolation and perfusion. Lungs from male CD-rats (Sprague Dawley, 350–400 g; Charles River, Sulzfeld, Germany) were isolated, ventilated and *ex-vivo*-perfused with Krebs-Henseleit buffer as previously described[14,15]. Perfusion pressure, ventilation pressure and weight of isolated organ were continuously registered.

Lungs selected for the study had a homogeneous white appearance without signs of hemostasis or edema formation; had pulmonary artery and ventilation pressures in the normal range; and were isogravimetric during a steady-state period of 30 min.

After the steady-state period, 75 μg LPS and 1000 U IFN-γ in a total volume of 5 ml were aerosolized into the afferent limb of the ventilator circuit with an ultrasound vaporizer (Portasonic; De Vilbiss, Langen, Germany) for 10 min. For high-challenge lung, an additional stretching maneuver was performed by increasing the end-expiratory pressure to 5 cm H_2O (normal is 3 cm H_2O) and repetitive doubling of tidal volume. Afterwards lungs were perfused and ventilated under standard conditions for 6 h. Control lung did not undergo these procedures. After termination of perfusion, the left lung was lavaged (see below) while the right lung was instilled with TissueTek via a cannula and snap frozen in liquid nitrogen.

Bronchoalveolar lavage. Aliquots (3 ml) of saline were instilled into the left lung and immediately reaspirated (total volume 18 ml). Cells were centrifuged at 1500 r.p.m., washed twice, and subsequently counted in a hemocytometer chamber. Viability was assessed by trypan blue exclusion and differential counting was performed (>97% alveolar macrophages, in all experiments). For direct performance of mRNA extraction, aliquots of lavaged alveolar macrophages (25,000–45,000 cells) were lysed in 250 μl homogenization buffer/2.5 μl β-mercaptoethanol of the MPG Guanidine Direct mRNA Purification Kit (CPG, Lincoln Park, New Jerey). For *in vitro* stimulation, alveolar macrophages were distributed to plastic Petri dishes and suspended in RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with 2% rat serum. Alveolar macrophages were allowed to adhere for 2 h at 37 °C and 5% CO_2.

***In vitro* stimulation.** After 2 h adherence, medium was exchanged with a mixture of 10 μg/ml LPS and 1000 U/ml IFN-γ. Alveolar macrophages were then incubated for 4, 6, 8, 10, and 14 h. For harvesting cells, medium was removed and alveolar macrophages were lysed in 250 μl homogenization buffer/2.5 μl β-mercaptoethanol. Cell lysate was transferred into a 1.5 ml reaction tube and snap frozen in liquid nitrogen for further RNA extraction.

mRNA extraction. mRNA extraction was carried out using the MPG Guanidine Direct mRNA Purification Kit based on magnetic separation of mRNA that is caught by attachment to oligo-dT fragments. According to the manufacturer's protocol, oligo-dT fragments were bound to streptavidin that is covalently coupled to the surface of supermagnetic glass particles. For each sample, 150 μg MPG–streptavidin were linked to 1.5 μl biotinylated oligo-dT. Isolated mRNA was finally solved in 20 μl DEPC-treated H_2O.

Cytospin sections, tissue sections and staining. For cytospin sections, one aliquot of lavaged alveolar macrophages was washed twice. After assessing viability by trypan blue exclusion and differential counting, cells were centrifuged in a 200-μl reaction tube at 1500 r.p.m. for 5 min, overlaid with TissueTek and snap frozen in liquid nitrogen. Next, 5-μm sections of tube and the TissueTek-embedded alveolar macrophages were prepared in a cryotome, mounted on glass slides (0.17-mm thickness), stained with hematoxylin for 45 s, rinsed twice in DEPC-water and subsequently immersed in 70%, 90%, and 100% ethanol. From tissue blocks of the TissueTek-fixed right lung, 5-μm cryosections were prepared in an identical manner.

Laser-assisted cell picking. The UV-laser microbeam (P.A.L.M., Wolfratshausen, Germany) used for microdissection consists of a nitrogen laser of high-beam precision (wavelength 337 nm), which is coupled to an inverted microscope (Axiovert 135; Zeiss, Jena, Germany) via the epifluorescence illumination path. Microscope stage and micromanipulator are digitally controlled and moved by computer mouse. After selecting cells of interest, adjacent cells were photolysed by laser beam (Fig. 3). A sterile needle linked to the micromanipulator served for picking the selected cell(s) via adhesive forces, with direct transfer into a reaction tube.

documented (Fig. 2*b*). Notably, both in low- and high-challenge lungs, the ratio of target to housekeeping mRNA approximated that calculated for the mRNA extracted from lavaged alveolar macrophages. Thus, the current technique of laser-assisted picking of a low number of isotypic cells from intact lung tissue and subsequent RT-PCR quantitation very reliably detected the manifold upregulation of TNF-α message, as documented by direct mRNA extraction from lavaged cells taken as reference and in spite of the considerable RNA stressing conditions of staining and lasering. Values for TNF-α/PBGD ratio in alveolar macrophages directly picked from intact lung tissues even surpassed the data obtained for lavaged cells secondarily cytospinned and embedded in TissueTek (to allow mimicry of the laser procedure). As the cytospin section is the most time-consuming preparation with the longest incubation of intact cells, this may give way to some overproportional loss of the unstable TNF-α mRNA (half-life of human TNF-α mRNA, ca. 20 min; refs. 12 &13) compared with the more stable PBGD mRNA.

When compared to approaches averaging large cell numbers, the current technique additionally allows the characterization of different subsets of morphologically indistinguishable cell types.

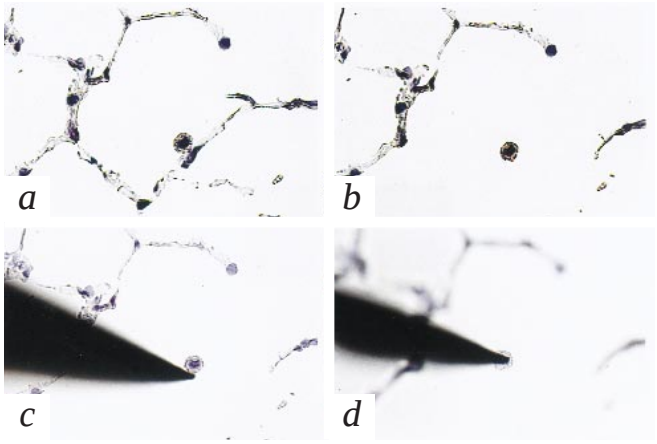

Fig. 3 Laser-assisted cell picking of alveolar macrophages from hematoxylin-stained frozen lung tissue sections. ***a***, Macrophages in alveolar lumen are selected that are distinguished from other cell types without doubt. ***b***, Adjacent cells are photolysed using laser microbeam. ***c***, Alveolar macrophage adheres to sterile needle that is approximated by mouse-driven micromanipulator. ***d***, Needle with adherent alveolar macrophage is lifted by micromanipulator for cell transfer into reaction tube.

Summary

The present study describes a technique for quantitation of mRNA in a few isotypic cells obtained from an intact organ structure by combining laser-assisted cell picking and real-time PCR. The microscopically controlled lasering of selected cells in stained tissue sections was applied to lung alveolar macrophages, which are unique in that they can alternatively be gathered as a pure cell population from intact lungs by bron-

RNA processing from picked cells. Needles with adherent cell(s) were transferred into a reaction tube containing 10 µl of first-strand buffer (FSB). FSB was prepared as described previously[16] with slight modifications: 4% RNase inhibitor (Perkin Elmer, Überlingen, Germany) was added to 52 mM Tris-HCl, pH 8.3, 78 mM KCl and 3.1 mM $MgCl_2$. Nonionic detergent was omitted. FSB and picked cells were incubated on ice for 5 min. Afterwards specimens were snap-frozen in liquid nitrogen.

cDNA synthesis. cDNA synthesis was done using products purchased from Perkin Elmer (10 × PCR-buffer II, $MgCl_2$, random hexamers, RNase inhibitor, MULV reverse transcriptase) and Eurobio (dNTPs; Raunheim, Germany). Tubes with picked cells as well as 10 µl of H_2O-diluted mRNA from lavaged alveolar macrophages were heated to 70 °C for 10 min and then cooled on ice for 5 min. For cDNA synthesis of extracted mRNA, 4 µl $MgCl_2$ (5 mM), 2 µl 10 × PCR-buffer II, 1 µl dNTP (10 mM each), 1 µl random hexamers (50 µM), 0.5 µl (10 U) RNase inhibitor and 1 µl (50 U) MULV reverse transcriptase were added to a total volume of 19.5 µl. For cDNA synthesis from picked alveolar macrophages, 4 µl H_2O, 1 µl dNTP (10 mM each), 1 µl random hexamers (50 µM), 0.5 µl (10 U) RNase inhibitor and 1 µl (50 U) MULV reverse transcriptase were added to a total volume of 17.5 µl. Samples were incubated at 20 °C for 10 min and 43 °C for 60 min. Reactions were stopped by heating to 99 °C for 5 min.

Real-time PCR. Cleavage of the sequence-specific probe by nuclease activity releases the reporter dye resulting in an emission increase of respective wavelength. PE ABI's Prism 7700 Sequence Detection System monitors emission intensity continuously. The signal is normalized to an internal reference (ΔRn) and the software sets the threshold cycle C_T, when ΔRn becomes equal to ten standard deviations of the baseline. C_T is used for quantitation of the input target number. For relative quantitation as used here, comparative C_T method normalizes the number of target gene copies to an endogenous reference called calibrator, for example, a suitable housekeeping gene. Based on the exponential amplification of target gene, as well as calibrator, the amount of amplified molecules at the threshold cycle is given by (also described in ref. 11):

$$X_T = X_0 \cdot (1+E_x)^{CT} = K_x$$

X_T: Number of molecules at threshold cycle; X_0: Initial number of molecules; E_x: Efficiency of target amplification; CT: Threshold cycle for amplification; and K_x: Constant.

Ratio of target gene copies (T) to standard gene copies (R, calibrator reference) at threshold cycle normalizes target gene expression for further comparison:

$$\frac{T_0}{R_0} \quad (1 + E)^{(CT,T \quad CT,R)} \quad K$$

Assuming same efficiency of target and reference gives the following expression:

$$\frac{T_T}{R_T} \quad \frac{T_0 \quad (1 + E_T)^{CT,T}}{R_0 \quad (1 + E_R)^{CT,R}} \quad \frac{K_X}{K_R} \quad K$$

and

$$\frac{T_0}{R_0} \quad K \quad (1 + E)^{(CT,R \; - \; CT,T)}$$

After cDNA synthesis, each sample of picked macrophages was divided for target gene and standard gene analysis into two aliquots of 8 µl. In case of separated mRNA, 1.5 µl of cDNA each were applied. The TaqMan PCR Reagent Kit (Perkin Elmer) was used according to the manufacturer's protocol with slight modifications: dUTP was replaced by dTTP at the same concentration, and incubation with AmpErase was omitted. $MgCl_2$ concentration at 4 mM and 0.5 µl (2.5 U) of AmpliTaq Gold Polymerase was tested to be optimal in pilot experiments. Oligonucleotide primers (Table 1) were added to a final concentration of 300 nM each and hybridization probe (Table 1) to a final concentration of 200 nM in a volume of 50 µl. Oligonucleotides were synthesized by PE ABI (Weiterstadt, Germany). Cycling conditions were modified to 94 °C for 2.45 min, followed by 60 cycles of 94 °C for 45 s, 62 °C for 45 s and 73 °C for 45 s.

choalveolar lavage as a reference technique. TNF-α was chosen as the transcriptionally inducible target gene to be quantified in alveolar macrophages of control rat lung, as well as low- and high-challenge lungs stimulated by endotoxin and IFN-γ nebulization. Online fluorescence detection for quantitation of the number of amplified copies was based on 5' nuclease activity of *Taq* polymerase cleaving a sequence-specific dual-labeled fluorogenic hybridization probe. A pseudogene-free sequence of PBGD served as an internal calibrator for comparative quantitation of target. A quick procedure and minimized loss of template were achieved by avoiding RNA extraction, DNase digestion and nested-PCR. Using this approach, we demonstrated dose-dependent manifold upregulation of the ratio of TNF-α mRNA copies per one copy of PBGD mRNA in alveolar macrophages of the challenged lungs. The quantitative data obtained from laser-picked alveolar macrophages were well matched with those of lavaged alveolar macrophages carried out in parallel. We suggest that this new combination of laser-assisted cell picking and real-time PCR has great promise for quantifying mRNA expression in a few single cells or oligocellular clusters in intact organs, allowing assessment of transcriptional regulation in defined cell populations.

Acknowledgments

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