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Bacteria Displaying Interleukin-4 Mutants Stimulate Mammalian Cells and Reflect the Biological Activities of Variant Soluble Cytokines

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We describe a novel procedure that allows the rapid determination of cytokine activity on cells that express their cognate receptor. The four-helix bundle cytokine interleukin-4 (IL-4) was inducibly expressed as a fusion with the E. coli *outer-membrane protein intimin, such that IL-4 was presented on the surfaces of the bacteria. Expression and accessibility of the cytokine on the cell exteriors were monitored by Western blotting and fluorescence microscopy, making use of two epitopes flanking the IL-4 component of the fusion protein. To demonstrate the biological activity of the immobilized cytokine, a Ba/F3-derived cell line stably transfected with both the bipartite human IL-4 receptor and an IL-4-specific luciferase reporter gene construct was employed. Bacterial cells displaying interleukin-4 elicited a specific, dose-dependent response in the reporter cells. Two variants of IL-4 with previously characterized (partial) antagonistic properties were also expressed as membrane-bound fusion proteins and were tested for their activity in the immobilized state. In comparison with bacteria displaying wild-type IL-4,* E. coli *clones presenting variants IL-4 Y124G and Y124D showed diminished or abolished activity, respectively, on murine reporter cells. The relative signaling potencies of the immobilized IL-4 variants thus closely mirror the agonistic properties of the corresponding soluble cytokines. This approach should be generally applicable for the mutational analysis of numerous signal mediators that trigger cellular responses through dimerization of transmembrane receptors.*

Introduction

The activity of cytokines and growth factors on responder cells through dimerizing transmembrane receptors is crucial for the coordination throughout the organism. Inadequate function of the underlying processes causes or accompanies numerous disease states, including immune disorders and cancer. Receptors for growth factors and cytokines thus constitute attractive targets for pharmaceutical interference.

Intense work on many receptor–ligand systems has been devoted to detailed understanding of activity-related protein–protein interactions. Such studies have frequently involved the generation of numerous ligand variants and subsequent testing of their biological activity on cells that express the respective cognate receptor.[1–4] In various cases, systematic functional characterization of mutant ligands led to the identification of antagonistic derivatives that bind, but do not activate, the receptor, and so are very interesting lead molecules for the development of therapeutic compounds.[5–8]

Extended mutational analysis of cytokines or growth factors is laborious, since in addition to the generation of expression constructs for every individual variant, all mutant proteins have to be purified from host cells for binding and activity tests. Here we have devised a way of facilitating this task by a bacterial cell surface display approach.

Display on the cell surfaces of bacteria has been developed into a powerful technology for the functional testing of proteins.[9] Moreover, the presentation of polypeptides by replicating entities has rendered the isolation of proteins from complex random libraries feasible on the basis of their affinity for a given target structure.[10–13] To achieve the exposure of heterologous proteins on the surfaces *of E. coli* cells in a native form, several outer membrane proteins have been successfully em-

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ployed as fusion partners and anchors; examples include the peptidoglycan-associated lipoprotein PAL,[14] the β-domain of *Neisseria gonorrhoeae* immunoglobulin A (IgA) protease,[15] or *E. coli* flagellin FliC.[16] Recently, we have shown that interleukin-4 can, like various other polypeptides, be efficiently displayed on the outer membranes of *E. coli* by C-terminally fusing it to the transporter domain of intimin EaeA from enterohemorrhagic *E. coli* O157:H7.[17]

We used this finding as a starting point to determine if cell-bound IL-4 is biologically active. For the first time, we show that bacterial cells exposing an immobilized cytokine are capable of eliciting a specific response in mammalian cells and, moreover, that the signaling properties of cytokine variants are testable by this approach. For the purposes of this study, an IL-4-specific reporter gene assay based on murine lymphocytes was set up and employed to quantitate IL-4 receptor activation in an automatable manner.

IL-4 is a typical cytokine that exerts its activity by interacting with two cytokine receptor subunits on target cells.[18] Dysregulation of IL-4 function is involved in the development of type I allergy.[19] IL-4 constitutes a particularly suitable model system for this study, since detailed information on the properties of mutant derivatives is available.[2,6] Substitution of Tyr124 for other amino acid residues results in variants that largely retain receptor binding, but show different extents of impairment in biological activity. We show here that the relative degrees of receptor activation by different IL-4 variants in mammalian cells can be readily monitored by employing *E. coli* cells that display the respective cytokines on their surface. The approach should be generally applicable to the rational screening of cytokine or growth factor variants for biological activity, without the necessity for prior protein purification.

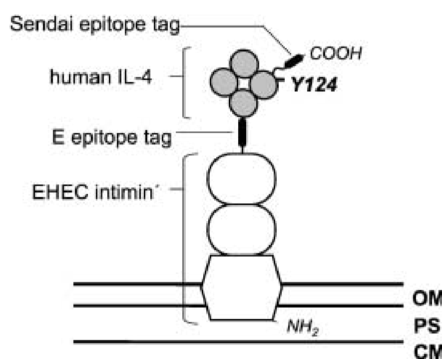

Figure 1. *Schematic representation of the fusion of truncated EaeA intimin (intimin′) and human interleukin-4 displayed on the surface of* E. coli. *Intimin′ resides in the outer membrane (OM) with its amino terminus extending into the periplasmic space (PS), but not into the cytoplasm membrane (CM). The four-helical cytokine interleukin-4 (represented by four circles) is fused to the carboxy terminus of intimin′ and flanked by two epitope tags ("E" and "Sendai") to permit detection of the passenger domain on the bacterial surface. Residue Y124 of IL-4 is located close to the C terminus of the cytokine and constitutes an important determinant of receptor activation.*

Results

Inducible presentation of interleukin-4 and interleukin-4 variants on the surface of *Escherichia coli*

We intended to test *E. coli* cells that display IL-4 on their surfaces for their potential to exert IL-4-specific biological activity. Moreover, we wished to assess the possibility of characterizing cell-bound IL-4 mutants with regard to impaired receptor activation. Therefore cDNAs encoding wild-type IL-4 as well as the two IL-4 mutants—Y124G and Y124D—were fused to a DNA sequence representing the transporter domain of enterohemorrhagic intimin EaeA and placed under the control of a tightly regulatable *tetA* promoter. Figure 1 schematically depicts the predicted structure of the membrane-anchored display constructs for IL-4 and variants. Two different epitope tags (E and Sendai) flanking the IL-4 component were included in the design as a means to detect surface expression and to obtain information on accessibility of the cytokine moiety.

These three constructs (pASKInt100-IL-4, pASKInt100-IL-4 Y124G, and pASKInt100-IL-4 Y124D) were transformed into *E. coli* BMH 71-18. As a positive control, the previously characterized expression plasmid pASKInt100-REI[17] encoding the Bence–Jones protein REI_v was used. Expression of intimin′ fusions was induced by anhydrotetracycline (AHT) and analyzed by Western blot. Probing of lysates from bacterial cultures with antibodies to both the E and Sendai epitopes showed that the intimin-IL-4 fusion proteins were expressed in an inducible manner (Figure 2).

Appearance of the IL-4 moieties on the cell surface was monitored by fluorescence microscopy, with antibodies to both the E and Sendai epitope tag again employed. Figure 3 shows that all IL-4 variants and also the REI_v protein become readily detectable on bacteria upon treatment with AHT. This result also indicates that the IL-4 portions of the fusion proteins are likely to have a native-like structure. The epitopes at their N and C termini are apparently not buried due to misfolding, but rather are accessible for antibodies.

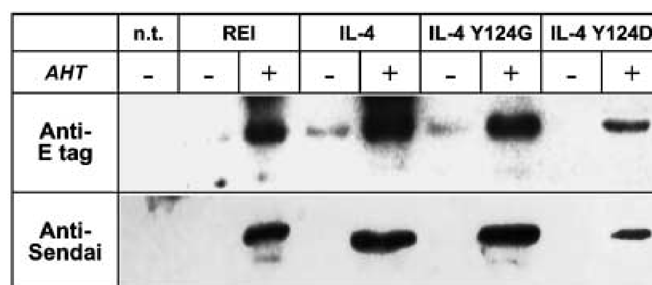

Figure 2. *Inducible expression of intimin′-fusions analyzed by Western Blot.* E. coli *BMH 71–18 clones transformed with plasmids encoding intimin′-fusions with IL-4, IL-4 mutants and REI_v (positive control) were grown to an OD_{600} of 0.2. Cultures of each clone were then divided into two aliquots and further grown for 1 h in the absence (−) or presence (+) of anhydrotetracycline (AHT) (0.2 $\mu g\,mL^{-1}$). Whole cell extracts were prepared as described in the Experimental Section and subjected to 10% SDS-PAGE followed by immunoblotting. Blots were probed with antibodies to either the E-tag* (top) *or the Sendai tag* (bottom)*. Lane "n.t." ("not transformed") represents a negative control experiment with parental* E. coli *BMH 71–18 cells.*

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	n.t.	REI		IL-4		IL-4 Y124G		IL-4 Y124D	
AHT	–	–	+	–	+	–	+	–	+
Anti-E tag									
Anti-Sendai									

Figure 3. Determination of intimin′-mediated cell surface display of IL-4 variants or Bence–Jones protein REI$_v$ through immunostaining and fluorescence microscopy. Images show fluorescence micrographs of non-transformed E. coli *BMH 71–18 (n.t.) and BMH 71–18 clones expressing fusions of intimin′ with REI$_v$ (positive control), IL-4, IL-4 Y124G, and IL-4 Y124D as indicated. Bacterial cultures were either left untreated (−) or incubated with anhydrotetracycline (AHT) (0.2 μg mL^{-1}) for 1 h (+). Staining of cells was achieved by incubation with antibody to E-tag (*top*) or Sendai tag (*bottom*), followed by probing with biotinylated anti-mouse antibody and subsequent incubation with streptavidin-R-phycoerythrin conjugate as detailed in the Experimental Section.*

line BAF-4α-pγ-S6RE-luc for 6 h. After this stimulation period, IL-4-dependent luciferase activity was determined. Figure 5 B shows that incubation of BAF-4α-pγ-S6RE-luc cells with non-induced *E. coli* clones resulted only in a marginal level of luciferase activity, largely irrespective of their plasmid contents. In contrast, induction of wild-type IL-4 display rendered *E. coli* cells a strong specific stimulus for the murine reporter cell line (stimulation index 18, about half the response elicited by a saturating concentration of soluble IL-4). *E. coli*-bound IL-4 mutant Y124D did not evoke a significant luciferase response, whereas mutant Y124G yielded a stimulation index of approximately 5. These results indicate that IL-4 exposed on the surfaces of bacteria is able to stimulate the activation of the IL-4 receptor complex on mammalian target cells. Moreover, the relative biological activity of bacterially dis-

Reconstitution of human IL-4 signaling in murine cells leading to STAT6-mediated reporter gene expression

To analyze biological activity of IL-4 fixed on the surfaces *of E. coli* in a sensitive and quantifiable fashion, we established a specific cellular readout system. On the basis of the factor-dependent murine pro-B cell line Ba/F3, a stable reporter cell line responding to human IL-4 by luciferase activity was generated. Ba/F3 cells were simultaneously transfected with expression plasmids encoding: i) the human IL-4 receptor α-chain (pKCR-4α), ii) the human γc receptor chain (pKCR-pγ), and iii) a luciferase gene under the control of a STAT6-dependent minimal promoter (p(STAT6RE)$_5$-TATAluc) (Figure 4 A). Clones were selected for resistance to G418 and were subsequently screened for IL-4-inducible luciferase activity. Three cell lines with stimulation indices (ratios of luciferase signal from IL-4-stimulated and non-stimulated cells) above 5 were subcloned by limiting dilution, and the clones obtained were again assayed for their degree of IL-4-inducible luciferase expression. One clone (termed BAF-4α-pγ-S6RE-luc) showing a maximum stimulation index of approximately 80 was identified. IL-4-dependent luciferase activity was dose-dependent, with an EC$_{50}$ of 100 pM (Figure 4 B). This result shows that inducible luciferase activity in BAF-4α-pγ-S6RE-luc cells reflects activation of the human IL-4 receptor complex in a readily detectable and quantifiable fashion.

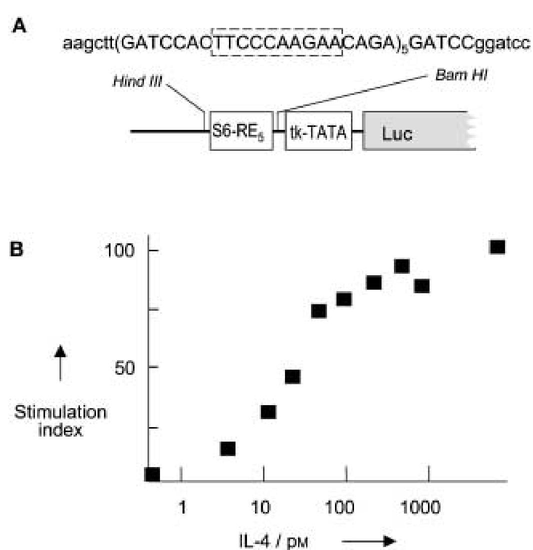

Figure 4. Generation of the IL-4 responsive reporter cell line BAF-4α-pγ-S6RE-luc. A) Structure of the IL-4-responsive luciferase reporter gene construct used in this study. Top*: Sequence of the synthetic pentameric STAT6 recognition element with flanking restriction sites (*Hind III *and* Bam HI*, indicated in lower case letters). The palindromic core STAT6 binding motif is emphasized by a dashed box.* Bottom*: Overall representation of the STAT6-responsive reporter gene construct. The IL-4-responsive region (S6-RE$_5$) was placed via* Hind III *and* Bam HI *upstream of the Herpes Simplex virus thymidine kinase TATA-box (tk-TATA)/luciferase gene (Luc) of plasmid pTATALuc$^+$ (compare Experimental Section). B) Dose dependence of luciferase expression by BAF-4α-pγ-S6RE-luc cells in response to stimulation with human IL-4. Samples of 10^5 cells were either left untreated or were stimulated with the indicated concentrations of human IL-4 for 8 h, after which cells were lyzed and luciferase activity was measured. The stimulation index represents the ratio of luciferase activity of IL-4-treated and unstimulated cells. The figure shows one representative experiment out of four independent ones.*

Specific and dose-dependent interleukin-4 receptor activation on reporter cells by IL-4-presenting bacteria

BAF-4α-pγ-S6RE-luc cells were used to test whether IL-4 displayed on the bacterial surfaces is able to elicit a specific cellular response. Figure 5 *A* schematically depicts the design of the experiment. Expression of wild-type or mutant variants Y124G and Y124D of human IL-4 and of REI$_v$ as a specificity control was induced by AHT in *E. coli* cultures harboring the respective expression constructs (compare Figures 2 and 3), after which bacteria were inactivated by treatment with gentamycin. Concentrations of 5×10^8 *E. coli* cells per mL from each inactivated bacterial culture were incubated with the murine reporter cell

Figure 5. *IL-4 receptor activation by* E. coli *cells displaying IL-4 and IL-4 mutants as fusions with intimin'.* A) *Schematic representation of the experimental design. A human IL-4 receptor complex consisting of the human IL-4 receptor α-chain (IL-4Rα) and the common γ-chain (γc) is stably expressed in Ba/F3 cells harboring a STAT6-responsive luciferase reporter gene construct (S6-RE5-luc) stably integrated into the genome. The resulting reporter cell line BAF-4α-pγ-S6RE-luc is incubated with* E. coli *BMH 71–18 surface-presenting IL-4 to activate the IL-4 receptor and ultimately to stimulate luciferase expression. B) BAF-4α-pγ-S6RE-luc reporter cells were left untreated (white bar), incubated with 1 nM IL-4 (black bar) or with 5×10^8* E. coli *cells per mL transformed with expression constructs for the "passenger" proteins indicated (gray bars).* E. coli *clones had previously been treated with anhydrotetracycline (AHT) (0.2 μg mL^{-1}) (+) to induce surface expression of passenger protein or kept in the absence of inducer (−). Growth inhibition of bacteria was achieved by incubation in PBS/gentamycin immediately before addition to IL-4 reporter cells as detailed in the Experimental Section.*

played IL-4 variants reflects the properties of the soluble cytokine proteins.

Lastly, we assessed the dose-dependency of the cellular reaction elicited by bacteria displaying IL-4. To this end, BAF-4α-pγ-S6RE-luc cells were stimulated with varying concentrations of *E. coli* cells surface-presenting wild-type IL-4 or the two mutants Y124G or Y124D, respectively. Luciferase activity was monitored after 6 h. As shown in (Figure 6A), reporter gene expression was clearly dependent on the number of IL-4-displaying bacterial cells employed. Reactivity followed a sigmoid pattern dependent on the concentration of bacteria with a half-maximum response in the range of 7×10^7 cells per mL and reaching a plateau at 10^8 cells per mL. Notably, reporter gene activity did not decrease at higher concentrations of bacterial cells up to 10^9 per mL. This finding indicates that gentamycin-inactivated *E. coli* cells do not interfere with the assay through toxic effects on Ba/F3 even at extremely high densities. Bacteria displaying IL-4 Y124G showed comparable saturation kinetics, with an IC_{50} around 7×10^7 *E. coli* cells per mL and saturation at 10^8 per mL (Figure 6B). The maximum response to Y124G was about 30% of that seen with wild-type IL-4. This ratio matches well with results obtained with soluble proteins.[6] Bacteria expressing IL-4 Y124D, an IL-4 antagonist according to work with purified cytokine,[6] did not evoke any significant response of Ba/F3 reporter cells (Figure 6C).

Discussion

We have shown that interleukin-4, a typical human cytokine, can be exposed on the surfaces of *E. coli* cells in a natively folded and biologically active state. Moreover, we have devised a readout system allowing quantitative assessment of (ant)agonistic properties of IL-4 mutants displayed on bacteria. This study underscores the wide potential of intimin-based display of polypeptides. We demonstrate here that proteins presented by bacteria as fusions with intimin can readily be analyzed with regard to their biological activity exerted on mammalian cells.

Importantly, the relative activities of bacterially displayed cytokine (IL-4) variants were similar to those observed with purified, soluble proteins. The maximum activity of cell-bound IL-4 was somewhat lower than that exerted by soluble cytokine (Figure 4), presumably due to steric constraints caused by the attachment of the molecules to the bacterial membrane. Fixing of IL-4 to bacteria requires compensation in concentration relative to a protein solution to achieve equivalent biological responses by target cells; 10^8 per mL IL-4-presenting *E. coli* cells yielded saturation in receptor activation on Ba/F3 cells. Given earlier findings that showed that about 10^4 copies of intimin fusion are displayed per *E. coli* cell,[17] this corresponds to a "chemical" IL-4 concentration of about 8 nM. Soluble recombinant IL-4 readily saturates the IL-4 receptor-mediated response at a concentration of 1 nM (Figure 4 and Ref.[20]). This difference can probably be attributed to the inhomogeneous distribution of ligand in the cell-bound state. Interaction of a bacterium with one target cell through a small number of displayed IL-4 molecules virtually prevents the remainder of ligand molecules from activating receptors on other target cells until the first contact ceases.

The interaction of cytokines and growth factors with their respective cognate receptors is of great medical importance. For the design of pharmaceutical compounds that interfere with ligand–receptor interactions, extended knowledge of the details of the mutual contact interfaces is required. High-resolution structural information is only available for a small number of cytokines or growth factors complexed with their receptors.[21–24] For other receptors that constitute attractive pharmaceutical targets (e.g., the receptor for interleukin-13), structures have not yet been resolved. In any event, structural data have to be substituted or complemented by results obtained through mutational analysis, in most cases a very laborious and time-consuming task. Consequently, experimental procedures that can accelerate the characterization of ligand–

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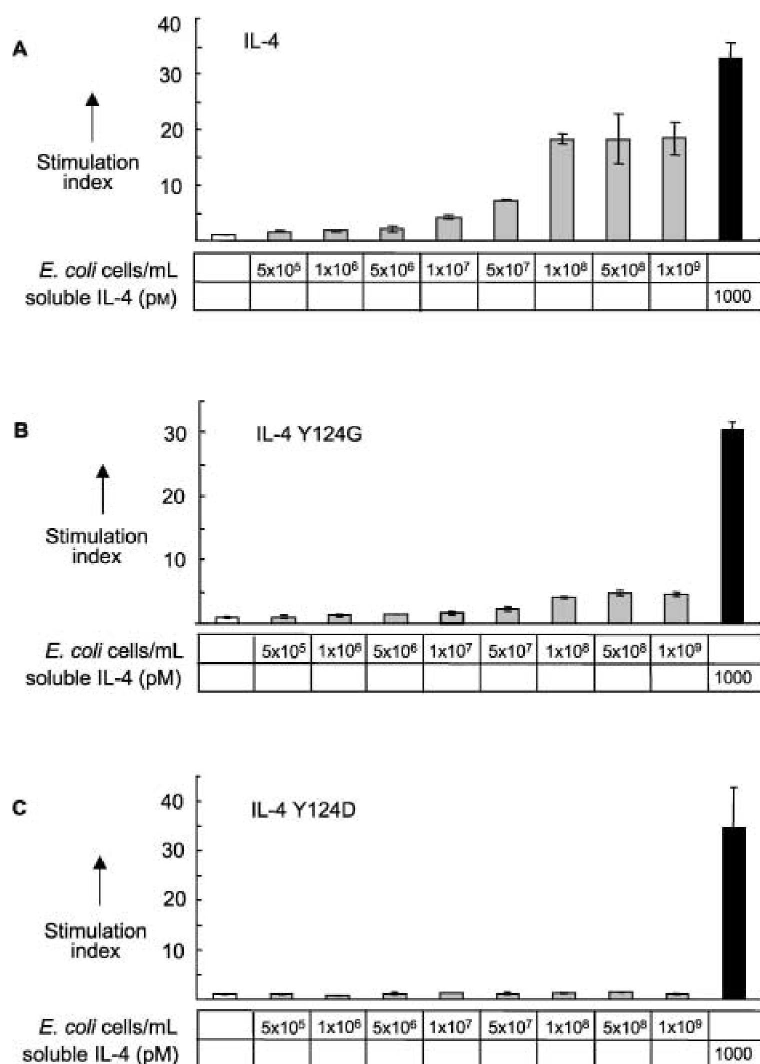

Figure 6. Activation of the IL-4 receptor on BAF-4α-pγ-S6REluc reporter cells in response to varying doses of E. coli *cells displaying IL-4 and variants. (*A*) BAF-4α-pγ-S6RE-luc reporter cells were left untreated (white bar), or incubated with 1000 pM IL-4 (black bar) or with the indicated concentrations of AHT-induced and subsequently inactivated* E. coli *cells displaying wild-type human IL-4 (gray bars). (*B*) Assay as in* A *with* E. coli *cells displaying IL-4 mutant Y124G. (*C*) Assay as in* A *with* E. coli *cells displaying IL-4 mutant Y124D.*

receptor interactions, with regard both to binding and to activation of signal release, are highly desirable.

The protocol presented in this work has been developed by employing IL-4 as an example for a cytokine that activates its receptor system by cross-linking the ectodomains of two receptor subunits. Intracellular signal release as a consequence of receptor subunit dimerization is a widespread mechanism in cytokine and growth factor biology, and the juxtaposed intracellular receptor domains determine the specificity of the cellular response. Numerous receptor chimeras in which ligand-dependent dimerization of ectodomains has been experimentally coupled to signal transduction through activation of genetically fused unrelated intracellular domains have been reported.[25–27] Making use of this possibility renders our approach of testing cell-bound cytokines and growth factor variants for their receptor activation potency generally applicable for many systems in which ligands induce receptor dimerization. To exploit this potential, we have fused the intracellular portion of the IL-4 receptor complex to the ectodomains of the bipartite human IL-13 receptor. These constructs mediated IL-13-specific transcription from the reporter gene plasmid used in this study (S.K. and K.F., unpublished results). Extending the scope of the assay to very distantly related receptor complexes, we have obtained ligand-dependent luciferase signals from the STAT6-specific reporter gene construct through activation of hybrid receptors in which the exodomains were derived from the heterodimeric Transforming Growth Factor-β (TGF-β) receptor (S.K. and K.F., unpublished results).

The intracellular segment of the interleukin-4 receptor is particularly well suited as a platform for the transmission and readout of dimerization signals, due to the specificity of its downstream signaling pathway. The ultimate result of IL-4 receptor activation is the transcription of target genes in which regulation occurs through transcription factor STAT6. STAT6 is unique among the family of STAT factors in that it recognizes a very specific DNA element with a four-nucleotide spacer between the palindromic half-sites TTC and GAA. All other STAT proteins bind to sequence elements with only three base pairs in this position.[28] This state of affairs results in a characteristic pattern of target gene expression in response to IL-4 and in a high expression specificity of reporter genes under the control of IL-4 target gene promoters.

It would be interesting to establish that the IL-4-presenting *E. coli* cells also induce other biological responses characteristic of this cytokine, such as upregulation of major histocompatibility complex class II or induction of proliferation in activated T cells. Such experiments, however, would pose particular difficulties as a result of the employment of bacteria: both proliferation tests and the measurement of MHC II induction would require the human cells to be incubated with bacteria for at least 24 h. Although we are able to prevent practically any *E. coli* growth for 6 h (the relatively short time span required for the performance of reporter gene assays) by inactivation with gentamycin, it is a complicated task to keep the mixture of murine/bacterial cells clear of any bacterial proliferation for such long periods of time.

The first advantage of the approach presented here lies in the great facilitation of mapping of cytokines and growth factors for receptor binding and activation determinants by rendering the purification of variant cytokines obsolete. Another attractive perspective is selection from mutant repertoires of cytokine variants that bind receptors expressed by Ba/F3 cells. We have shown earlier that *E. coli* clones displaying peptides with affinity for a specific target could be readily selected for by panning procedures.[17] By fluorescence-activated cell sorting (FACS), it should be possible to isolate Ba/F3-cells with at-

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tached bacteria through the reactivity of the latter with antibodies to epitope tags that label the displayed proteins. Subsequent testing of obtained *E. coli* clones for biological activity of the cell-bound cytokines on Ba/F3-derived reporter cells should then lead to the rapid identification of antagonistic cytokine variants that bind but do not activate their cognate receptors.

Experimental Section

Bacterial and mammalian cells: *Escherichia coli* strain BMH 71–18[29] and the interleukin-3-dependent murine pro-B cell line Ba/F3[30] have been described.

Reagents, antibodies, and oligonucleotides: Restriction and DNA-modifying enzymes were purchased from New England Biolabs. Pfu polymerase was from Promega. Antibiotics (G418, gentamycin) were purchased from Gibco/Invitrogen. Biotinylated goat anti-mouse antibody and peroxidase-coupled (POD-coupled) goat anti-mouse antibody were obtained from Sigma. The monoclonal antibody to a 13-residue C-terminal epitope of Sendai virus L protein (DGSLGDIEPYDSS)[31] was a gift from H. Einberger and H.P. Hofschneider (Max Planck Institute of Biochemistry, Martinsried, Germany). Anti-E monoclonal antibody was from Pharmacia Biotech. A streptavidin R-phycoerythrin conjugate was purchased from Molecular Probes. Oligonucleotides were purchased from MWG Biotech, Ebersberg or JenaBioscience, Jena, Germany. Recombinant human interleukin-4 was a gift from Walter Sebald, Biocenter, University of Würzburg.

DNA procedures: Recombinant DNA work was performed by standard procedures.[32]

Bacterial expression constructs for intimin′-IL-4 and intimin′-REI$_v$ fusions: Expression plasmids pASKInt100-IL-4 and pASKInt100-REI for the surface display of wild-type IL-4 and Bence–Jones protein REI$_v$, respectively, have been described.[17] These constructs encode fusions of the 659 N-terminal amino acids of EHEC intimin with human interleukin-4 or REI$_v$. Plasmids pASKInt100-IL-4 Y124G and pASKInt100-IL-4 Y124D, encoding epitope-tagged intimin′ fusions with IL-4 mutants, were generated as follows: PCR fragments were obtained by employing primers IL-4-up (5′-GCGCCCCGGGCA-CAAGTGCGATATCACC-3′) and IL-4Y124G-rev (5′-CTGAGATCTGCTC-GAACACTTTGAACCTTTCTC-3′) or IL-4Y124D-rev (5′-CTGAGATCT-GCTCGAACACTTTGAATCTTTCTC-3′), respectively, with plasmid pASKInt100-IL-4 as template. The resulting fragments were digested with *Sma* I and *Bgl* II and ligated into similarly cleaved pASKInt100-REI. All nucleotide sequences were confirmed by nucleotide sequence analysis.

Bacterial surface expression of intimin′ fusions: *E. coli* BMH 71–18 clones harboring expression plasmids were grown overnight at 37 °C in dYT medium supplemented with chloramphenicol (25 μg mL^{-1}) and subcultured at 1:200 until they reached an optical density at 600 nm (OD$_{600}$) of 0.2. Expression was induced by addition of anhydrotetracycline (AHT) to a final concentration of 0.2 μg mL^{-1}. After 60 min of further incubation at 37 °C, cells from 1 mL aliquots were pelleted by centrifugation in a tabletop centrifuge for 1 min, re-suspended in phosphate-buffered saline (PBS) such that an OD$_{600}$ of 1 was reached, and stored at 4 °C for analysis and stimulation experiments as described below.

Western Blot: Starting from *E. coli* cell suspensions with an OD$_{600}$ of 1 (see above), aliquots (20 μL) were pelleted and re-suspended in denaturing protein sample buffer (20 μL, 62.5 mM Tris-HCl, pH 6.8, 2% (*w/v*) sodium dodecyl sulfate (STS), 20% (*v/v*) glycerol, 100 mM dithiothreitol, 0.2% (*w/v*) bromophenol blue). After boiling for 5 min, samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 9% acrylamide-bisacrylamide (30:0.8) gels and with immunoblotting as described.[20] Blots were probed with a 1:3000 dilution of anti-E tag or a 1:500 dilution of anti-Sendai (hybridoma supernatant). Detection of bound antibodies was achieved with POD-coupled anti-mouse IgG at a dilution of 1:20000. Immunoreactive bands were visualized by enhanced chemoluminescence (Amersham).

Antibody staining of *E. coli* cells and visualization by fluorescence microscopy: *E. coli* cell suspensions (200–400 μL, OD$_{600}$ approximately 1) were centrifuged in a tabletop centrifuge. Pellets were re-suspended in antibody solutions (1:10, 10 μL) (anti-E tag or anti-Sendai) and incubated at room temperature for 5 min. Cells were pelleted, washed in PBS and re-suspended in a dilution of biotinylated goat anti-mouse antibody (1:10, 10 μL) followed by 5 min incubation at room temperature. After washing in PBS, cells were treated with a dilution of a streptavidin-R-phycoerythrin conjugate (1:10, 10 μL). Stained cells were visualized with a Zeiss Axiovert fluorescence microscope (filter set No. 15).

Mammalian expression constructs for receptor hybrids and luciferase: Expression plasmids pKCR-4α and pKCR-pγ, encoding human IL-4 receptor α-chain and γc-chain, respectively, have been described previously.[20,27] Luciferase reporter gene plasmid p(STAT6RE)$_5$-TATAluc, comprising a luciferase gene under the control of an IL-4-responsive promoter, was constructed by insertion of five consecutive recognition elements for Signal Transducer and Activator of Transcription 6 (STAT6) (…TTCCCAAGAA…) upstream of the thymidine kinase minimal promoter of luciferase expression plasmid pTATALuc+.[33] This was achieved by ligation of a synthetic *Hind III–Bam HI* fragment representing the STAT6 cognate elements separated spacers into the respective restriction sites of pTATA-Luc+. The latter fragment was generated by hybridization of oligonucleotides 5xSTAT6RE-up (5′-AGCTGATCCACTTCCCAAGAACA-GAGATCCACCTTCCCAAGAACAGAGATCCACTTCCCAAGAACAGAGAT-CCACTTCCCAAGAACAGAGATCCACTTCCCAAGAACAGAGATCCG-3′) and 5xSTAT6RE-lo (5′-GATCCGGAGCTCTGTTCTTGGGAAGTGGATCTC-TGTTCTTGGGAAGTGGATCTCTGTTCTTGGGAAGTGGATCTCTGTCTTT-GGGAAGTGGATCTCTGTTCTTGGGAAGTGGATC-3′). All nucleotide sequences were verified by DNA sequencing.

Culture and transfection of Ba/F3 cells: Ba/F3 cells and derivatives were cultured as described.[20] Stable transfection of Ba/F3 cells by electroporation and isolation of antibiotic-resistant clones was performed as described previously.[20] Briefly, 24 h post-electroporation cells were transferred to growth medium supplemented with G418 (1 mg mL^{-1}). The cell suspension was distributed into the wells of 24-well cell culture plates and cultivated for about two weeks. G418-resistant cells from individual wells were further propagated and tested for IL-4-dependent luciferase activity as described below.

Stimulation of BAF-4α-pγ-S6 Reluc cells with bacteria displaying IL-4, reporter gene assay: *E. coli* BMH 71–18 cells suspended in PBS at an OD$_{600}$ of 1 were inactivated by incubation with gentamycin (Gibco) (0.5 μg mL^{-1}) overnight at room temperature. Bacterial suspensions were then either used directly for cell stimulation experiments or first diluted with PBS or further concentrated by centrifugation and re-suspension in an appropriate volume of PBS.

BAF-4α-pγ-S6RE-luc cells were cultured in medium devoid of IL-3 for 16 h. Cells were divided at this stage into samples of 2×10^5 cells and incubated in individual wells of 24-well cell culture plates.

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In a total volume of 200 μL, cell aliquots were treated variously with medium alone, with medium containing 1 nM human IL-4, or with medium supplemented with dilutions of inactivated *E. coli* cells (1:10) for 6 h at 37 °C. Subsequently, luciferase activity was measured in a microplate luminometer (Berthold) after lysis of cells in a standardized cell lysis reagent (Promega) (60 μL) as described previously.[34]

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Keywords: bacterial surface display · cytokines · IL-4 · immobilization · signal transduction

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