Inhibition of Tumor Necrosis Factor mRNA Translation by a Rationally Designed Immunomodulatory Peptide*

Received for publication, November 16, 1999, and in revised form, February 1, 2000
Published, JBC Papers in Press, March 15, 2000, DOI 10.1074/jbc.M909219199

Suhasini Iyer‡§, Dimitris Kontoyiannis‡, Dominique Chevrier‡, Jacky Woo‡, Nancy Mori‡,
Marie Cornejo‡, George Kollias‡, and Roland Buelow‡

From ‡SangStat, The Transplant Company, Fremont, California 94555 and the ‡Laboratory of Molecular Genetics,
Hellenic Pasteur Institute, Athens 11521, Greece

Based on sequences of immunomodulatory peptides derived from the heavy chain of HLA Class I, novel immunomodulatory peptides with increased potency were developed by computer-aided rational design. Allotrap 1258 was characterized in detail and shown to inhibit cell-mediated immune responses in vitro and in vivo. Immunomodulatory activity was associated with the capability of the peptides to modulate heme oxygenase (HO) activity. In this study we analyzed the effect of Allotrap 1258 on cytokine expression. Allotrap 1258 inhibited concanavalin A- and lipopolysaccharide-induced human and mouse tumor necrosis factor (TNF) production in vitro and in vivo but had no effect on interleukin (IL)-1, IL-2, IL-4, IL-6, or IL-10 expression. Experiments with HO-1/KO and iNOS/KO mice showed that Allotrap 1258-mediated inhibition of TNF was independent of HO-1 and iNOS. Quantitation of TNF protein expression and mRNA steady state levels demonstrated that Allotrap 1258-mediated inhibition occurred at the translational level. Deletion of the AU-rich element in the 3'-untranslated region (UTR) of TNF mRNA, a region known to be involved in TNF mRNA translation, had minimal effect on Allotrap 1258-mediated inhibition. However, replacement of the TNF 3'-UTR with the human globin 3'-UTR rendered the peptide inactive. This demonstrates that besides AU-rich elements, other sequences in the 3'-UTR of TNF mRNA are involved in translational control of TNF expression. Such sequences are necessary for Allotrap 1258-mediated inhibition of TNF production.

Peptides derived from the heavy chain of the HLA Class I molecule have been shown to modulate immune responses in vitro and in vivo (1–7). In vitro, these peptides inhibited cytotoxicity and differentiation of cytotoxic T cells (1). In vivo, they were shown to prolong allograft survival in rodents (2–7). Based on these HLA-derived peptide sequences, novel immunomodulatory peptides were developed by computer-aided rational design (8). Initially, the biological activity of a panel of 19 peptides derived from peptide 2702.75–84 (HLA-B2702, amino acids 75–84) as well as other major histocompatibility complex peptides derived from peptide 2702.75–84 (HLA-B2702, amino acids 75–84) as well as other major histocompatibility complex molecules was assessed in a heterotopic mouse allograft model. Allotrap 1258 was characterized in detail and shown to inhibit concanavalin A- and lipopolysaccharide-induced cytokine expression and mRNA steady state levels demonstrated that Allotrap 1258-mediated inhibition occurred at the translational level. Deletion of the AU-rich element in the 3'-untranslated region (UTR) of TNF mRNA, a region known to be involved in TNF mRNA translation, had minimal effect on Allotrap 1258-mediated inhibition. However, replacement of the TNF 3'-UTR with the human globin 3'-UTR rendered the peptide inactive. This demonstrates that besides AU-rich elements, other sequences in the 3'-UTR of TNF mRNA are involved in translational control of TNF expression. Such sequences are necessary for Allotrap 1258-mediated inhibition of TNF production.

I The abbreviations used are: HO, heme oxygenase; ConA, concanavalin A; IL, interleukin; GalN, galactosamine; UTR, untranslated region; TNF, tumor necrosis factor; TNFRI, tumor necrosis factor receptor I; iNOS, inducible nitric-oxide synthase; HPLC, high pressure liquid chromatography; IFN, interferon; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; mAb, monoclonal antibody; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TEPM, thioglycolate-elicited peritoneal macrophage; ARE, AU-rich element; SAPK, stress-activating protein kinase.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: SangStat, The Transplant Company, 6300 Dumbarton Circle, Fremont, CA 94555. Tel.: 510-789-4508; Fax: 510-789-4206; E-mail: siyer@sangstat.com.
studied in a galactosamine (GalN/LPS mouse model that has been characterized as a model for systemic inflammatory response syndrome (15, 16). A detailed analysis of TNF production in the presence of peptide demonstrated that peptide-mediated inhibition of TNF production occurs at the translational level. Experiments with macrophages from transgenic and targeted animals expressing modified TNF genes demonstrated that AU-rich elements (AREs) in the mRNA 3′-UTR sequences appear to play a moderate role in mediating the suppressive effects of the peptides, but other as yet unidentified sequences in the 3′-UTR are more dominant in mediating the inhibition by the peptide of TNF translation.

TNF exerts a key role in the pathogenesis of many inflammatory diseases such as sepsis, inflammatory bowel disease, rheumatoid arthritis, and Crohn’s disease (17–20). Although originally described as a molecule that could reduce the size of malignant tumors, a massive up-regulation of TNF at a systemic level such as occurs in global infections or severe tissue injury leads to vasodilation, intravascular coagulation, and multiple organ failure (21). A therapeutic benefit of inhibiting TNF in such inflammatory conditions is readily apparent. Clinically, various approaches to reduce TNF levels in patients have been explored, including the use of the phosphodiesterase inhibitor, pentoxifylline, and anti-TNF antibodies. In particular, protein based therapies (i.e. anti-TNF antibody and soluble receptors) have shown considerable clinical success (22). To our knowledge, Allotrap 1258 is the first low molecular weight compound inhibiting TNF production at a translational level.

MATERIALS AND METHODS

Mice—C57BL/6, CBA, and inducible nitric-oxide synthase (iNOS) knock-out mice were purchased from Jackson Laboratory (Bar Harbor, ME). HO-1 knock-out mice were kindly provided by Dr. Poss (23). Mice were housed according to the Animal Welfare Guidelines of the California Department of Health. Tg1278 and Tg197 human TNF transgenic mice (24) and homozygous TNFαRKO targeted animals in a TNF-FR-deficient background (25, 26) were bred and maintained on mixed 129SvxC57BL/6 or CBAXC57BL/6 genetic backgrounds at the animal facilities of the Helenic Pasteur Institute under specific pathogen-free conditions.

Peptide Synthesis—Peptides (Allotrap 1258: rnlrlrlrlngly-CONH2, D2RP: rvlplirly-CONH2) were synthesized using Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry, purified by HPLC, and shown to be >90% homogenous by analytical reverse phase HPLC (SynPep, Dublin, CA). Peptides were stored lyophilized at 4 °C and were dissolved fresh before use in a 5% mannitol in water solution.

Effects on Survival—In a preliminary experiment, the kinetics of cytokine production following intravenous injection of ConA (20 mg/kg) were analyzed. Groups of mice (n = 3/group) were sacrificed 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 7 h post-injection. Plasma was collected and analyzed for IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, TNF, and IFNγ levels. Based on this kinetic analysis, the time point with maximum plasma cytokine concentration was chosen to study the effect of peptide. Mice (n = 3/group) were injected intravenously with 20 mg/kg ConA and 0, 5, 10, or 20 mg/kg peptide in 5% mannitol. Animals were given simultaneous injections of GalN at 800 mg/kg and LPS at 0.1 mg/kg (intraperitoneal). One group each was also treated with Allotrap 1258 or negative control peptide at 10 mg/kg (intraperitoneal) at the time of GalN/LPS injection. Animal survival was monitored twice daily.

Cell Culture—RAW264.7 cells, a mouse macrophage cell line, and THP-1 cells, a human monocyte/macrophage cell line, were obtained from ATCC (Manassas, VA) and were routinely cultured in RPMI 1640 containing 10% fetal bovine serum in 5% CO2/95% air at 37 °C. RAW264.7 cells were seeded at a density of 3.5 × 105/ml and stimulated for TNF production by the addition of LPS (10 μg/ml). THP-1 cells were seeded and stimulated in the presence of LPS (15 μg/ml) and IFNγ (10 units/ml). For experiments with transgenic mouse macrophages, total exudate peritoneal macrophages were isolated by peritoneal lavage with phosphate-buffered saline from 10-week-old animals 3 days after a single intraperitoneal injection of 1.0 ml of 4% thioglycolate broth (Difco Laboratories) as described previously (27). Cells were seeded at a density of 5 × 105/ml and stimulated for TNF production by the addition of LPS (1 μg/ml) in the presence or absence of Allotrap peptides. Peptides were added at various concentrations ranging from 3 to 100 μM. Cells were incubated in 5% CO2/95% air at 37 °C for 24 h. At the indicated times, culture medium was collected and stored frozen until use. TNF concentrations were determined by sandwich ELISA using anti-mouse or anti-human TNF mAb (see above).

Immunoprecipitation—THP-1 cells were seeded at a density of 5 × 106 cells/ml in methionine-deficient RPMI spiked with [35S]Met (3 μCi/ml) and were stimulated for TNF production with LPS (5 μg/ml) and IFNγ (100 units/ml). After 24 h, cells were separated from culture medium and were washed in ice-cold phosphate-buffered saline. Cell pellets were resuspended in lysis buffer consisting of 20 mM sodium phosphate, pH 7.5, 500 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 200 mM sodium chloride (imunoprecipitation buffer). Insoluble material was discarded after centrifugation. Monoclonal anti-TNF antibody (clone mAb1) (3 μM) was added to culture medium as well as cell extracts. In identically treated control cells, TP-25 (anti-Class I mAb) or mouse anti-human IgG (3 μM) were added to cultures to supplement and control extracts. The samples were incubated overnight at 4 °C. 10 μl of protein G-agarose was added to each sample, and the mixture was incubated for 2 h at 4 °C. After centrifugation, the agaroase beads were washed three times in ice-cold lysate buffer and counted in a scintillation counter (TopCount, Packard Instrument, Meridan, CT).

Northern Blots and RT-PCR—Trizol reagent (Life Technologies, Inc.) was used to extract total RNA from 5 × 106 THP1 cells for each condition and time point used in the LPS stimulation assay. The RNA from each sample was adjusted to an equal concentration according to their A260 values. RNA was reverse transcribed at 42 °C for 1 h using oligo(DT)12-18 and avian myeloblastosis virus reverse transcriptase (Promega). Quantitative PCR for TNF and GAPDH was performed using a commercially available kit and according to the instructions of the manufacturer (BIOSOURCE). To allow sample comparisons, results were expressed in number of TNF mRNA copies per GAPDH mRNA copy. The TNF mRNA quantitation shown in this study is the result of two independent quantitative PCRs from the same cDNA.

TNF mRNA from transgenic macrophage cultures was extracted using guanidinium isothiocyanate (28) and subsequently treated with RQ1 RNase free DNASE (1 unit of μg of cellular RNA, Promega). Total RNA was then synthesized from 5 μg of cellular RNA at 37 °C for 1 h in a final volume of 20 μl containing 5 μg oligo (dT)18 (5′-CCAGCGAGGAGGAGGAGG) and 1 μl each dNTP (New England Biolabs) in the presence of 200 units of Moloney murine leukemia virus reverse transcriptase (Promega). Primer sequences and PCR conditions were identical to those described recently (25). Data were normalized relative to β-actin values. For Northern blot of total RNA from targeted mutant macrophages, 15 μg of RNA were resolved in 1.2% formaldehyde agarose gel, blotted onto Hybond N +
RESULTS

Allotrap 1258-mediated Effects on Cytokine Production in Vivo—Mice challenged with a single intravenous dose of ConA (20 μg/kg) showed up-regulation of circulating cytokine levels that peaked at various time points. 5 mg/kg Allotrap 1258 caused a 75% reduction of TNF plasma levels measured 60 min after treatment. Higher doses of Allotrap 1258 had a similar effect. Similarly, administration of 5–20 mg/kg Allotrap 1258 caused a 50% reduction of IFNγ plasma levels measured 360 min after treatment. In contrast, administration of Allotrap 1258 at 5 mg/kg had little effect on IL-12 plasma levels (30% reduction), whereas 20 mg/kg Allotrap 1258 resulted in a 70% reduction. Other cytokines studied, namely IL-1β, IL-2, IL-4, IL-6, and IL-10, showed no differences in response to peptide treatment (data not shown). For all cytokines, the effect of Allotrap 1258 was studied at their respective peak time points. Treatment with Allotrap 1258 alone (in the absence of ConA stimulation) did not result in detectable levels of any of the studied cytokines (Fig. 1).

Allotrap 1258-mediated Effects on TNF Production in Vivo—Allotrap 1258 administration effectively inhibited the production of TNF in mice challenged with GalN and LPS (Table I). Treatment with GalN alone at 800 mg/kg had no effect on serum TNF levels in all strains of mice studied. Challenge with a low dose of LPS (0.1 mg/kg) following GalN, however, resulted in significantly higher TNF levels at 60 min. (Table I). Simultaneous administration of Allotrap 1258 prevented the rise in TNF levels in C57BL/6 and CBA mice, which were undetectable. Similar inhibition was observed in iNOS/KO and HO-1/KO mice as well as in the respective wild type controls (Table I). Inhibition of LPS induced TNF production correlated with increased survival. Mice (C57BL/6 or CBA) challenged with GalN/LPS died because of endotoxic shock 12–24 h post-injection (Table I). Those treated with Allotrap 1258 at 10 mg/kg showed significantly higher survival rates with 80–100% animals surviving at 24 h. Negative control peptide D2RP had no protective effects (Table I). Allotrap 1258 treatment in iNOS/KO animals similarly challenged with GalN/LPS resulted in protection of these animals from death (70% survival at 24 h). Because of the limited number of HO-1/KO animals available and given their extreme sensitivity to endotoxin in general (23), survival studies were not done in this group.

Peptide-mediated Inhibition of TNF in Vitro—The mechanism of peptide-mediated inhibition of TNF production was investigated in vitro experiments. The human monocyte cell line THP-1 was labeled with 51Cr and incubated at 37 °C in the presence or absence of Allotrap 1258, or vehicle control for 4 and 24 h. 51Cr released into the medium by labeled cells cultured in the presence of Allotrap 1258 was not different from the spontaneous release of 51Cr by untreated cells either at 4 or 24 h (data not shown). This demonstrated that the peptides were not cytotoxic and did not induce cell lysis.

Neither THP-1 cells nor the mouse macrophage cell line RAW264.7 produced detectable amounts of TNF in normal tissue culture. In the presence of LPS (10 μg/ml), TNF levels in the RAW264.7 cell culture medium increased continuously over 24 h to a level of 2.46 ng/ml (Fig. 2A). Similarly, THP-1 cells stimulated with LPS (5 μg/ml) and IFNγ (100 units/ml) for 24 h produced 3.04 ng/ml TNF. (Fig. 2B). In the presence of Allotrap 1258 a dose-dependent inhibition of TNF production was seen both in RAW264.7 cells (Fig. 2A) as well as in THP-1 cells (Fig. 2B). The IC50 values obtained was 20 μM. Negative control peptide had no effect on LPS-mediated TNF levels in either cell line.

To evaluate whether peptide-mediated inhibition of TNF production was on a translational or transcriptional level, we quantitated the amount of TNF protein and mRNA produced in the presence or absence of peptide. Protein production was measured by immunoprecipitation of [35S]Met-labeled TNF. Neither THP-1 cells nor the mouse macrophage cell line RAW264.7 produced detectable amounts of TNF in normal tissue culture. In the presence of LPS (10 μg/ml), TNF levels in the RAW264.7 cell culture medium increased continuously over 24 h to a level of 2.46 ng/ml (Fig. 2A). Similarly, THP-1 cells stimulated with LPS (5 μg/ml) and IFNγ (100 units/ml) produced 3.04 ng/ml TNF. (Fig. 2B). In the presence of Allotrap 1258 a dose-dependent inhibition of TNF production was seen both in RAW264.7 cells (Fig. 2A) as well as in THP-1 cells (Fig. 2B). The IC50 values obtained was 20 μM. Negative control peptide had no effect on LPS-mediated TNF levels in either cell line.
Upon stimulation with LPS or LPS, amounts of TNF (closed triangle) after 24 h was 2.492 increase in TNF concentration was observed. The TNF concentration (b) THP-1 (open circles) resulted in a dose-dependent inhibition of TNF production. Addition of Allotrap 1258 (closed square) had no effect. (43447 ± 17054 cpm). However, Allotrap 1258 had no effect on the incorporation of [35S]Met into other proteins. In particular, peptide did not change production of HLA class I immunoprecipitated with mAb TP25. Equivalent amounts of class I were immunoprecipitated from stimulated cultures in-dependent of the presence of Allotrap 1258 or D2RP (61808 ± 2993 cpm). Similar results were obtained with cell lysates (Fig. 3B). However, as expected, the amount of TNF in the supernatant was larger than the amount of TNF in the cell lysate, and the opposite was true for HLA class I.

The amount of TNF mRNA was quantitated by RT-PCR. RNA was isolated from THP-1 cells at 2, 4, and 24 h after addition of LPS ± Allotrap 1258. Following reverse transcription, both TNF and GAPDH cDNAs were quantitated by PCR and ELISA (Biosource). Irrespective of the addition of Allotrap 1258 at 100 μg/ml, TNF mRNA levels increased in all samples after stimulation (Fig. 4). Relative to GAPDH mRNA copy numbers, the average number of TNF transcripts increased from 0.66 in unstimulated cells to 9.07 and 7.43 in the presence of LPS alone or with Allotrap 1258, respectively. This minor difference was not statistically significant and could not explain the complete inhibition of TNF protein in the presence of Allotrap 1258. Stimulated cells in this set of experiments produced 96.81 pg/ml TNF. Addition of Allotrap 1258 resulted in complete inhibition of TNF production, and TNF was undetectable. In the presence of a control peptide and LPS, cells produced normal amounts of TNF (117 pg/ml) that did not differ from control LPS-stimulated cells. Allotrap 1258-mediated inhibition of TNF translation requires 3’-ARE-dependent and ARE-independent mechanisms

The post-transcriptional regulation of TNF mRNA has been reported to rely on mechanisms that utilize elements residing on the 3’-UTR of this message (25, 29–31). To determine whether the Allotrap 1258-mediated suppression of TNF translation requires elements in the TNF 3’-UTR, the effect of this peptide on TNF biosynthesis by a 3’-modified human TNF transgene was analyzed. Thioglycolate-elicited peritoneal macrophages (TEPMs) were isolated from two separate transgenic lines: (a) Tg1278, which contained a wild type human TNF-3’-UTR transgene, and (b) Tg197, which contained a 3’-modified human TNF transgene bearing the 3’-UTR of the β-globin gene (24). As in the case of macrophage cell lines, addition of Allotrap 1258 to Tg1278 TEPM cultures inhibited LPS induced hTNF (Fig. 5A) and endogenous mTNF (not shown) production in a dose-dependent manner with a half-maximal inhibition at a concentration of 6.2 μg/ml. No change in the levels of the corresponding mRNAs were detected, via semi-quantitative RT-PCR, at 2 h after LPS + Allotrap 1258 stimulation, confirming that the peptide inhibits translation. In sharp contrast, similar treatment of Tg197 TEPM, which affected endogenous mTNF production (not shown), had no effect on either TNF secretion or mRNA accumulation, demon-

| Strain          | Treatment     | Dose (mg/kg) | TNF (ng/ml) | Survival (n) |
|-----------------|---------------|--------------|-------------|--------------|
| CBA             | None          | 0            | 3.6 ± 2.16  | 0 (5)        |
| CBA             | D2RP          | 10           | 2.0 ± 1.1   | 0 (5)        |
| CBA             | Allotrap 1258 | 10           | <0.3        | 3 (5)        |
| C57Bl/6         | None          | 0            | 8.5 ± 11.3  | 1 (5)        |
| C57Bl/6         | D2RP          | 10           | 8.8 ± 7.46  | 4 (25)       |
| C57Bl/6         | Allotrap 1258 | 10           | 0.12 ± 0.26 | 24 (25)      |
| iNOS K/O        | D2RP          | 10           | 2.5 ± 0.95  | 2 (10)       |
| iNOS K/O        | Allotrap 1258 | 10           | 0.53 ± 0.92 | 7 (10)       |
| HO-1 WT         | None          | 0            | 1.8 ± 0.46  | NT           |
| HO-1 K/O        | Allotrap 258  | 10           | <0.3        | NT           |
| HO-1 K/O        | None          | 0            | 5.3 ± 5.02  | NT           |
| HO-1 K/O        | Allotrap 1258 | 10           | 0.53 ± 0.71 | NT           |

a Mean ± S.D.
b WT, wild type.
c NT, not tested.

| Strain          | Treatment     | Dose (ng/ml) | TNF (ng/ml) |
|-----------------|---------------|--------------|-------------|
| CBA             | None          | 0            | 0.26        |
| CBA             | D2RP          | 10           | 2.16        |
| CBA             | Allotrap 1258 | 10           | 0.92        |
| C57Bl/6         | None          | 0            | 5.02        |
| C57Bl/6         | D2RP          | 10           | 0.46        |
| C57Bl/6         | Allotrap 1258 | 10           | 0.26        |
| iNOS K/O        | D2RP          | 10           | 0.95        |
| iNOS K/O        | Allotrap 1258 | 10           | 0.46        |
| HO-1 WT         | None          | 0            | 0.71        |
| HO-1 K/O        | Allotrap 258  | 10           | 0.3         |
| HO-1 K/O        | None          | 0            | 7.46        |
| HO-1 K/O        | Allotrap 1258 | 10           | 0.71        |

Mean ± S.D.
Stratifying a requirement for the 3'-UTR in the Allotrap 1258-mediated suppression of TNF translation.

To further define whether the minimal ARE that resides in TNF 3'-UTR is necessary for the inhibitory effect of Allotrap 1258 on TNF translation, the effect of this peptide on TNF production from mutant mouse macrophages lacking TNF ARE was examined. To eliminate the possibility that the chronic inflammation that occurs in TNF ARE mice affects the efficacy of the peptide in inhibiting TNF translation, TEPM were isolated from disease-free TNF ARE/D ARE/2 TNFRI2/2 TEPM as controls (25). Significantly reduced levels of TNF protein were measured from LPS-induced TNFα1/1 3TNFRI2/2 TEPM in the presence of Allotrap 1258 reaching a $90\%$ inhibition at $25\,\text{mg/ml}$ (Fig. 6A). As before, no reduction in the levels of steady state TNF mRNA was observed. Interestingly, similar treatment of TNF ARE/ARE × TNFRI−/− TEPM with Allotrap 1258 resulted in a moderate 50–60% inhibition of TNF protein production, indicating that translational repression could still take place in the absence of ARE elements (Fig. 6A and B). Taken together, these results point toward a minor, if any, role of the 3'-ARE in Allotrap 1258-mediated inhibition and indicate that 3' sequences other than

**Fig. 3. Immunoprecipitation of [35S]methionine labeled proteins.** THP-1 cells (5 × 10⁶ cells/ml) in methionine-deficient medium were spiked with [35S]Met and stimulated for TNF production with LPS and IFNγ. After 24 h, cells were separated from culture medium and lysed. TNF was immunoprecipitated from culture medium (a) and cell lysates (b) using mAb to human TNF. Monoclonal antibody against human Class I (TP25) and an isotype control (goat-anti human IgG) were used in identically treated cells as negative controls. Cells alone (open bars) produced insignificant levels of TNF. Stimulated cells (hatched bars) produced significantly higher levels of TNF that were inhibited in the presence of Allotrap 1258 (solid bars). Negative control peptide had no effect on stimulated cells (dotted bars).

**Fig. 4. Quotiation of TNF mRNA in LPS/IFNγ stimulated THP-1 cells.** THP-1 cells were stimulated with LPS/IFNγ in the presence or absence of Allotrap 1258 (50 μM), and mRNA was isolated after 2 (closed bar), 4 (open bar), and 24 (striped bar) h. Following reverse transcription, quantitative PCR for TNF and GAPDH was performed using a commercially available kit. Results are expressed as relative number of copies of TNF compared with GAPDH.

**Fig. 5. Effect of TNF 3’-UTR absence on the Allotrap 1258-mediated inhibition of the expression of human TNF transgenes.** A, TNF protein secretion by transgenic Tg1278hTNF 3’-UTR (left panel) and Tg197hTNF 3’globin (right panel) peritoneal macrophages stimulated with LPS (1 μg/ml) in the presence of increasing doses of Allotrap 1258 (closed triangles) or control peptide D2RP (open circles). B, semi-quantitative RT-PCR analysis of total RNA isolated from pooled (n = 5) Tg1278 and Tg197 transgenic macrophage cultures in the presence of LPS, LPS + Allotrap 1258, or LPS + D2RP.

≈90% inhibition at 25 μg/ml (Fig. 6A). As before, no reduction in the levels of steady state TNF mRNA was observed. Interestingly, similar treatment of TNFARE/ARE × TNFRI−/− TEPM with Allotrap 1258 resulted in a moderate 50–60% inhibition of TNF protein production, indicating that translational repression could still take place in the absence of ARE elements (Fig. 6A and B). Taken together, these results point toward a minor, if any, role of the 3’-ARE in Allotrap 1258-mediated inhibition and indicate that 3’ sequences other than
Peptide-mediated Inhibition of TNF mRNA Translation

FIG. 6. Effect of the TNF 3′-ARE absence on Allotrap 1258-mediated inhibition of mTNF mRNA translation. A, TNF protein secretion by wild type (TNF+/+) and targeted mutant (TNFΔARE/ΔARE) peritoneal macrophages stimulated with LPS (1 μg/ml) in the presence of increasing doses of Allotrap 1258 (closed triangles) or control peptide D2RP (open circles). In B, a comparison of peptide-mediated inhibition in TNF+/+ (closed circles) and TNFΔARE/ΔARE (closed triangles) macrophages is shown. Values are shown as percentages of LPS alone. Results from a representative experiment with macrophages derived from individual mice (n = 5 mice/group) *, p < 0.001. C, Northern analysis and quantitation of total RNA from TNFRI+/+ and TNFΔARE/ΔARE pooled (n = 5) macrophage cultures incubated in the presence of LPS, LPS + Allotrap 1258, or LPS + D2RP for 2 h and hybridized with probes specific for mouse TNF or β-actin.

ARE are required to impose the full inhibitory effect of Allotrap 1258 on TNF translation.

DISCUSSION

In this study we continued the characterization of rationally designed immunomodulatory peptides. We demonstrated that peptide Allotrap 1258 did not induce cytokine production in mice. Allotrap 1258 inhibited ConA- and LPS-induced production of TNF in vitro and in vivo. In addition, a partial but significant inhibition of IFNγ and IL-12 synthesis was observed. Allotrap 1258 therapy also resulted in significant and efficient protection from endotoxic shock following LPS challenge. Survival in all groups was closely correlated with circulating TNF levels. Given the rapid kinetics of TNF, complete inhibition of a TNF burst by Allotrap 1258 treatment may have been responsible for higher survival. This also indicates that the inhibitory activity of the peptide may be a result of direct effects and may not involve synthesis of other cellular proteins. However, systemic changes that may determine survival and occur at later time points were not analyzed in our study.

We have previously reported that Allotrap 1258 modulates heme oxygenase activity in vitro and in vivo (8, 12). In animals, peptide therapy resulted in up-regulation of heme oxygenase, and we have speculated that the immunomodulatory activity of Allotrap 1258 may be mediated via effects on HO-1. The degradation of heme by hemeoxygenase results in the production of carbon monoxide, which has been shown to directly affect the activity of guanylate cyclase and therefore cGMP production (32–34). The secondary messenger cGMP has been implicated in cell growth arrest and the production of TNF. Based on these observations one may speculate that the effect of the peptide on TNF production may also involve heme oxygenase. This hypothesis is supported by the extreme sensitivity of HO-1 knock-out mice to LPS challenge (23). In addition, up-regulation of HO-1 was shown to inhibit inflammation, whereas inhibition of HO exacerbated it (14). Up-regulation of HO-1 following administration of hemoglobin was also shown to protect rats from LPS-induced death, whereas inhibition of HO-1 reversed the protective effect of hemoglobin administration (38). These results demonstrate that up-regulation of HO-1 provides protection against inflammatory insults including those induced by LPS. Furthermore, CoPP, an inducer of HO-1, was shown to inhibit TNF production by RAW264.7 cells (13), supporting the hypothesis that the protective effect of elevated HO-1 includes inhibition of TNF synthesis. On the other hand, we have seen that the LPS-induced rise in serum TNF levels in mice was rapid (60 min), and it is unclear whether a small up-regulation of HO-1 expression can effectively inhibit TNF production. To test the above hypothesis, further studies in HO-1 knock-out animals were carried out. The inhibition of TNF synthesis in Allotrap 1258-treated HO-1-deficient animals was as effective as in normal mice. This indicates that the protective effects of Allotrap 1258 on TNF levels are not mediated via direct effects on HO-1.

Another closely related molecule, NO, generated by the activity of iNOS and shown to be involved in cGMP-mediated TNF production was studied using iNOS knock-out animals. Similar to the result with HO-1/KO mice, Allotrap 1258 therapy resulted in significantly lower levels of TNF in iNOS/KO mice and protected these animals from death. This further supports our hypothesis that Allotrap 1258 exerts direct effects on TNF production, independent of the presence of HO-1 or iNOS.

The mechanism of inhibition of TNF production was investigated in vitro using macrophage cell lines or those from mice expressing various TNF transgenes. Allotrap 1258 inhibited effectively the production of TNF by mouse and human macrophages. Quantitative RT-PCR analysis and Northern blot analysis of TNF mRNA levels demonstrated that addition of Allotrap 1258 had little or no effect on human and mouse TNF mRNA levels. However, under those conditions the synthesis of TNF was completely suppressed indicating that the peptide inhibited TNF mRNA translation. Addition of peptide resulted in a complete inhibition of TNF synthesis but had little effect on overall protein synthesis as measured by [35S]methionine incorporation. The degree of peptide-mediated inhibition was similar in cell extracts and culture medium, indicating that the peptides did not influence the enzyme-mediated release of TNF into the medium.

It is well established that elements in the TNF mRNA 3′-UTR are involved in the regulation of TNF mRNA translation (25, 30, 31, 35). In particular it has been demonstrated that an ARE in the TNF 3′-UTR mediates both alleviation and reinforcement of message destabilization and translational silencing. In addition, in the absence of ARE, TNF mRNA is no longer responsive to translational modulation by the stress-activated protein kinases p38/SAPK and JNK/SAPK. Absence of the ARE from TNF mRNA rendered the effect of Allotrap 1258-mediated inhibition of TNF mRNA translation less effective but did not eliminate it. In contrast, replacement of the TNF mRNA 3′-UTR completely abolished the inhibitory effect of the peptide. Furthermore, Allotrap 1258 had little effect on the expression of IL-1β, whose mRNA contains ARE. Similarly, Allotrap 1258-mediated inhibition of IFNγ, another mRNA containing ARE, was much less effective than Allotrap 1258-mediated inhibition of TNF expression. Taken together these results clearly demonstrate that the peptide inhibits TNF mRNA translation via
mechanisms involving besides the ARE, other as yet unidentified elements in the 3'-UTR of TNF mRNA. Such elements have been described previously (30) by analysis of the translation efficiency of a reporter gene linked to various deletion mutants of the TNF 3'-UTR. Hel et al. (37, 38) further identified a distinct and novel region in the 3'-UTR shown to be different from the ARE and characterized cytosolic proteins that formed complexes with the regulatory elements. At this point, we cannot attribute direct binding of Allotrap 1258 to the regulatory elements in the 3'-UTR of TNF mRNA, as the potential mechanism of inhibition. Further studies are necessary to determine whether mechanism of inhibition by translation of Allotrap 1258 involves other cellular protein(s).

These observations pose the following possibilities: (a) that mechanisms affected by Allotrap modulation act positively (and in an additive fashion to the ARE functions), to activate TNF translation or (b) Allotrap inhibition acts through a novel negative enforcing mechanism. In either case it seems that the ARE-independent modulation may not involve the utilization of SAPK modules because it has been shown that these modules act through the ARE (39, 40).

The ARE-dependent translational modulation of TNF mRNA has been demonstrated to rely on SAPK signals, namely through the p38 and the JNK modules (25, 39, 40). It can therefore be speculated that the ARE-dependent, Allotrap 1258-mediated, translational inhibition may result from the modulation of these kinases. However, the ARE-independent control may not utilize similar signaling pathways because the ARE dependence for SAPK modulation of the TNF message seems, at least in macrophages, to be absolute. The application of the Allotrap 1258 peptide for the characterization of new 3' element(s) as well as the corresponding signaling modules via which these elements of ARE modulated will provide novel insight into the modes of control of TNF mRNA translation.

TNF is an important cytokine that has been vastly studied for both its inflammatory as well as immunoregulatory properties. TNF is a mediator of inflammation and tissue damage and appears to be involved in the pathology of several diseases including sepsis syndrome (41), rheumatoid arthritis (42, 43), inflammatory bowel disease (44, 45), and cachexia (46). Recently, anti-TNF monoclonal antibody therapy was shown to be effective in patients suffering from Crohn’s disease (a particular form of inflammatory bowel disease) (22). Similar observations have been made in rheumatoid arthritis patients (36). The application of a small non-immunogenic molecule like Allotrap 1258 that effectively lowers TNF serum levels could be of clear clinical benefit in similar situations.

In conclusion our results show that (a) the immunomodulatory peptide Allotrap 1258 displays a strong suppressive capacity on the in vivo and in vitro production of potent pro-inflammatory mediators and predominantly of TNF; (b) peptide-mediated inhibition of TNF biosynthesis is independent of the NO and HO pathways and occurs at the level of TNF mRNA translation; and (c) translational inhibition targets cis-elements residing on the 3'-UTR of TNF mRNA; more specifically, Allotrap 1258-mediated inhibition of TNF translation requires the function of both ARE-dependent and ARE-independent mechanisms, establishing novel non-ARE elements that modulate translation of TNF mRNA.
Inhibition of Tumor Necrosis Factor mRNA Translation by a Rationally Designed Immunomodulatory Peptide

Suhasini Iyer, Dimitris Kontoyiannis, Dominique Chevrier, Jacky Woo, Nancy Mori, Marie Cornejo, George Kollias and Roland Buelow

J. Biol. Chem. 2000, 275:17051-17057.
doi: 10.1074/jbc.M909219199 originally published online March 15, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M909219199

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 16 of which can be accessed free at http://www.jbc.org/content/275/22/17051.full.html#ref-list-1