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Concomitant Tumor and Autoantigen Vaccination Supports Renal Cell Carcinoma Rejection

Nicolás Herbert,*† Axel Haferkamp,‡ Hubertus F. Schmitz-Winnenthal,§ and Margot Zöller*†

Efficient tumor vaccination frequently requires adjuvant. Concomitant induction of an autoimmune response is discussed as a means to strengthen a weak tumor Ag-specific response. We asked whether the efficacy of dendritic cell (DC) vaccination with the renal cell carcinoma Ags MAGE-A9 (MAGE9) and G250 could be strengthened by covaccination with the renal cell carcinoma autoantigen GOLGA4. BALB/c mice were vaccinated with DC loaded with MHC class I-binding peptides of MAGE9 or G250 or tumor lysate, which sufficed for rejection of low-dose RENCA-MAGE9 and RENCA-G250 tumor grafts, but only retarded tumor growth at 200 times the tumor dose at which 100% of animals will develop a tumor. Instead, 75–100% of mice prevaccinated concomitantly with Salmonella typhimurium transformed with GOLGA4 cDNA in a eukaryotic expression vector rejected 200 times the tumor dose at which 100% of animals will develop tumor. In a therapeutic setting, the survival rate increased from 20–40% by covaccination with S. typhimurium-GOLGA4. Autoantigen covaccination significantly strengthened tumor Ag-specific CD4+ and CD8+ T cell expansion, particularly in peptide-loaded DC-vaccinated mice. Covaccination was accompanied by an increase in inflammatory cytokines, boosted IL-12 and IFN-γ expression, and promoted a high tumor Ag-specific CTL response. Concomitant autoantigen vaccination also supported CCR6, CXCR3, and CXCR4 upregulation and T cell recruitment into the tumor. It did not affect regulatory T cells, but slightly increased myeloid-derived suppressor cells. Thus, tumor cell eradication was efficiently strengthened by concomitant induction of an immune response against a tumor Ag and an autoantigen expressed by the tumor cell. Activation of autoantigen-specific Th cells strongly supports tumor-specific Th cells and thereby CTL activation.

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Abbreviations used in this paper: BMC, bone marrow cell; DC, dendritic cell; IL-1β, β form of pro-IL-1; LN, lymph node; LNC, lymph node cell; MAGE9; MAGE-A9; MDSC, myeloid-derived suppressor cell; MHC-I, MHC class I; MHC-II, MHC class II; NA, not applicable; NCBI, National Center for Biotechnology Information; RCC, renal cell carcinoma; SC, spleen cell; SEREX, serological identification of Ags by recombinant expression cloning; SL, Salmonella typhimurium avΩ strain SL7207; SL-empty, Salmonella typhimurium avΩ strain SL7207 transformed with the empty vector; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T cell.
The attenuated *S. typhimurium* strain SL7207 (SL) (43) was grown in Luria-Bertaini medium and has been transfected with human MAGE9 (31, 41) or hG250 (42) cDNA fragment, which was cloned into the eukaryotic expression plasmid pcDNA3.1 (SL-G250) or pcDNA3.1 containing a GOLGA4 cDNA fragment of 1694 bp (SL-GOLGA4). For insertion of the GOLGA4 cDNA fragment, which was isolated from a phage library of the human RCC line KTCL28, the cDNA were cloned into the pcDNA3.1 vector using HindIII/XhoI (G250 cDNA) and EcoRI/BamHI (MAGE9 cDNA). Transfection of *S. typhimurium aroA* with the eukaryotic expression plasmid pcDNA3.1 containing *GOLGA4* was performed by electroporation (2500 V, 25 μF) in Luria-Bertaini medium and transformed by electroporation (2500 V, 25 μF) in Luria-Bertaini medium and transformed by electroporation (2500 V, 25 μF). After overnight cultures in Luria-Bertaini medium, OD600 was determined (OD600 1 corresponds to OD600 1). Bacteria were centrifuged at 6000 rpm, washed twice with PBS, and resuspended in PBS/10% bicine (pH 8.3–8.5) to neutralize the acid pH of the stomach.

**Abs**

Hybridoma supernatant of anti-CD4, anti-CD8, anti-CD11b (European Association of Animal Cell Cultures, Salisbury, U.K.), and anti-CD-2 and-4 (44) were purified by affinity chromatography. Unlabeled, biotinylated, or dye-labeled anti-CD25, -CD40, -CD69, -CD80, -CD86, -CD152, -CD154, -Gr1, -Foxp3, -β form of pro-IL-1 (IL-1β), -IL-2, -IL-4, -IL-6, -IL-10, -IL-12, -IFN-γ, -CCL1, -CCL19, -CCL20, -CCR6, -CCR7, -CXC, and -CXC R and biotinylated or dye-labeled secondary Abs and streptavidin were obtained commercially (BD Pharmingen, Heidelberg, Germany; Dianova, Hamburg, Germany; Biotrend, Köln, Germany; Bender Medsystems, Vienna; Austria; and Santa Cruz Biotechnology, Santa Cruz, CA).

### Lymphocyte preparation

Mice were bled by puncture of the vena facialis and sacrificed by cervical dislocation. BMCs were collected from femur and tibia by flushing the bones with 5 ml PBS. Spleen, lymph nodes (LN), and the tumor were teased through fine gauze. Tumor infiltrating lymphocytes (TILs) were isolated from the dispersed tumor tissue by Ficol-Hyphaque centrifugation. BMCs, spleen cells (SCs), LN cells (LNCs), and TILs were washed and counted. Where indicated, cells were CFSE labeled (Invitrogen, Karlsruhe, Germany).

### In vivo generation and expansion of DC

BMCs (2 × 10⁵) were cultured in 10-cm diameter petri dishes in 10 ml RPMI 1640 supplemented with 10 ng/ml recombinant murine GM-CSF and 2 ng/ml recombinant murine IL-4. On day 3 of culture, an additional 10 ml medium was added, and half of the medium was exchanged on day 6. At day 8, loosely adherent cells were harvested and seeded in new petri dishes in 10 ml medium adding 0.25 μg/ml LPS for 24 h to induce DC maturation. Matured DCs were harvested on day 9, washed, and resuspended in serum-free RPMI 1640 containing 10 μg/ml synthetic 9-mer H-2Kb-binding peptides of G250, MAGE9, GOLGA4, or synthetic 15-mer H-2Aβa-binding peptides of GOLGA4. Peptides are listed in Table I. Alternatively, DCs were loaded with RENCA-MAGE9 or RENCA-G250 lystate (lysate of three cells/one DC). DC loading with peptides was terminated after 2 to 3 h and with tumor lystate after overnight incubation at 37°C by washing (45).

### Flow cytometry

Cells (5 × 10⁵) were stained according to routine procedures. For intracellular staining (cytokines, chemokines), cells were fixed and permeabilized in advance. Samples were processed in an FACSCalibur using the Cell Quest program for analysis (BD Pharmingen).

### Trogocytosis analysis protocol

Loaded DCs (10 × 10⁵) were resuspended in PBS containing 1 mg/ml SulfoBiotin-X-NHS (Calbiochem, Darmstadt, Germany) and incubated for 10 min at 25°C. After adding an equivalent volume of FCS, cells were incubated for an additional 10 min at 4°C. After extensive washing, biotinylated DCs were cocultured with LNCs at a ratio of 1:1 for 1 to 2 h at 37°C in 96-well plates. After washing in 2 ml EDTA/PBS, cells were resuspended in PBS for staining. Alternatively, biotinylated DCs (2 × 10⁵) were injected s.c. into vaccinated BALB/c mice. Draining LNs were excised after 24 h, LNCs were stained with anti-CD4 and anti-CD8, and trogocytosis was evaluated by flow cytometry (46).

### Cell proliferation

LNCs and SCs were titrated (2 × 10⁵–2.5 × 10⁶/well) in the absence or presence of 10¹¹ DCs loaded with MAGE9, G250, or GOLGA4 peptide or tumor cell lysate in 96-well plates. Cells were cultured for 3 d, adding [³H]thymidine (10 μCi/ml) during the last 16 h. Plates were harvested, and [³H]thymidine incorporation was evaluated in a β-counter.

### Cytotoxicity assays

CTL activity was evaluated after in vitro restimulation of LNC by [³H]thymidine release from labeled (12 h, 10 μCi/ml) targets (10⁷/well), which were seeded on uitered numbers (10 × 10⁵–6 × 10⁶) of effectors in 96-well plates. After 6 h at 37°C, plates were harvested, and radioactivity was determined in a β-counter. Cytotoxicity is presented as percent cytotoxicity = 100 × (counts in test wells − counts in control wells)/(total counts – counts in control wells). The spontaneous release of tumor cells ranged between 6 and 12% and of lymphoblasts (ConA-stimulated LNCs) between 12 and 20%. SD of triplicates was 3–5%.

### Cytokine ELISA and ELISPOT

Standard sandwich ELISA procedures were used to measure cytokine secretion. IFN-γ-secreting cells were evaluated by ELISPOT. Plates were coated with 10 μg/ml anti-IFN-γ (overnight, 4°C). After washing and blocking, LNCs (1 × 10⁵) were plated in triplicate and restimulated with DCs at an effector/stimulator ratio of 10:1 (48, 37°C). Cells were decanted, plates were washed (PBS/0.01% Tween 20), and biotinylated anti–IFN-γ (2 μg/ml in PBS/0.5% BSA) was added. Postincubation (2 h, 37°C), washing (6 ×, PBS/0.01% Tween 20) and streptavidin–alkaline phosphatase enzyme-conjugate incubation (45 min, room temperature). BCIP (Calbiochem/NBT (Sigma, Munich, Germany) substrate was added. The reaction was stopped by washing in tap water. Membranes were dried, and spots were counted.
or an oral application of 1 to 2 \times 10^6 \text{SL-} \text{GOLGA4} \text{H-2A}^d-\text{binding peptides} and formed a tumor that started to grow with significant delay after 12–16 wk. Instead, only one out of eight DC-G250 cells developed a tumor and were sacrificed according to the protocol of each with 8–10 mice per group. Mean ± SD of in vivo experiments are derived from two experiments (in vivo assays) or the Student's t test (in vitro assays). Mean values ± SD of in vitro experiments are derived from two experiments each with 8–10 mice per group. Ex vivo experiments were repeated three to five times with two to three mice per group. Mean ± SD of in vitro experiments, repeated three to four times, are based on triplicates.

**Table I. MAGE9, G250, GOLGA4 H-2D^d-binding, and GOLGA4 H-2A^d-binding peptides**

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<tr>
<th>Position</th>
<th>Sequence</th>
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<tr>
<td>MAGE9, G250, and GOLGA4 H-2D^d-binding peptides</td>
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</tr>
<tr>
<td>MAGE9 141–149</td>
<td>NYKRYFPV1</td>
</tr>
<tr>
<td>MAGE9 160–168</td>
<td>IFUDVRKV1</td>
</tr>
<tr>
<td>MAGE9 237–245</td>
<td>FGYEPRLKL</td>
</tr>
<tr>
<td>MAGE9 281–289</td>
<td>SYRKYVINYL</td>
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<tr>
<td>MAGE9 299–307</td>
<td>CYPSLYEBV</td>
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<tr>
<td>G250 172–180</td>
<td>AFRCPFALRL</td>
</tr>
<tr>
<td>G250 219–227</td>
<td>EYRALQLHFL</td>
</tr>
<tr>
<td>G250 258–266</td>
<td>AFAVRDEAL</td>
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<tr>
<td>G250 288–296</td>
<td>AYEQLLSR1</td>
</tr>
<tr>
<td>G250 323–331</td>
<td>RYFQYEESL</td>
</tr>
<tr>
<td>GOLGA4 1–9</td>
<td>MFKKLKQKI</td>
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<tr>
<td>GOLGA4 67–75</td>
<td>LRVPSEESL</td>
</tr>
<tr>
<td>GOLGA4 152–160</td>
<td>SYRKGKSESL</td>
</tr>
<tr>
<td>GOLGA4 564–572</td>
<td>TYRTRILEL</td>
</tr>
<tr>
<td>GOLGA4 2110–2118</td>
<td>TQLAQKTTL</td>
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<tr>
<td>GOLGA4 H-2A^d-binding peptides</td>
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<td>GOLGA4 1171–1195</td>
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<tr>
<td>GOLGA4 1478–1492</td>
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*Protein identification/National Center for Biotechnology Information (NCBI) reference sequence: NP_005356.1.
*Protein identification/NCBI reference sequence: CAB82444.
*Protein identification/NCBI reference sequence: NP_002069.2.
*Protein identification/NCBI reference sequence: NP_002069.2.
*Protein identification/NCBI reference sequence: NP_005356.1.
*Protein identification/NCBI reference sequence: CAB82444.
*Protein identification/NCBI reference sequence: NP_002069.2.

**Immunohistology**

Sections (5 \mu m) of snap-frozen tumor were fixed (chloroform/acetone 1:1, 4 min) and treated with levamisole solution to ablate tissue alkaline phosphatase activity. Nonspecific binding was blocked using an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA) and 2% normal serum with biotinylated DCs loaded correspondingly to the DCs used for immunization. Significantly more CD4^+ and CD8^+ LNCs underwent antigen-specific T-cell expansion. The latter was also seen with untransformed (data not shown) or vector-transformed SL (SL-empty). Tumor Ag-specific T cells in response to tumor peptide- or lysate-pulsed DCs induced an anti-RCC response.

**Tumor growth**

BALB/c mice received 1 \times 10^4, 2 \times 10^4, or 1 \times 10^5 tumor cells s.c. into the right flank. Tumor growth was controlled (mean diameter) twice per week. Mice were later killed when the s.c. tumor mass reached a mean diameter of 2.5 cm (survival time). For vaccination, mice received 2 \times 10^6 tumor peptide- or tumor lysate-loaded DCs s.c., starting 20 d before tumor cell application. Where indicated, mice received concomitant DC loaded with H2-Ad–binding GOLGA4 peptides or an oral application of 1 to 2 \times 10^6 \text{SL-} \text{GOLGA4} DC application was repeated in 10-d and SL application in 14-d intervals. Animal experiments were approved by the governmental authorities for animal health care, Baden-Wuerttemberg, Germany.

**Statistical analysis**

Significance of differences was calculated according to the Wilcoxon rank sum test (in vivo assays) or the Student's t test (in vitro studies). Mean values ± SD of in vivo experiments are derived from two experiments each with 8–10 mice per group. Ex vivo experiments were repeated three to five times with two to three mice per group. Mean ± SD of in vitro experiments, repeated three to four times, are based on triplicates.

**Results**

SEREX screening has provided evidence for abundance of autoantibodies against GOLGA4 in patients with RCC (32). Based on previous work indicating that concomitant vaccination with protein/peptide-loaded DCs and transformed SL can strengthen the antitumor response (22, 23, 26), we examined in this study whether limiting activation of Th cells by vaccination with tumor peptide-loaded DCs (Table I) may be overcome by covaccination with GOLGA4. We used the murine RENCA line, which expresses GOLGA4, transfected with the human RCC-associated Ags G250 or MAGE9 (31, 32, 42).

**Tumor peptide- or lysate-pulsed DCs induce an anti-RCC response**

All BALB/c mice receiving s.c. two times the dose at which 100% of animals will develop tumor (1 \times 10^6 \text{RENCA-MAGE9} or RENCA-G250 cells) developed a tumor and were sacrificed according to the protocol of each with 8–10 mice per group. Mean ± SD of in vivo experiments are derived from two experiments each with 8–10 mice per group. Ex vivo experiments were repeated three to five times with two to three mice per group. Mean ± SD of in vitro experiments, repeated three to four times, are based on triplicates.

**Preferential expansion of tumor Ag-specific CD8^+ T cells in response to tumor peptide- or lysate-loaded DCs**

The trogocytosis analysis protocol assay provides an estimate on the frequency of Ag-specific T cells by the transfer of proteins/membrane fragments from DCs to T cells, called trogocytosis (47). In the first setting, LNCs from vaccinated mice were cocultured with biotinylated DCs loaded correspondingly to the DCs used for vaccination. Significantly more CD4^+ and CD8^+ LNCs underwent trogocytosis postvaccination with peptide- or lysate-pulsed than with unloaded DCs. Trogocytosis was more efficient postvaccination with lysate- than peptide-loaded DCs. Covaccination with SL-\text{GOLGA4} did not affect trogocytosis by CD4^+ LNCs and to a minor degree by CD8^+ LNCs. The latter was also seen with untransformed (data not shown) or vector-transformed SL (SL-empty). Tumor Ag-specific...
CD8+ cells were strongly increased and CD4+ cells were slightly increased after repeated vaccination with tumor Ag-loaded DCs (shown for DC-G250 lysate). However, in the tumor-free mouse, covaccination with SL-GOLGA4 exerted no significant effect even upon repeated vaccination (Fig. 2A, Supplemental Fig. 1A).

Autoantigen covaccination becomes efficient only in the tumor-bearing mouse

A different picture of in vivo trogocytosis was seen in vaccinated tumor-bearing mice challenged with biotinylated DCs with/without concomitant SL-GOLGA4 vaccination. Mice were sacrificed after 24 h. A significantly higher percentage of T cells had trogocytosed the biotin from peptide- or lysate-loaded DCs than from unloaded DCs. Furthermore and distinct to tumor-free mice, the percentage of biotinylated T cells was significantly increased in mice covaccinated with DC plus SL-GOLGA4. CD4+ and CD8+ LNCs from RENCA-G250-bearing mice trogocytosed biotin from G250-loaded DCs when vaccinated with DC-G250 peptide and from DC-GOLGA4 when vaccinated with SL-GOLGA4. When vaccinated with both DC-G250 peptide and SL-GOLGA4, biotin uptake from DC-G250 was increased compared with LNCs from mice vaccinated exclusively with DC-G250 (Fig. 2D).

These findings show that repeated SL-GOLGA4 vaccination promotes autoantigen-specific T cell activation. In addition, help for tumor Ag-specific T cell activation is provided when the autoantigen is expressed by the tumor cell. Exploration of the T cell activation state supported this interpretation.

T cell activation by tumor Ag and autoantigen covaccination

A slightly increased percentage of CD4+ cells was seen in DC-vaccinated mice and of CD8+ cells in DC- plus SL-GOLGA4–vaccinated mice. In addition, a higher number of LNCs expressed the accessory molecules CD69 and CD154 in DC-vaccinated mice, the percentage of CD154+ cells being further increased by SL-GOLGA4 covaccination. The percentage of CD40+ cells was more
strongly increased postvaccination with peptide- than lysate-loaded DCs and was further increased after covaccination with peptide-loaded DCs plus SL-GOLGA4. On the contrary, expression of immune response silencing CD152 and its preferential ligand CD80 was only augmented postvaccination with lysate- but not peptide-loaded DCs (Fig. 3A).

Covaccination with SL-GOLGA4 but also with nontransformed SL exerted a strong effect on cytokine expression. Peptide-pulsed more efficiently than lysate-pulsed DCs induced a strong upregulation of IL-12 and IFN-γ expression that was further increased by SL-GOLGA4 and, albeit less pronounced, by SL (Fig. 3B). Nonetheless, the increase in IFN-γ-expressing cells after SL vaccination was unexpected. According to previous reports (30), we assumed that only the GOLGA4 autoantigen might strengthen the efficacy of the tumor vaccine via heterospecific help. Therefore, we evaluated whether SL and SL-GOLGA4 preferentially induced IFN-γ expression in CD4+ or CD8+ cells. In draining LNCs of mice vaccinated with peptide-pulsed DCs, the increase in IFN-γ-expressing cells was mostly restricted to CD4+ cells, and only SL-GOLGA4 promoted a further increase. On the contrary, the percentage of IFN-γ-expressing CD8+ cells was increased in SL-GOLGA4 as well as in SL-vaccinated RENCA-G250 or RENCA-MAGE9 tumor-bearing mice (Fig. 3C, Supplemental Fig. 2).

Last, it should be noted that neither peptide- nor lysate-pulsed DC vaccination had a major impact on immunoregulatory cytokine expression. Instead, both SL-GOLGA4 and SL covaccination stimulated inflammatory IL-6, but also IL-1β and IL-10, expression (Fig. 3B), which can stimulate myeloid-derived suppressor cell (MDSC) and Treg expansion.

Taken together, vaccination with tumor peptide- or lysate-loaded DCs supported T cell activation. Accessory molecule and Th cytokine expression were strengthened by SL-GOLGA4 covaccination, most efficiently in DC-peptide–vaccinated mice. SL-GOLGA4, but not SL, strengthened Th cell activation, whereas both SL-GOLGA4

FIGURE 2. Trogocytosis of G250-loaded DCs by CD4+ and CD8+ cells: impact of concomitant autoantigen vaccination. A, Tumor-free mice were vaccinated with DCs and SL-GOLGA4 as described in Fig. 1. Draining LNs were excised, and lymphocytes were cocultured with biotinylated tumor peptide- or lysate-loaded DCs for 2 h. The percentage of biotinylated CD4+ and CD8+ LNCs was evaluated by flow cytometry. An example and mean values ± SD of triplicates are shown. B, Two weeks after s.c. tumor cell application, vaccinated mice received an s.c. application of biotinylated DCs. After 24 h, draining LNs were excised, and LNCs were stained with streptavidin-allophycocyanin and anti–CD4-FITC or anti–CD8-FITC. Mean values ± SD of triplicates are shown. C, Representative example of in vivo trogocytosis by CD4+ and CD8+ LNCs of RENCA-G250 tumor-bearing mice after repeated vaccination with peptide- or tumor lysate-loaded DCs with/without SL-GOLGA4 covaccination. D, Mice were vaccinated with SL-GOLGA4, G250 peptide-loaded DCs, or both. Two weeks after s.c. tumor cell application, draining LNs were excised and incubated with biotinylated G250 or GOLGA4 peptide-loaded DCs. After 2 h, LNCs were stained with streptavidin-allophycocyanin and anti–CD4-FITC or anti–CD8-FITC. Mean values ± SD of triplicates are shown. A, B, and D, A significant increase in the percentage of biotinylated CD4+ or CD8+ cells as compared with cocultures with biotinylated, unloaded DCs, or LNCs from nonvaccinated mice is indicated with * (tumor Ag) or "s" (GOLGA4); a further increase in biotinylated T cells after covaccination with SL or SL-GOLGA4 is indicated with +. Tumor lysate- and peptide-loaded DCs as well as autoantigen-transformed SL support Ag-specific CD4+ and CD8+ T cell expansion/activation. The superiority of lysate- compared with peptide-loaded DCs is due to the lysate containing the tumor Ag and the autoantigen. The efficacy of covaccination with autoantigen-transformed SL relates to both activation of autoantigen- and support of tumor Ag-specific T cells. Empty vector-transformed SL provide a minor unspecific stimulus.
and SL supported activation of CD8+ cells, likely via the SL-induced inflammatory milieu. As vaccination with lysate-loaded DCs also supported CD152 expansion, and SL and SL-GOLGA4 application was accompanied by increased IL-1β and IL-10 expression, it became demanding to explore the impact of vaccination on immunoregulatory cell and factor stimulation.

The impact of covaccination on T<sub>reg</sub> and MDSC

We first controlled the serum level of IL-1β and IL-10 in vaccinated tumor-bearing mice. IL-1β and IL-10 were increased in tumor-bearer sera. No additional increase was seen in DC-vaccinated mice. Instead, IL-10 and IL-1β were slightly increased in the serum of SL- or DC- and SL-vaccinated mice. This was irrespective of whether the mice received nontransformed SL or SL-GOLGA4 (Fig. 4A). Despite the slight increase in CD152<sup>+</sup> cells in lysate-vaccinated mice and in IL-10 expression/secretion in SL and SL-GOLGA4 vaccinated mice, we did not observe a significant expansion of T<sub>reg</sub> (Fig. 4B, Supplemental Fig. 3A). Instead, an increase in MDSCs was seen in the spleen of DC-/lysate–vaccinated mice covaccinated with SL-GOLGA4 (Fig. 4C). This is in line with the upregulated IL-1β expression and secretion, which strongly promotes MDSC expansion (49, 50).

Accordingly, CD25<sup>high</sup> (T<sub>reg</sub>) were very rarely detected in the tumor tissue, few cells mostly being seen in DC- plus SL-GOLGA4–vaccinated mice. CD11b<sup>+</sup> and Gr-1<sup>+</sup> cells were rare in the tumors of nonvaccinated mice, but were slightly increased in tumors of DC-peptide–vaccinated mice (Fig. 4D).

These findings confirmed that activation of immunosuppression is not dominating in tumor- and autoantigen-covaccinated mice. Although both tumor lysate and SL/S-L-GOLGA4 promoted to a measurable, but not excessive, degree MDSC expansion, recruitment into the tumor was low. The vaccination protocols obviously do not support T<sub>reg</sub> expansion.

Tumor Ag and autoantigen covaccination supports Ag-specific T cell expansion

An analysis of T cell proliferation of naive versus vaccinated tumor-free mice confirmed a strong increase by DC vaccination that was strengthened by in vitro restimulation with G250-loaded DCs (Fig. 5A). DC vaccination also stimulated the proliferative response of tumor-bearing mice LNCs. Covaccination with SL-GOLGA4 resulted in a significantly increased proliferative response, particularly when mice had been vaccinated with DC-peptide (Fig. 5B, Supplemental Fig. 4A). The latter was confirmed by evaluating fluorescent dye dilution in CD4<sup>+</sup> and CD8<sup>+</sup> LNCs. Whereas the synergistic effect of SL-GOLGA4 covaccination was not significant in CD4<sup>+</sup> and CD8<sup>+</sup> LNCs of lysate-pulsed DCs, which showed a higher percentage of proliferating T cells independent of covaccination, the percentage of both proliferating CD4<sup>+</sup> and CD8<sup>+</sup> cells became nearly doubled by DC-peptide and SL-GOLGA4 covaccination (Fig. 5C, Supplemental Fig. 4B).

To control for the Ag specificity of response, LNCs from G250 peptide-loaded DCs or G250 peptide-loaded DCs plus SL-GOLGA4–vaccinated RENCA and RENCA-G250 tumor-bearing mice were restimulated in vitro with unloaded or G250 peptide- or RENCA lysate (GOLGA4<sup>+</sup>)– or RENCA-G250 lysate (GOLGA4<sup>+</sup>,
G250\(^+\) -loaded DCs. RENCA tumor-bearer LNCs responded only to lysate-loaded DCs, with no difference being observed between RENCA and RENCA-G250 lysates. Instead, RENCA-G250 tumor-bearer LNCs also responded to G250 peptide-loaded DCs, and the response toward RENCA-G250 lysate-loaded DCs was stronger than the response toward G250 peptide-loaded DCs (Fig. 5D). Notably, when tumor-bearing mice were vaccinated with SL-GOLGA4 or SL vaccination provided an unspecific stimulus for IFN-\(\gamma\) secretion, a further increase in IFN-\(\gamma\)-secreting LNCs from peptide-loaded DC-vaccinated mice was only seen after SL-GOLGA4 covaccination (Fig. 6A, Supplemental Fig. 5A).

DC vaccination induced a strong CTL response that did not markedly differ in mice vaccinated with peptide- versus lysate-loaded DCs. Cytotoxic LNC activity was increased after covaccination with SL-GOLGA4. As LNCs from vaccinated mice also displayed slightly increased cytotoxic activity against the parental RENCA cells and, at a low level, against syngeneic BALB/c blasts (Fig. 6B, Supplemental Fig. 5B), we controlled whether vaccination with transformed SL stimulates NK/lymphokine-activated killer activity. Except for the low-level cytotoxic activity of LNCs from nonvaccinated tumor-bearing mice, cytotoxic activity was strongly reduced in the presence of an anti-CD8 or an anti–H-2\(^d\) Ab, indicating that cytotoxic activity was mostly CTL-mediated (Fig. 6C, Supplemental Fig. 5C).

The latter finding argues for the increased cytotoxic activity against RENCA cells after covaccination with SL-GOLGA4 to be directed against GOLGA4. This was confirmed by evaluating cytotoxic activity of LNCs from SL-GOLGA4 (restimulated with DC-GOLGA4) and SL-GOLGA4 plus DC-G250 peptide (restimulated with DC-GOLGA4 plus DC-G250)–vaccinated mice against RENCA-G250-GOLGA4kd cells. Irrespective of the vaccination regimen, RENCA-G250-GOLGA4kd cells were hardly lysed (Fig. 6D). Instead, the same effector cells efficiently lysed RENCA-G250 target cells and lysis of RENCA-G250-GOLGA4kd cells by effector cells from SL-GOLGA4–vaccinated mice was reduced, not abolished (Fig. 6E). Thus, SL-GOLGA4 supported the activation of CTL specific for the tumor cell’s autoantigen and the tumor Ag.

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Taken together, tumor Ag-specific CTL activation by DC vaccination becomes significantly strengthened by SL-GOLGA4 application. The latter, besides strengthening the tumor Ag-specific response, supports activation of autoantigen-specific CTL.
Covaccination and recruitment of TIL

Contrasting the poor recovery of MDSCs and Treg within the tumor tissue, there has been a strong recruitment of CD4+ and CD8+ cells in DC-vaccinated mice. T cell recruitment was strengthened by concomitant SL-GOLGA4 application, in which only islets of tumor cells between clusters of T cells were seen in the few mice that developed a tumor (Fig. 7A). Importantly, a considerable percentage of TILs trogocytosed biotin from G250 and GOLGA4 peptide-pulsed DCs, which implies that particularly Ag-specific T cells became recruited into the tumor.

In line with the immunogenicity of GOLGA4, some GOLGA4-specific TILs were even recovered from tumors of nonvaccinated mice, whereas G250-specific TILs were only detected in vaccinated mice. SL-GOLGA4 covaccination led to a significant increase in G250- and GOLGA4-specific CD4+ and CD8+ TILs (Fig. 7B). The high recovery of Ag-specific TILs of mice covaccinated with DCs and SL-GOLGA4 suggested that covaccination could affect chemokine receptor expression. CCR3, CCR5, and CCR7 expression were unaltered (data not shown). Instead, CCR6, CXCR3, and CXCR4 expression, increased in draining LNCs of DC-vaccinated mice, was further augmented by SL-GOLGA4 co-vaccination (Fig. 7C, Supplemental Fig. 6).

Thus, DC vaccination, strongly enhanced by covaccination with an autoantigen, promoted T cell infiltration into the tumor. Covaccination was also advantageous for induction of chemokine receptor expression that supported T cell and DC recruitment toward the tumor.

Combined peptide-loaded DC and SL-GOLGA4 vaccination efficiently prevents RCC growth

To evaluate the in vivo efficacy of tumor Ag and autoantigen covaccination, mice received 200 times the dose at which 100% of animals will develop tumor. In nonvaccinated mice, palpable tumor nodules were observed after 2 wk. After 5–7 wk (RENCA-MAGE9) and 4 to 5 wk (RENCA-G250), mice were sacrificed due to the tumor burden. As far as DC-vaccinated mice developed a tumor, tumor growth was significantly delayed. By covaccination with DC-lysate plus SL-GOLGA4, the survival rate of RENCA-G250—
RENCA-MAGE9–bearing mice increased from five to seven out of eight mice, which was statistically NS. Covaccination with SL-GOLGA4 was more efficient in DC-peptide–vaccinated mice. Without SL-GOLGA4, five and four out of eight mice developed RENCA-MAGE9 and RENCA-G250 tumors; with covaccination, RENCA-G250 grew only in two out of eight mice, and RENCA-MAGE9 and RENCA-G250 tumors; with covaccination, the growth rate was further reduced, differing significantly even from the growth rate of
DC-G250 peptide-vaccinated mice, and 40% of the mice did not develop a tumor (Fig. 8 G,8H, Table II).

Both prophylactic and therapeutic vaccination provided evidence that autoantigen covaccination retards tumor growth and strengthens the tumor rejection rate, SL-GOLGA4 being more efficient than GOLGA4 peptide-loaded DCs.

Discussion
Immunotherapy of cancer has become an attractive alternative, particularly for immunogenic cancer entities like RCC (2, 3, 46, 48). However, clinical success still does not meet expectations (3). This is discussed to be a consequence of insufficient Th cell activation by weakly immunogenic tumor Ags (51, 52), but could also be due to immunosuppression (53). Both obstacles also account for DC-based vaccines. Tumor lysate-loaded DCs may not present efficient amounts of immunogenic epitopes, and a deviation toward immunosuppression has repeatedly been described (54, 55). Peptide loading of DCs avoids these drawbacks, but DCs, mostly loaded with MHC-I–binding peptides, may not efficiently activate Th cells. For these reasons, we focused on how to support Th cell activation when vaccinating with peptide-loaded DCs. Concomitant vaccination with peptide-loaded DCs and autoantigen-transformed SL significantly improved suppression of tumor growth. Evidence is presented that in the tumor-bearing host, Th cells, recognizing an autoantigen expressed by the tumor cells, support tumor Ag- and autoantigen-specific CTL activation. Vaccination with transformed SL provides an additional inflammatory stimulus, which strengthens tumor-specific T cell activation by promoting chemokine receptor expression recruitment into the tumor.

The choice of the Ags and route of vaccination
RENA cells were transfected with the well-established human RCC Ag G250 (42) or with human MAGE9, which could serve as an alternative immunogen in G250+ RCC (41). In many studies, the first choice for tumor vaccination has been DCs as the classical APCs (5, 6, 9). We used this established protocol to compare the efficacy and possible drawbacks of MHC-I–binding peptide- versus tumor lysate-loaded DCs.

As most tumor Ags are weakly immunogenic, tumor vaccination frequently requires adjuvant. One mode to strengthen a tumor-specific immune response has been covaccination with an autoantigen, which is expressed by the tumor cell (27, 30). In fact, SEREX and serum Ab detection array revealed the majority of Abs detected in tumor patients’ sera to be autoantibodies (35, 56, 57). These detection
systems only recognizing high-avidity IgG Abs, the corresponding autoantigens will be strongly immunogenic. As GOLGA4-specific Abs have been detected in all tested RCC patients’ sera (32), and RENCA cell express GOLGA4, this autoantigen appeared well suited to examine whether autoantigen-specific Th cells support tumor Ag-specific CTLs, provided the tumor cell expresses the autoantigen.

We chose transformed, attenuated SL typhimurium for autoantigen covaccination, as eukaryotic vector-transformed SL has repeatedly been demonstrated to be a potent vaccine (22, 24–26). Expectedly, draining LNCs and TILs of SL-\textit{GOLGA4}–vaccinated mice contained GOLGA4-specific Th cells and CTLs. Besides induction of this autoantigen-specific response, covaccination with SL-\textit{GOLGA4} efficiently supported the tumor Ag-specific response (see below).

However, whereas we and other groups (24–26) did not observe a response against the eukaryotic vector, SLs themselves are immunogenic (58) and are prone to deviating an immune response toward immunosuppression in favor of their survival (59). Although the latter feature is largely circumvented by using a eukaryotic expression vector (23), we noted a slight upregulation of IL-1β, IL-10, and MDSC in SL-fed mice. These immunoregulatory features are not dominating, as covaccination with SL-\textit{GOLGA4} supported tumor growth retardation/tumor rejection. Nonetheless, we tried to circumvent this minor drawback by covaccination with GOLGA4 peptide (MHC-II–binding)-loaded DCs. However, GOLGA4 peptide-loaded DCs were a weaker costimulus than SL-\textit{GOLGA4}. The superiority of SL-\textit{GOLGA4} likely is due to induction of inflammatory cytokine and IFN-γ secretion. SL-\textit{GOLGA4} being the superior adjuvant, we suggest coping with the SL-induced minor deviation of immune response by all-transretinoic acid, which efficiently drives MDSCs into differentiation (50).

Taken together, covaccination with an autoantigen via transformed SL appeared a good choice. In addition, distinct to DC vaccination, transformed attenuated SL could be a cheap and easy-to-handle vaccine that would not require a patient’s hospitalization.

The mode whereby covaccination with an autoantigen supports tumor Ag-specific T cells

Tumor- and autoantigen-covaccinated mice rejected a low dose of RENCA-MAGE9 or RENCA-G250 tumor cells. At a higher tumor dose, the rejection rate increases from 37.5 to 100%, respectively, from 50 to 75%, and in a therapeutic setting from 20 to 40% by covaccination. Trying to unravel the underlying mechanism, we emphasized the comparison between tumor lysate- versus peptide-loaded DCs and the adjuvant effect of SL-\textit{GOLGA4}.

By repeated vaccination of tumor-free mice with lysate-loaded DCs, in vivo trogocytosis (60) increased from <2% to >10% of CD4+ and >20% of CD8+ draining LNCs. Covaccination with SL-
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NA, not applicable.
or SL-GOLGA4 led to a further increase in the percentage (>25%) of biotin-binding CD8* cells. In tumor-free mice, vaccination with peptide-loaded DCs was less efficient, and covaccination was ineffective. On the contrary, in the tumor-bearing mouse, trogocytosis by both CD8* and CD4* cells was strongly increased by covaccination with peptide-loaded DCs and SL-GOLGA4.

We interpret these findings in the sense that tumor lysates are processed to be presented in MHC-I and MHC-II molecules, which can explain Th cell binding, albeit at a low level. Furthermore, tumor lysates contain the autoantigen GOLGA4. Thus, as demonstrated by the increased proliferative activity of LNCs from RENCA-bearing mice vaccinated with RENCA lysate-pulsed DCs, DCs will present tumor Ag- as well as autoantigen-derived peptides, which amplifies Th cell activation and thereby CTL activity (61). In addition, SLs themselves are a strong immunogen (58) and will first be presented by MHC-I on peritoneal macrophages (24, 25). Thus, it is possible that increased trogocytosis of CD8* cells in tumor-free mice is, at least partly, tumor Ag and autoantigen independent and relates to the vaccine carrier (SL)-induced inflammatory response. Instead, a strong increase in trogocytosis after peptide-loaded DC and SL-GOLGA4 covaccination only in tumor-bearing mice points toward heterospecific help (30) and is compatible with the finding that coimmunization with a Th1 peptide and a tumor peptide strengthens tumor-specific CTL activation (62).

Our interpretation that autoantigen covaccination via SL-GOLGA4 provides a powerful adjuvant, which supports tumor Ag-specific Th cell activation, was validated by an increase in the percentage of CD154* T cells, CD40* cells, IFN-γ-secreting cells, the steep increase in the percentage of proliferating CD4* cells, and the high cytotoxic activity against RENCA-G250 and RENCA-MAGE9 cells. Evaluating proliferative activity in response to DC-GOLGA4 and the cytotoxic activity toward RENCA-G250-GOLGA4-kd tumor cells confirmed that vaccination with SL-GOLGA4 induced an autoantigen-specific proliferative and cytotoxic response. However, the CTL response of DC-G250 plus SL-GOLGA4–vaccinated mice toward RENCA-G250-GOLGA4-kd targets significantly exceed the response of CTL from DC-G250–vaccinated mice. These findings provide direct evidence for the contribution of autoreactive Th cells in tumor Ag-specific T cell activation. One likely explanation could be that in the tumor-bearing mouse, Th cells and CTLs in the draining LNs become restimulated by DCs presenting both tumor debris or tumor exosome-derived GOLGA4 and MAGE9 or G250 (64–66). The high survival rate of mice prevaccinated with peptide-loaded DCs and SL-GOLGA4 as well as the significantly prolonged survival time of mice receiving a therapeutic DC plus SL-GOLGA4 covaccination support this hypothesis.

Finally, after lysate-loaded DC vaccination, CD152 and CD80 expression was slightly upregulated, and a higher percentage of MDSC was seen in the spleen and within the tumor. The reasons for this mild tumor-lysate induced immunosuppression could be: 1) immunosuppressive features of RENCA cells (67); 2) competition between immunogenic and tolerogenic peptides in the crude tumor lysate (68–70); or 3) stimulation of MDSCs by tumor lysate-pulsed DCs, in which immunogenicity may correlate with induction of suppression (68, 71–73). However, immunosuppression did not become dominating in the proposed covaccination protocols.

**SL covaccination supports effector cell recruitment into the tumor**

In mice covaccinated with SL-GOLGA4, the tumors showed a high density of infiltrating T cells, which was likely a consequence of SL vaccination contributing to leukocye migration. CCL1 expression (data not shown), which supports the recruitment of DCs (74), was strengthened by vaccination with SL-GOLGA4. SL-GOLGA4 covaccination promoted CCR6, CXCXR3, and CXCR4 expression. CXCXR3, predominantly expressed by Th1-polarized T cells, has been associated with their recruitment in autoimmune disease and tissue damage (75). CCR6, expressed by immature DCs and effector/memory T cells, becomes attracted by its ligand CCL20 (76), which is also expressed by RCC (77). CXCR4 plays an important role in T cell activation and migration (78), and both CCR6 and CXCR4 are, at least transiently, expressed in DCs and involved in DC traffic (79). These features are well in line with their importance in autoimmune disease and, correspondingly, their induction by vaccination with an autoantigen. Thus, costimulation with SL-GOLGA4 supports the recruitment of DC and effector T cells by upregulation of chemokine/chemokine receptor expression. Taken together, peptide-loaded DCs, although less efficient than lysate-loaded DCs, may be the superior vaccine, provided they are supported by vaccination with an autoantigen that is expressed by the tumor cell. Thereby, Th cells become activated, which strengthens the tumor-specific CTL response. Vaccination with peptide-loaded DCs can circumvent the drawback of concomitant activation of immunosuppression by tumor lysate vaccination. Autoantigen-transformed SL stimulates an autoimmune response against the tumor’s autoantigen, provides efficient help for tumor Ag-specific T cells, and stimulates an inflammatory milieu with high-level cytokine and chemokine/chemokine receptor expression that further supports tumor-specific T cell expansion and recruitment of effector T cells into the tumor.

**Acknowledgments**

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

CONCOMITANT TUMOR AND AUTOANTIGEN VACCINATION


Supplementary Figure Legends

Supplementary Figure 1 Trogocytosis of MAGE9-loaded DC by CD4⁺ and CD8⁺ cells. (A) Mice were vaccinated with DC and SL-\textit{GOLGA4} as described in Figure 1. Draining LN were excised and lymphocytes were co-cultured with biotinylated MAGE9-peptide or RENCA-MAGE9 lysate-loaded DC for 2h. (B) Two weeks after s.c. RENCA-MAGE9 application, vaccinated mice received a s.c. application of biotinylated MAGE9 peptide or lysate-loaded DC. After 24h draining LN were excised and LNC were stained with Streptavidin-APC and anti-CD4-FITC or anti-CD8-FITC. (A,B) Mean values±SD of triplicates of the percentage of biotinylated CD4⁺ or CD8⁺ LNC as evaluated by flow cytometry are shown. A significant increase in the percentage of biotinylated CD4⁺ or CD8⁺ cells as compared to co-cultures with biotinylated, unloaded DC is indicated by *; a further increase in biotinylated T cells by co-vaccination with SL or SL-\textit{GOLGA4} is indicated by +.

Supplementary Figure 2 Induction of cytokine expression by concomitant MAGE9 and SL-\textit{GOLGA4} vaccination. Mice were vaccinated as described in Figure 1 and received 10⁶ RENCA-MAGE9 cells after the second challenge. DC vaccination was repeated in 10d and SL vaccination in 14d intervals. Mice were sacrificed 3wk after tumor cell application Draining LNC from vaccinated RENCA-MAGE9 tumor-bearing mice were excised and the percentage of CD4⁺/IFNγ⁺ and CD8⁺/IFNγ⁺ LNC was evaluated by flow cytometry. The mean±SD of triplicates is shown. Significant differences between LNC of non-vaccinated versus DC vaccinated mice are indicated by *; significant differences by vaccination with SL are indicated by s; significant differences by co-vaccination with SL-\textit{GOLGA4} are indicated by +.

Supplementary Figure 3 The impact of MAGE9 and GOLGA4 vaccination on immunoregulatory cells. Mice were vaccinated as described in Figure 1 and received 10⁶ RENCA-MAGE9 cells after the second challenge. DC vaccination was repeated in 10d and SL vaccination in 14d intervals. Mice were sacrificed 3wk after tumor cell application. (A) The % of T_{reg} (CD4⁺CD25⁺FoxP3⁺) in draining LNC and (B) the % of MDSC (CD11b⁺Gr-1⁺) in the spleen was evaluated by flow cytometry. (A,B) The mean±SD of triplicates is shown. Significant differences between LNC of non-vaccinated versus DC vaccinated mice are indicated by *; significant differences by co-vaccination with SL-\textit{GOLGA4} are indicated by +.
Supplementary Figure 4 **Th activation by MAGE9-loaded DC and SL-GOLGA4 vaccination in tumor-bearing mice:** (A) $^3$H-thymidine incorporation is shown for draining LNC and SC of MAGE9 and MAGE9 plus SL-GOLGA4 vaccinated mice at day 5 after the last challenge and restimulation *in vitro* with MAGE9-pulsed DC (LNC:DC = 10:1). (B) LNC from vaccinated RENCA-MAGE9-bearing mice were CFSE-labeled. Cells were cultured for 24h in the presence of peptide- or lysate-loaded DC. Proliferation was evaluated by flow cytometry after counterstaining with anti-CD4-APC or anti-CD8-APC. The percentage of proliferating CD4$^+$ and CD8$^+$ cells in comparison to all CD4$^+$, respectively, CD8$^+$ cells is shown. (A,B) The mean±SD of triplicates is shown. Significant differences between LNC / SC of non-vaccinated versus DC vaccinated mice are indicated by *; significant differences by co-vaccination with SL-GOLGA4 are indicated by +.

Supplementary Figure 5 **Induction of IFN$\gamma$ secretion and CTL activation by MAGE9-loaded DC and SL-GOLGA4 vaccination in RENCA-MAGE9-bearing mice:** (A) The number of IFN$\gamma$ secreting cells was evaluated by ELISpot in LNC of tumor-bearing mice that have repeatedly been stimulated with MAGE9 peptide- or lysate-loaded DC with/without SL-GOLGA4 co-vaccination. The mean±SD of triplicates is shown. Significant differences between LNC of non-vaccinated versus DC vaccinated mice are indicated by *; significant differences by vaccination with SL are indicated by s; significant differences by co-vaccination with SL are indicated by +. (B) LNC of non-vaccinated and vaccinated RENCA-MAGE9-bearing mice were restimulated *in vitro* with correspondingly loaded DC or were maintained (non-vaccinated) in medium supplemented with 20U/ml IL-2. Cells were harvested after 5d, counted and seeded at the indicated ratios on $^3$H-thymidine-labeled RENCA-MAGE9 cells. (C) LNC were restimulated as described above and were seeded at a 1:50 ratio on $^3$H-thymidine-labeled RENCA-MAGE9 cells. Cultures contained in addition 10µg/ml rat IgG or anti-H-2d or anti-CD8. (B,C) Cytotoxicity was evaluated after 8h of co-culture. The mean±SD of triplicates is shown. (B) Significant differences between LNC of non-vaccinated versus DC vaccinated mice are indicated by *. Significant differences by co-vaccination with SL are indicated by +. (C) Significant inhibition of cytotoxic activity by anti-H-2d or anti-CD8 is indicated by *.

Supplementary Figure 6 **The impact of MAGE9-DC and SL-GOLGA4 co-vaccination on chemokine receptor expression.** Mice were vaccinated as described in Figure 1 and received 10⁶ tumor cells after the second challenge. DC vaccination was repeated in 10d and SL vacci-
nation in 14d intervals. Draining LNC of 3wk tumor-bearing mice were stained with the indicated chemokine receptor-specific antibodies. The percentage of stained cells was evaluated by flow cytometry. The mean±SD of triplicates is shown. Significant differences between LNC of non-vaccinated versus DC vaccinated mice are indicated by *; significant differences by co-vaccination with SL-GOLGA4 are indicated by +.
A  DC co-culture with LNC from vaccinated, tumor-free mice

B  ex vivo analysis of vaccinated, tumor-bearer LNC

Suppl. Fig. 1
Suppl. Fig. 2
Supp Fig. 3

Supp Fig. 4
Suppl. Fig 5