Anti-CD3/Anti-CD28 Monoclonal Antibody–Coated Suture Enhances the Immune Response of Patients With Head and Neck Squamous Cell Carcinoma

Terry Y. Shibuya, MD; Wei-Zen Wei, PhD; Michelle Zormeier, MD; John Ensley, MD; Wael Sakr, MD; Robert H. Mathog, MD; Robert J. Meleca, MD; George Yoo, MD; Carl H. June, MD; Bruce Levine, PhD; Lawrence G. Lum, MD

**Objective:** To test whether anti-CD3/anti-CD28 (αCD3/αCD28) monoclonal antibodies could be coated on surgical suture and used to enhance T-cell immune function in patients with advanced-stage head and neck squamous cell carcinoma (HNSCC).

**Design:** αCD3/αCD28 monoclonal antibodies at varying concentrations and ratios were coated on surgical sutures and tested on peripheral blood mononuclear cells from normal donors to identify the optimal stimulating condition. Immune-enhancing properties of αCD3/αCD28 monoclonal antibody suture were tested on peripheral blood mononuclear cells and regional lymph node mononuclear cells isolated from patients with advanced HNSCC and on normal donor peripheral blood mononuclear cells. Proliferation, T-cell phenotype, and cytokines were measured during 8-day in vitro stimulation with αCD3/αCD28 suture and compared with αCD3/αCD28-coated tissue culture plastic, a previously recognized carrier.

**Results:** Optimal stimulation was observed with monoclonal antibodies at the concentration of 2 µg/mL and a 1:1 ratio for 18 hours at 37°C. Strong proliferation of peripheral blood mononuclear cells and lymph node mononuclear cells in patients with HNSCC was induced by αCD3/αCD28 suture. There was no difference in maximal proliferation between αCD3/αCD28 plastic and suture. On day 6 after αCD3/αCD28 suture stimulation, T-cell subpopulations expressing CD3, CD4, CD8, CD28, and CD45RO were enhanced. Suture stimulation significantly enhanced interleukin 2 secretion when compared with plastic stimulation (P = .01). Both αCD3/αCD28 suture and plastic stimulated interferon γ secretion.

**Conclusions:** To our knowledge, this study is the first to report the modification of surgical suture to create an immunomodulant. αCD3/αCD28-coated suture expanded T cells from patients with HNSCC and induced a Th1 immune response, which may be a useful therapeutic tool in the treatment of HNSCC and other diseases.


**From the Departments of Otolaryngology–Head and Neck Surgery (Drs Shibuya, Zormeier, Mathog, Meleca, and Yoo), Immunology/Microbiology (Dr Wei), Medicine (Dr Ensley), and Pathology (Dr Sakr), Wayne State University School of Medicine, and the Karmanos Cancer Institute (Drs Wei, Ensley, and Sakr), Detroit, Mich; the Naval Medical Research Institute, Bethesda, Md (Drs June and Levine); and the Immunotherapy Research and Treatment Institute, St Luke’s Medical Center, Milwaukee, Wis (Dr Lum).**
PATIENTS AND METHODS

HUMAN SUBJECTS

All individuals participating in this study provided informed consent, and a detailed explanation of the procedure, risks, and alternatives was given to the individuals. Human Investigation Committee and Institutional Review Board approval for this study has been granted by Wayne State University, Detroit, Mich.

PATIENTS WITH HNSCC

Patients with advanced-stage HNSCC were studied because of the known immunosuppression that occurs in this population of cancer patients.\(^\text{16-19}\) The mean age of the patients studied was 51.7 years (range, 38-65 years). All patients had advanced stage III (n = 3) or IV (n = 8) HNSCC. The site of the HNSCC primary cancers included the oropharynx (n = 5), the larynx (n = 4), the oral cavity (n = 1), and an unknown primary cancer (n = 1).

PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood was drawn from healthy volunteers or patients with HNSCC before surgery. Blood was suspended in an equal volume of phosphate-buffered saline (PBS), and PBMCs were isolated by centrifugation over a Ficoll-Hypaque (Pel-Freez, Brown Deer, Wis) density gradient (10 minutes at 2800 rpm).

LYMPH NODE MONONUCLEAR CELLS

Lymph nodes were harvested at the time of surgery and placed in balanced salt solution with 20% heat-inactivated fetal calf serum (Hyclone); glutamine (Gibco), RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and 1% amphotericin B. Lymph nodes were then minced, filtered through a nylon mesh, and washed twice in balanced salt solution with 5% fetal calf serum, 1% penicillin potassium and streptomycin sulfate, and 1% amphotericin B. Lymph node mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation. Only pathologically confirmed negative lymph nodes were used in this study.

MoAbs AND SUTURES

αCD3 MoAb was purchased from Caltag Corp, Burlingame, Calif. αCD28 (9.3) was provided by one of us (C.H.J.). The sutures used were nylon, polyglactin, chromic gut, plain gut, and silk, all purchased from Ethicon Inc, Somerville, NJ.

LYMPHOCYTE ACTIVATION

Lymphocytes were incubated in triplicate with uncoated control suture (Figure 1, A), αCD3-coated suture, αCD3/αCD28-coated suture (Figure 1, B), uncoated plastic, αCD3-coated plastic, and αCD3/αCD28-coated plastic for 8 days. Peripheral blood mononuclear cells (1.5 \times 10^5) were cultured in 200 µL of culture media in 96-well flat-bottom plates (Costar, Cambridge, Mass). Culture media consisted of RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (Hyclone); glutamidate (Gibco), 2 mmol/L; penicillin (Gibco), 100 U/mL; streptomycin (Gibco), 100 µg/mL; and amphotericin B (Gibco), 100 U/mL. For phenotyping and cytokine quantification, 7.5 \times 10^5 PBMCs or LNMCs were cultured in 2.0 mL of culture media in 24-well flat-bottom plates for 6 days. All cultures were performed at 37°C in a 5% carbon dioxide atmosphere.

SUTURE COATING AND STIMULATION

αCD3-coated suture was prepared by incubating sterile suture in PBS containing αCD3 at various concentrations for 18 hours at 37°C. αCD3/αCD28-coated suture was prepared by incubating sterile suture in PBS at various concentrations and ratios of the antibodies for 18 hours at 37°C. The types of suture studied included nylon, polyglactin, chromic gut, plain gut, and silk. The suture was washed 3 times in PBS before culturing with mononuclear cells. A single 1-cm suture was placed in each 96-well culture, and 3 sutures per well were used for 24-well cultures.

PLASTIC STIMULATION

The 96-well microwell plates were precoated with 100 µL of varying concentrations of αCD3 or αCD3/αCD28 and incubated at 37°C for 18 hours. The 24-well plates were similarly precoated with 1 mL of varying concentrations of αCD3 or αCD3/αCD28. After incubation, the plate was washed 3 times with PBS before cell culture.

3[H]-THYMIDINE INCORPORATION BY PBMCs

On days 2, 4, 6, and 8, the cultures were pulsed with 7.4 \times 10^4 Bq of 3[H]-thymidine (tritium-thymidine) for 4 hours and harvested onto glass fiber disks using a cell harvester (PhD cell harvester; Cambridge Technology, Cambridge, Mass). The glass fiber disks were counted in vials containing 6 mL of scintillation-counting cocktail in a scintillation counter (Beckman, Fullerton, Calif).

PHENOTYPE

On days 0 and 6 after incubation, cell suspensions were prepared from LNMC or PBMC cultures and stained with the following MoAbs: anti–CD3-phycocerythin (PE), anti–CD4-PE, anti–CD8-PE, anti–CD28-PE, or anti–CD45RO-PE (PharMingen, San Diego, Calif) as previously described.\(^\text{20}\) The expression of surface markers was measured by flow cytometry (FACScan; Becton Dickinson, San Jose, Calif).

CYTOKINE ANALYSIS

Cell culture supernatants were harvested on days 2, 4, 6, and 8. The quantity of interleukin (IL) 2, IL-4, tumor necrosis factor α, IL-12, and interferon γ (IFN-γ) present in the supernatant was determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn).

STATISTICAL ANALYSIS

The paired t test was used to compare between groups using the Sigma Stats statistical package (Jandel Scientific, San Rafael, Calif).
were immobilized on sutures, and the activation of peripheral blood mononuclear cells (PBMCs) from normal donors was measured.

To further test the immune-enhancing properties of \(\alpha\)CD3/\(\alpha\)CD28-coated suture, PBMCs and lymph node mononuclear cells (LNMCs) from 11 patients with advanced head and neck squamous cell carcinoma (HNSCC) were incubated in vitro with antibody-coated suture, and the patients’ responses were measured. Patients with HNSCC were chosen because of the known immunosuppression that exists in this patient population, whereby immune responses are maximally suppressed in tumor-infiltrating lymphocytes, followed by proximal lymph node lymphocytes, distal lymph node lymphocytes, and peripheral blood lymphocytes.\(^{16-19}\) The immune responses measured were proliferation, cytokine production, and cellular phenotype during an 8-day period. This article examines a new method of T-cell activation with \(\alpha\)CD3/\(\alpha\)CD28–coated suture.

**RESULTS**

**MoAb COATING OF SUTURE AND T-CELL ACTIVATION**

To test if MoAbs could be immobilized on surgical suture, \(\alpha\)CD3 at varying concentrations was incubated with 6 types of sutures and PBMCs from normal donors were stimulated in vitro for 6 days with coated sutures. Monofilament nylon, chromic gut, and plain gut sutures were found to consistently activate T cells after coating with \(\alpha\)CD3 MoAbs (Figure 2, A, B, and C). Uncoated suture did not stimulate normal PBMCs (Figure 1, A). All 3 sutures stimulated maximal proliferation on day 4 (Figure 2, A, B, and C).

Because \(\alpha\)CD3-coated nylon, chromic gut, and plain gut sutures stimulated consistently, the efficacy of coating with \(\alpha\)CD3/\(\alpha\)CD28 was examined (Figure 2, A, B, and C). Nylon suture exhibited the strongest MoAb carrier function (Figure 1, B). Proliferation of PBMCs induced by \(\alpha\)CD3/\(\alpha\)CD28-coated nylon exceeded that by \(\alpha\)CD3-coated suture (Figure 2, A). \(\alpha\)CD3/\(\alpha\)CD28 MoAbs were tested at varying concentrations and ratios; the optimal coating condition was achieved by incubating nylon suture with \(\alpha\)CD3/\(\alpha\)CD28 (1:1), 2 µg/mL, for 18 hours at 37°C (data not shown).

**IMMUNE-STIMULATING EFFECT OF \(\alpha\)CD3/\(\alpha\)CD28-COATED SUTURE**

To further define the immune-stimulating properties of \(\alpha\)CD3/\(\alpha\)CD28-coated nylon suture, PBMCs and LNMCs from 11 patients with HNSCC were stimulated for 8 days, and cell proliferation, lymphocyte phenotype, and cytokine production were measured.

To compare the stimulating capacity of coated suture to a known \(\alpha\)CD3/\(\alpha\)CD28 carrier, proliferative responses of PBMCs and LNMCs from patients with HNSCC were measured after \(\alpha\)CD3/\(\alpha\)CD28-coated suture (Figure 3) or tissue culture plastic. Tissue culture plastic is an established in vitro carrier for \(\alpha\)CD3/\(\alpha\)CD28 MoAbs.\(^{17}\) Responses peaked on day 6 for suture and plas-
Peripheral Blood, %

CD45RO (expression of all cytokines screened (bent assay. Unstimulated cultures revealed minimal ex-
quantity of IL-2, IL-4, IL-12, IFN-
culture supernatants were harvested on day 6 and the

To characterize the immunologic response en-
acted after αCD3/αCD28 suture stimulation, T cells were characterized before activation and on day 6 after
stimulation. A statistically significant increase occurred
by day 6 in the CD3 (P<.001), CD4 (P=.003), CD8
(P=.04), CD28 (P=.04), and CD45RO (P<.001)
populations (all determined by t test) (Table 1). A similar
increase of T cells was observed in stimulated PBMCs, with
statistically significant increases occurring by day 6 in the
CD3 (P=.002), CD4 (P=.01), CD28 (P=.005), and
CD45RO (P<.001) populations (all determined by t test).
The increase of the CD8 population was not significant
(P=.06). Stimulation with αCD3/αCD28-coated plastic did
not permit adequate expansion of lymph node and pe-
ripheral blood T cells to allow phenotyping.

To identify from the lymph node the T-cell popula-
tion expanded after αCD3/αCD28 suture stimulation, T
cells were characterized before activation and on day 6 af-

Table 1. Phenotype of T Cells on Days 0 and 6 After Anti-CD3/Anti-CD28 Nylon Suture Stimulation

<table>
<thead>
<tr>
<th>Lymph Node, %</th>
<th>Peripheral Blood, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>Day 0</td>
</tr>
<tr>
<td>CD3</td>
<td>46 ± 21</td>
</tr>
<tr>
<td>CD4</td>
<td>27 ± 13</td>
</tr>
<tr>
<td>CD8</td>
<td>11 ± 7</td>
</tr>
<tr>
<td>CD28</td>
<td>39 ± 13</td>
</tr>
<tr>
<td>CD45RO</td>
<td>21 ± 12</td>
</tr>
</tbody>
</table>

All data are given as the mean ± SD; n = 11 unless otherwise specified.
†Significant increase on day 6 (t test). Specific P values are given in the
"Immune-Stimulating Effect of αCD3/αCD28-Coated Suture" subsection of the
"Results" section.

Figure 3. Proliferative responses on day 6 of 11 patients with head and neck
squamous cell carcinoma (HNSCC) and 6 normal subjects. PBMC indicates
peripheral blood mononuclear cell; LNMC, lymph node mononuclear cell;
large bar, PBMC mean; large circle, normal control PBMC mean; and large
triangle, LNMC mean.

To our knowledge, this study is the first to report the
modification of surgical suture to create an immuno-
modulant. Suture was coated initially with αCD3 MoAbs,
and our results revealed that nylon, chromic gut, and plain
gut sutures were reasonable carriers. Further testing of
αCD3/αCD28 plastic stimulation increased expression of IFN-γ
and tumor necrosis factor α above unstimulated con-
trols (P=.002 and P=.03, respectively). There was not a
significantly higher quantity of IFN-γ generated by
αCD3/αCD28 plastic compared with suture (P=.11).

Comment

To characterize the immunologic response enhanced
by αCD3/αCD28-coated suture and plastic, cell culture supernatants were harvested on day 6 and the
quantity of IL-2, IL-4, IL-12, IFN-γ, and tumor necrosis
factor α was measured by enzyme-linked immunosor-
ent assay. Unstimulated cultures revealed minimal ex-
pression of all cytokines screened (Table 2). Stimulation
of LNMCs with αCD3/αCD28 suture enhanced the
secretion of IL-2. This was significantly higher than the
quantity induced by αCD3/αCD28 plastic (t test, P=.001)
or the unstimulated control response (P<.001) (Table
2). Costimulation of LNMCs with αCD3/αCD28 suture
and plastic induced IFN-γ production to significantly
higher levels than the unstimulated control levels (P<.001
for both suture and plastic) (Table 2). Production of IL-12
by αCD3/αCD28 plastic was significantly higher than
the unstimulated control expression (P=.004) (Table 2). No
other significant differences in LNMC cytokine expres-
sion were identified (P>.05). Therefore, stimulation with
αCD3/αCD28 suture resulted in a Th1 cytokine expres-
sion in the LNMCs studied. Stimulation of PBMCs with
αCD3/αCD28 suture also induced significantly higher
production of IL-2 (P=.02) and IFN-γ (P<.001) than
unstimulated controls (Table 2). Suture-stimulated
PBMCs also produced significantly higher levels of IL-2
(P=.02) than plastic stimulations (Table 2). Stimulation
with αCD3/αCD28 suture resulted in a Th1 cyto-
kine expression in the PBMCs studied.

Normal donor PBMCs were stimulated in vitro for
6 days, and cytokine expression was measured by enzyme-
linked immunosorbent assay (Table 3). αCD3/αCD28
suture stimulation significantly increased expression of
IFN-γ (P=.002) above unstimulated controls. αCD3/
αCD28 plastic stimulation increased expression of IFN-γ
and tumor necrosis factor α above unstimulated con-
trols (P=.002 and P=.03, respectively). There was not a
significantly higher quantity of IFN-γ generated by
αCD3/αCD28 plastic compared with suture (P=.11).
ery. Peripheral blood mononuclear cells stimulated with αCD3/αCD28 suture also revealed significant enhancement in all T-cell subpopulations (CD3, CD4, CD28, and CD45RO) with the exception of CD8. The largest increase was noted in the CD45RO or memory cell population of T cells. Suture- and plastic-coated αCD3/αCD28 enhanced IFN-γ levels significantly above unstimulated controls. αCD3/αCD28-coated suture generated a Th1 immune response in LNMCs and PBMCs. Suture stimulation of LNMCs and PBMCs induced IL-2 to levels above those obtained by αCD3/αCD28-coated plastic stimulations. It is not clear how suture induced higher levels of IL-2. These 2 surfaces may differ in binding capacity, or the physical orientation of the MoAbs may vary between surfaces. Either of these differences could affect the MoAb stimulation of T cells.

Normal donor PBMCs were also tested with αCD3/αCD28-coated suture. Proliferative responses (Figure 3) and secretion of IFN-γ (Table 3) were enhanced. These findings support the potential use of this immune-stimulating suture in other diseases and cancers. Interleukin 2 and tumor necrosis factor α data were not available for complete cytokine profiling.

The induction of Th1 cytokines (IL-2 and IFN-γ) by αCD3/αCD28-coated suture is of significance. Th1 cytokines have been associated with cytotoxic immune responses against cancer. There are 2 active Food and Drug Administration–approved adoptive immunotherapeutic trials using αCD3/αCD28-coated beads as immunostimulants for the treatment of human immunodeficiency virus (C.H.J.) and advanced end-stage solid cancers (L.G.L.). αCD3/αCD28-coated surgical suture as a carrier may potentially allow cellular activation to be performed in vivo, bypassing the need for ex vivo expansion and reinfusion. Targeted areas could be the primary cancer site or regional lymph nodes. Further in vivo animal studies are necessary to test this treatment option.

The modification of surgical suture into an immunomodulant is a new therapeutic strategy. Cotton suture laden with Klebsiella pneumoniae has been previously reported as an infectious source in an animal model.22 The antigenicity of catgut has also been studied.22–24 Our findings showed that a monofilament nylon suture had minimal immune-stimulating properties (Figure 1, A), while αCD3/αCD28-coated suture had strong stimulatory effects (Figure 1, B). To our knowledge, this is the first report to describe a suture as an immune modulator.

Manipulation of the immune system with modified suture has tremendous clinical potential. It may be possible to inject a finely minced immunomodulating suture into regional lymph nodes before cancer surgery to generate tumor regression and increase antitumor T cells. Activated T cells may then be harvested, purified, expanded, and reinfused into the patient at a later date to kill residual or recurrent cancer. An immunomodulating suture may also be placed in the wound after surgical resection of the cancer to enhance the local immune response postoperatively, possibly stimulating memory T cells against residual or recurrent cancer. Flow cytometric data revealed that the CD45RO or memory population of T cells was expanded by suture stimulation. Immune stimulation via antibody-coated suture may be added to existing treatment regimens to generate greater anticancer immune responses. It may be possible to use αCD3/αCD28 suture stimulation in conjunction with anticancer vaccines to bolster the immune response. Another clinical application may use suture coated with immunosuppressants to prevent rejection after organ transplantation. We have been able to coat suture with αCD3, which is used to prevent rejection after renal transplantation.

### Table 2. Cytokines Present on Day 6 After Stimulation of Patients With HNSCC With αCD3/αCD28 Nylon Suture or Plastic

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Unstimulated</th>
<th>αCD3/αCD28 Suture</th>
<th>αCD3/αCD28 Plastic</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>0 ± 0</td>
<td>647 ± 736††</td>
<td>19 ± 59</td>
</tr>
<tr>
<td>IL-4</td>
<td>15 ± 36</td>
<td>16 ± 15</td>
<td>21 ± 29</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>60 ± 167</td>
<td>1264 ± 642‡</td>
<td>997 ± 703†</td>
</tr>
<tr>
<td>IL-12</td>
<td>12 ± 19</td>
<td>57 ± 58</td>
<td>107 ± 82‡</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0 ± 0</td>
<td>66 ± 141</td>
<td>21 ± 44</td>
</tr>
</tbody>
</table>

*All data are given as the mean ± SD; n = 11. HNSCC indicates head and neck squamous cell carcinoma; αCD3, anti-CD3; αCD28, anti-CD28; LNMC, lymph node mononuclear cell; PBMC, peripheral blood mononuclear cell; IL, interleukin; IFN, interferon; and TNF, tumor necrosis factor.
†Significant increase above unstimulated control (t test). Specific P values are given in the “Immune-Stimulating Effect of αCD3/CD28-Coated Suture” subsection of the “Results” section.
‡Significant increase above αCD3/αCD28 plastic stimulation. Specific P values are given in the same section as noted in the second footnote.

### Table 3. Cytokines Present on Day 6 After Stimulation of Controls With αCD3/αCD28 Nylon Suture or Plastic

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Unstimulated</th>
<th>αCD3/αCD28 Suture</th>
<th>αCD3/αCD28 Plastic</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>7 ± 18</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>IL-4</td>
<td>5 ± 2 (n = 3)</td>
<td>3 ± 5 (n = 3)</td>
<td>332 ± 656 (n = 4)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0 ± 0</td>
<td>1984 ± 678†</td>
<td>3139 ± 1482†</td>
</tr>
<tr>
<td>IL-12</td>
<td>304 ± 337 (n = 5)</td>
<td>4695 ± 458 (n = 5)</td>
<td>324 ± 336 (n = 5)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0 ± 0 (n = 5)</td>
<td>NT</td>
<td>227 ± 178 (n = 5)</td>
</tr>
</tbody>
</table>

*All data are given as the mean ± SD; n = 6 unless otherwise specified. αCD3 indicates anti-CD3; αCD28, anti-CD28; PBMC, peripheral blood mononuclear cell; IL, interleukin; NT, not tested; IFN, interferon; and TNF, tumor necrosis factor.
†Significant increase above unstimulated control (t test). Specific P values are given in the “Immune-Stimulating Effect of αCD3/αCD28-Coated Suture” subsection of the “Results” section.
Our method of modifying suture into an immuno-modifier is relatively simple. The clinical applications of such a substance are quite broad. Further investigations are planned in animal models before the development of a phase 1 trial to study suture as an immune response modifier.

CONCLUSIONS

This study has the following conclusions: (1) αCD3/αCD28 MoAbs can be coated on monofilament nylon suture. (2) αCD3/αCD28 suture stimulates LNMCs and PBMCs from patients with HNSCC and results in T₃₁ cytokine production with greater IL-2 expression than that induced by αCD3/αCD28 plastic. (3) αCD3/αCD28 suture enhanced T-cell populations of CD3, CD4, CD8, CD28, and CD45RO. (4) Surgical suture may be developed into an immune response modifier, which may be useful in the treatment of HNSCC and other diseases.

Accepted for publication May 14, 1999.

This study was supported by grant T32DC00026 from the National Institutes of Health and grant IN-162 from the American Cancer Society.

Reprints: Terry Y. Shibuya, MD, Department of Otolaryngology—Head and Neck Surgery, Wayne State University School of Medicine, 5-E UHC 540 E Canfield, Detroit, MI 48201 (e-mail: ShibuyaT@Karmanos.org).

REFERENCES