Bioactive Suture: A Novel Immunotherapy for Head and Neck Cancer

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ABSTRACT

Purpose: We have proposed to characterize the mechanism through which bioactive surgical sutures generate a Th1 immune response and to define the immune-stimulating half-life of the sutures.

Experimental Design: Bioactive sutures of interferon γ (IFNγ), interleukin 2 (IL-2), anti-CD3/CD28, anti-CD3/CD28 + IL-2, or anti-CD3/CD28 + IFNγ sutures were used to stimulate lymphocytes from normal donors and from head and neck cancer patients in vitro over a 24-day period. Cell supernatants were analyzed by ELISA, and T cells were phenotyped to characterize the immune response generated. Intracellular cytokine staining was performed to measure the expansion of flu-specific T cells. Electromobility shift assay and supershift assay were used to measure the intranuclear DNA binding activity of nuclear factor-κB, which was blocked by MG-132, and the p65 subunit in T cells activated by sutures in the presence and absence of a proteasome inhibitor, MG-132.

Results: Anti-CD3/CD28, anti-CD3/CD28 + IL-2, or anti-CD3/CD28 + IFNγ generated a prolonged Th1 immune response for 18 days in vitro. Anti-CD3/CD28 expanded flu-specific T cells. Activated T cells demonstrated enhanced CD40 ligand (CD40L) expression within 72 hours of stimulation, which stimulated other cells to secrete IL-12. Stimulated T cells demonstrated increased intranuclear expression of nuclear factor-κB, which was blocked by MG-132, and also reduced CD40L and IL-12 expression.

Conclusions: This is the first report to demonstrate that bioactive surgical sutures can generate a prolonged Th1 immune response and expand flu-specific T cells. Bioactive sutures, which are primarily a T-cell stimulant, also stimulated other cells to secrete IL-12 and prolonged the immune response. Sutures may provide a novel in situ stimulating strategy for enhancing the immune system of cancer patients.

INTRODUCTION

Immunotherapeutic treatment strategies for cancers have used numerous delivery methods, such as intravenous infusion, local injections, adoptive cellular infusions, and gene therapy injections. For head and neck squamous cell carcinoma (HNSCC), there have been only a modest number of immunotherapeutic trials to date (1–3). The use of cytokines, such as interleukin 2 (IL-2), have, in some instances, demonstrated good initial clinical responses in up to 65% of patients tested. Because of these positive responses, additional strategies have combined IL-2 with chemotherapy and shown clinical responses ranging from 30 to 100% (4–6). Unfortunately, these initial responses could not be sustained long-term and cytokine-based therapy for HNSCC has met with only limited success. However, such results should not be surprising, because many of the trials were done to evaluate toxicity and maximal tolerated doses of cytokines (phase I trials). In addition, these trials were done on patients with advanced, inoperable, end-stage head and neck cancer, and were not designed to answer the question of efficacy or restoration of immune function.

From these initial trials, several problems have become very clear, one problem has been the systemic toxicities of fever, malaise, hypotension, pulmonary edema, and shock associated with the systemic infusion of IL-2 (4–12). The problem with locally administering IL-2 has been the need for repeated (daily or weekly) injections. As an example, in several studies, injections were done up to 10 times a day (13–16). A method of delivery that localizes an immune stimulant and extends its half-life would be very helpful. The ideal carrier would be one that binds a variety of factors such as cytokines, proteins, or antibodies and extends their half-life. If such a carrier could be identified and a minimally invasive procedure for placement in situ could be developed, this would be extremely valuable for manipulating the immune system. In addition, if the stimulant could be removed without any surgical intervention, this would be greatly beneficial and in the case of an adverse reaction, could be a life-saving maneuver. Unfortunately, local injections of cytokines, proteins, or genes are not easily retrieved.
Previously, our laboratory (University of California Irvine Medical Center, Orange, CA) has reported the transformation of surgical suture into an immune-stimulant by coating anti-CD3/CD28 monoclonal antibodies onto monofilament nylon sutures (17) and covalently linking IL-2 or interferon γ (IFNγ) to polyester sutures (18). There is a significant advantage and novelty for using a suture as an immune delivery device. First, it is a medium that has long been recognized for its safety in humans and is FDA approved. Second, it is inexpensive. Third, it can be used to carry a variety of cytokines, proteins, and antibodies. And finally, sutures can be placed in vivo with a modified Seldinger technique whereby a needle with trochar is introduced to the desired location by palpation or an image guidance system (ultrasound or computerized tomography).

Next, the trochar is removed and the suture is passed to the end of the needle barrel. Next, the needle is removed, and the suture is left in place. The suture is kept long and taped to the skin surface, similar to a surgical drain. The suture can be changed, removed, or replaced by a substitution at any time; and in the event of an adverse reaction, the stitch can be immediately removed along with the linked stimulant, thereby averting or reducing an adverse reaction.

The transcription factor nuclear factor (NF)-κB transcribes cellular genes that are important in immunity, inflammation, and apoptosis (19). NF-κB exists as a heterodimeric or homodimeric complex formed by five distinct subunits, RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52). RelA, RelB and c-Rel are transcriptionally active members of the NF-κB family, whereas p50 and p52 are mainly DNA-binding subunits. The most abundant form of NF-κB is a heterodimer of p50 and p65. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form through interaction with the IκB inhibitory proteins (including IκBα, IκBβ, and IκBε), of which the best studied is IκBα. On stimulation, IκBα is phosphorylated by the serine-specific IκB kinase (IKK) and then degraded by the ubiquitin/26S proteasome way, which allows NF-κB to translocate to the nucleus and regulate gene expression (19, 20).

The immune response mediated by T cells can be divided into type 1 (T_{H1}, cellular immune response) or type 2 (T_{H2}, humoral immune response). A T_{H1} immune response generates expression of IL-2, IFNγ, IL-12, and tumor necrosis factor α (TNFα), which enhances a cytotoxic immune response, whereas a T_{H2} response stimulates IL-4, IL-5, and IL-10 and promotes a humoral or antibody immune response. In prior studies from our laboratory, we have characterized the immune response generated by several bioactive sutures as being a T_{H1} response (17, 18). The objectives of this study were to define the mechanism through which bioactive sutures generated a prolonged T_{H1} immune response and to estimate the effective immune-stimulating half-life of sutures. The development in vitro of an effective combination of sutures may provide support for future clinical trials to test in vivo bioactive sutures in head and neck cancer patients.

### MATERIALS AND METHODS

**Human Subjects.** All of the individuals participating in this study gave informed consent, and a detailed explanation of the procedure, risks, and alternatives was given. The Human Investigation Committee and Institutional Review Board (University of California Irvine College of Medicine) granted approval for this study. Twelve advanced-stage HNSCC patients were studied because of the known immunosuppression that occurs in this population of cancer patients (1–3). Mean age of patients studied was 60.1 years with a range of 36–82 years. All of the patients had advanced stage III (n = 1) or IV (n = 11) HNSCC. Primary sites of HNSCC included oral cavity (n = 6), oropharynx (n = 5), and larynx (n = 1). In addition, six normal donors participated in this study. Patient and donor testing were limited by the quantity of lymphocytes obtained from each study participant.

**Peripheral Blood Mononuclear Cells.** Peripheral blood was drawn from patients before surgery and from normal donors. Blood was suspended in an equal volume of potassium-buffered saline (PBS) and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll-Hypaque (Pel-Freez, Brown Deer, WI) density gradient (10 minutes at 2,400 rpm).

**Lymph Node Mononuclear Cells.** Lymph nodes were harvested at the time of surgery and placed in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 1% penicillin/streptomycin (penicillin/streptomycin) and 1% fungizone. Lymph nodes were minced, were filtered through wire mesh cups, and were washed twice in RPMI 1640 with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% fungizone. Lymph node mononuclear cells (LNMCs) were obtained by Ficoll-Hypaque density centrifugation. Only pathologically confirmed negative lymph nodes were used in this study.

**T-Cell Purification.** Regional LNMCs and PBMCs were purified by using human T-cell enrichment columns as specified by R&D Systems (Minneapolis, MN).

**Covalent Linking of IL-2 or IFNγ to Suture.** The method of covalently linking IL-2 and IFNγ to sutures has been described previously in detail (18).

**Coating of Anti-CD3/CD28 to Suture.** The method of coating anti-CD3/CD28 onto suture has been described previously in detail (17).

**Suture Stimulation of Lymphocytes.** To measure immune-stimulating half-life, stimulations were done with uncoated control, IFNγ, IL-2, anti-CD3/CD28, anti-CD3/CD28 + IFNγ, or anti-CD3/CD28 + IL-2 sutures for up to 24 days. During this period of stimulation, cell supernatants were harvested every other day for 24 consecutive days. Sutures were removed from culture every 6 days, rinsed in PBS, and then reused to stimulate fresh lymphocytes from the same donor in fresh medium. This was done to simulate the conditions in vivo in which cells would traffic in and out of the area where sutures were placed. Because the body has fresh serum, blood, and plasma perfusing the suture bed, there are no limitations in nutrients or food. However, with in vitro testing, this is a limiting factor. To compensate for this limitation, the medium and PBMCs were changed every 6 days. This process was done for 4 cycles (24 days). The effective half-life was defined as a reduction in cytokine expression by one half of the maximum quantity measured during the initial 6-day stimulation (first cycle). For IFNγ or IL-2, 5–0 polyester sutures were used. For anti-CD3/CD28 suture, 3–0 monofilament nylon sutures were used. Stimulations were performed in 24-well flat-bottomed microtiter plates, three stitches (each 1 cm in length) per 1.0 ×
Fig. 1 Quantity of IL-2, IL-4, IL-5, IL-12, and IFN-\( \gamma \) secreted from normal donor PBMCs (\( n = 6 \), A–C) and HNSCC patient PBMCs (\( n = 4 \), D–F) and LNMCs (\( n = 3 \), G–I) on days 2, 4, and 6 after stimulation with control (Unstim), IFN-\( \gamma \), IL-2, anti-CD3/CD28, anti-CD3/CD28 + IFN-\( \gamma \), or anti-CD3/CD28 + IL-2 sutures.

Table 1 Quantity of IL-12 produced by T cells or PBMCs over 6-day suture stimulations

<table>
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<tr>
<th>Suture stimuli</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
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<tr>
<td></td>
<td>T cells</td>
<td>PBMCs (pg/mL)</td>
<td>T cells</td>
</tr>
<tr>
<td>Control</td>
<td>0 ± 0</td>
<td>0.33 ± 0.58</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Anti-CD3/CD28</td>
<td>0 ± 0</td>
<td>383.7 ± 181.8</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Anti-CD3/CD28 + IFN-( \gamma )</td>
<td>0 ± 0</td>
<td>409.3 ± 159.4</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Anti-CD3/CD28 + IL-2</td>
<td>0 ± 0</td>
<td>382.3 ± 105.2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Anti-CD3/CD28 + MG-132 (inhibitor)</td>
<td>NT</td>
<td>5.3 ± 5.0</td>
<td>NT</td>
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NOTE. \( n = 3 \) normal donors.
Abbreviation: NT, not tested.
10⁶ cells were performed for each single stimulus (control, IFNγ, IL-2, anti-CD3/CD28). For combined stimulations (anti-CD3/CD28 + IFNγ or anti-CD3/CD28 + IL-2), three stitches of each respective stimulus were incubated per 1.0 × 10⁶ cells. For all samples, uncoated suture controls were tested. As an internal quality control to confirm that IFNγ, IL-2, anti-CD3/CD28, anti-CD3/CD28 + IFNγ, or anti-CD3/CD28 + IL-2 sutures were made correctly, a control PBMC was stimulated in parallel with patient samples, and immune response characterization was performed by measuring the quantity of IL-2, IL-4, IL-5, IL-12, and IFNγ from cell culture supernatants on days 2, 4, and 6 by ELISA (R&D Systems). IL-12 quantities were also measured prestimulation and 24, 48, 72, and 144 hours poststimulation in the presence and absence of MG-132 proteasome inhibitor.

**Phenotypic Analysis.** Cell suspensions were prepared from LNMC or PBMC cultures and were stained with monoclonal antibodies to CD3, CD4, CD8, CD40L, CD28, or CD5RO conjugated to phycoerythrin or fluorescein isothiocyanate (PharMingen, San Diego, CA) as described previously (17, 18, 21). Surface marker expression was measured by flow cytometry with a Becton Dickinson FACScan measuring 1 × 10⁶ events. Cells were also double stained before stimulation and 24, 48, 72, and 144 hours after stimulation for CD3/CD40L, CD4/CD40L, and CD8/CD40L expression. IFNγ-producing cells were identified by intracellular cytokine staining and surface expression for CD3, CD4, or CD8 as described previously (22). Stained cells were then fixed in 4% formaldehyde for 10 minutes on ice, washed with PBS, and made permeable by incubation in staining buffer containing 0.1% saponin. Nonspecific monoclonal antibody binding was blocked after saturation with Gam-immune (Bayer Healthcare, Research Triangle Park, NC) diluted in saponin for 10 minutes, and anti-IFNγ monoclonal antibody was added (0.1 μg/10⁵ cells) for 30 minutes (22). Double-positive staining cells were then counted.

**Electromobility Shift Assay and Supershift Assay.** Nuclear extracts were prepared as described previously (23). After treatments, 1.5 mL of ice-cold PBS was added to each culture dish, and cells were scraped and centrifuged. Cell pellets were suspended in 400 μL of ice-cold buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.150 mmol/L MgCl₂, 0.5 mmol/L dithiothreitol, 0.2 mmol/L phenylmethylsulfonyl fluoride] and were allowed to swell on ice for 10 minutes. Next, 25 μL of a 10% NP40 solution were added, and cells were lysed by vortexing for 10 seconds. After 30 seconds of centrifugation, the pellet nuclei were suspended in 25 μL of buffer C (20 mmol/L HEPES, 20% glycerol, 0.420 mol/L citric acid buffer. The occurrence of the covalent linking reaction was confirmed by the presence of oxidized O-phenylenediamine, measured by spectrometry at 490 nm light. Anti-CD3/CD28 suture stimulations were also preformed in the presence of MG-132, a proteasome inhibitor at a concentration of 0.5 μg/mL.

**Flu-Specific Stimulations.** To measure the antigen-specific immune response, one donor and two cancer patients who were HLA-A2+ and had adequate numbers of PBMCs and LNMCs were studied. Lymphocytes (1 × 10⁶ per well) were stimulated for in vitro days 1 to 3 under the following conditions: (a) unstimulated suture control (three stitches per well); (b) flu-peptide (50 μg/ml); (c) anti-CD3/CD28 suture (three stitches per well); and (d) anti-CD3/CD28 (three stitches per well) + flu-peptide (50 μg/mL). Cells were then washed and rested in fresh medium on days 4 to 6, and then were pulsed with flu-peptide (50 μg/mL) for 24 hours and were analyzed by flow cytometry for intracellular expression of IFNγ. Cells were double stained for CD3/IFNγ, CD4/IFNγ, or CD8/IFNγ; and total double-positive expressing cells were counted.

**Cytokine Analysis.** For half-life studies, the quantity of IL-12, IFNγ, and IL-5 present in the supernatant was determined by ELISA (R&D Systems) for 24 days. Immune response characterization was performed by measuring the quantity of IL-2, IL-4, IL-5, IL-12, and IFNγ from cell culture supernatants on days 2, 4, and 6 by ELISA (R&D Systems). IL-12 quantities were also measured prestimulation and 24, 48, 72, and 144 hours poststimulation in the presence and absence of MG-132 proteasome inhibitor.

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NaCl, 0.150 mmol/L MgCl$_2$, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 0.2 mmol/L phenylmethylsulfonyl fluoride) and incubated on ice with shaking for 15 minutes. After centrifugation for 2 minutes, the extract was removed and diluted with 75 mmol/L NaCl, 0.150 mmol/L MgCl$_2$, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 0.2 mmol/L phenylmethylsulfonyl fluoride) and stored at −70°C. For the electromobility shift assay, a NF-κB probe, which is a synthetic double-stranded oligonucleotide containing the κB binding site of the immunoglobulin κ promoter (5′-GATCCAGGAGCTTCCATG-3′) was end-labeled by T4 kinase. Next, the binding reaction mixture was added, containing 20 mmol/L HEPES, 50 mmol/L KCl, 0.5 mmol/L EDTA, 1.0 mmol/L dithiothreitol, and 0.2 mmol/L phenylmethylsulfonyl fluoride) and stored at −70°C. For the electromobility shift assay, a NF-κB probe, which is a synthetic double-stranded oligonucleotide containing the κB binding site of the immunoglobulin κ promoter (5′-GATCCAGGAGCTTCCATG-3′) was end-labeled by T4 kinase. Next, the binding reaction mixture was added, containing 20 mmol/L HEPES, 50 mmol/L KCl, 1.0 mmol/L dithiothreitol, 1.0 mmol/L EDTA, 5% glycerol, 1 μg of poly(deoxyinosinic-deoxycytidylic acid), and 0.4 ng of labeled oligonucleotide, and the new mixture was incubated with nuclear extract samples (10 μg) at room temperature for 15 minutes in a total of 20-μL volume. The protein-DNA complexes were separated by electrophoresis on a 5% nondenaturing polyacrylamide gel 0.5× Tris-borate EDTA. Dried gels were subjected to autoradiography. For supershift assay, antibody against p65 was added 20 minutes before the labeled probe.

**Materials.** Both 3–0 monofilament nylon and 5–0 polyster sutures (Ethicon Inc, Somerville, NJ), as well as IFNγ (InterMune Pharmaceuticals, Inc., Brisbane, CA), IL-2 (Chiron, Emeryville, CA), and anti-CD3 monoclonal antibody (OrthoBiotech), were purchased. Anti-CD28 (9.3) monoclonal antibody was tested (gift C. H. June, University of Pennsylvania Cancer Center, Philadelphia, PA). Flu-peptide was purchased (Influenza A Matrix peptide 58–66, GILGFVFTL, Quality Controlled Biochemicals, Hopkinton, MA).

**Statistical Analysis.** For five subjects, T cells were stimulated for 24 days with each of six stimulants (uncoated control, IFNγ, IL-2, anti-CD3/CD28, anti-CD3/CD28 + IFNγ, and anti-CD3/CD28 + IL-2). Measured phenotypic outcomes included the expression of CD3, CD4, and CD8, and the ratio of CD4 to CD8, CD28, and CD45RO. Nonparametric repeated-measures ANOVA was used to examine the differences in rank means among sutures, time points, and suture-time interactions (24). For comparisons between pairs of stimulants, adjustment for multiple comparisons was made with the Bonferroni method. Because 15 paired comparisons were made for each phenotypic outcome, a comparison-wise significance level of 0.0033 was used to maintain an overall significance level of 0.05. All of the statistical tests were two-sided. All of the analyses were done with Statistical System software (Version 8.2, SAS Institute Inc., Cary, NC).

### RESULTS

**Defining the Immune-Stimulating Mechanism.** To define the immune-stimulating mechanism, the type of immune response (T$_{H1}$ or T$_{H2}$) generated by the various suture combinations was characterized. Cell culture supernatants were harvested from the PBMCs of six normal donor, the PBMCs of four HNSCC patients, and the LNMCs of three HNSCC patients over a 6-day period. The quantity of IL-2, IL-4, IL-5, IL-12, and IFNγ was measured by ELISA on days 2, 4, and 6. Cytokines peaked in value by day 6 and expression profiles favored a T$_{H1}$ immune response (Fig. 1A–I), with enhanced levels of IL-12, IFNγ, and IL-2.

To further characterize the source of IL-12, a key cytokine in generating a T$_{H1}$ immune response, we also performed stimulations with PBMCs or T lymphocytes alone, and IL-12 levels were compared between groups. PBMCs or T cells were stimulated for 6 days with suture combinations of anti-CD3/CD28 alone, anti-CD3/CD28 + IFNγ, or anti-CD3/CD28 + IL-2, which all generated high levels of IL-12. T cells alone did not generate any IL-12, whereas PBMCs produced high levels of IL-12 (Table 1).

### CD40L Expression.

T cells were phenotyped before and after anti-CD3/CD28 stimulation at 24, 48, 72, and 144 hours for CD3/CD40L, CD4/CD40L, and CD8/CD40L expression. CD40L expression was elevated within 72 hours of stimulation, with 10.2 to 15.6% of CD3+ cells expressing CD40L after stimulation with anti-CD3/CD28, anti-CD3/CD28 + IFNγ, or anti-CD3/CD28 + IL-2 (Table 2; Fig. 2A–F). To confirm that CD40L expression was important for elevated levels of IL-12, we also stimulated PBMCs with anti-CD3/CD28 suture in the presence of MG-132. MG-132 is a proteasome inhibitor that stabilizes IκBα protein, and then inhibits its NF-κB p65 subunit intranuclear translocation and its transcription activity (25). Intranuclear translocation of NF-κB p65 subunit is required for CD40L expression in T cells (23, 25–28). Stimulation of PBMCs in the presence of MG-132 reduced the expression of CD40L of T lymphocytes (Fig. 2G–I) and the quantity of IL-12 measured in culture (Table 1).

To further confirm that MG-132 was inhibiting the intranuclear translocation of the NF-κB p65 subunit within the T cell, we performed electromobility shift and supershift assays. T lymphocytes were stimulated with anti-CD3/CD28 in the presence and absence of MG-132, and the intranuclear DNA binding activity of NF-κB was measured. MG-132 inhibited the intranuclear translocation of the NF-κB p65 subunit (Fig. 3).

### Suture Half-Life Studies and Cytokine Expression.

To define the effective immune-stimulating half-life of sutures, PBMCs were stimulated for 24 days in vitro. Kinetics studies, measuring the quantity of IL-12 (T$_{H1}$), IFNγ (T$_{H1}$), and IL-5
(Th2) in supernatant, were performed, and the type of immune response generated was characterized as either Th1 or Th2. The mean value of IL-12, IFNγ/H9253, and IL-5 that was secreted by the PBMCs of four individuals (three normal donors and one HNSCC patient) over a 24-day period is shown (Fig. 4A–F). Six suture conditions were tested (uncoated control, IFNγ, IL-2, anti-CD3/CD28, anti-CD3/CD28/H11001 IFNγ, and anti-CD3/CD28/H11001 IL-2 sutures). The maximum quantity of IL-12 generated

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<tr>
<td>24 h</td>
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</tr>
<tr>
<td>Anti-CD3/CD28 + IFNγ</td>
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<tr>
<td>3.9 ± 3.6</td>
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<tr>
<td>2.3 ± 1.5</td>
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<tr>
<td>0.4 ± 0.4</td>
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<tr>
<td>90.2 ± 1.0</td>
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<tr>
<td>Anti-CD3/28 + IL-2</td>
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<tr>
<td>2.6 ± 2.3</td>
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<tr>
<td>2.0 ± 1.8</td>
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<tr>
<td>0.2 ± 0.1</td>
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<td>87.3 ± 0.8</td>
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Fig. 2 Expression of CD3/CD40L, CD4/CD40L, and CD8/CD40L at 48 hours after stimulation with control suture (A–C), anti-CD3/CD28 suture (D–F), and anti-CD3/CD28 suture (G–I) in the presence of a 0.5 μg/mL concentration of MG-132 inhibitor (n = 3 normal donor PBMCs). (PE, phycoerythrin; FITC, fluorescein isothiocyanate.)
during the first stimulation cycle (days 0–6) for anti-CD3/CD28, anti-CD3/CD28 + IFNγ, and anti-CD3/CD28 + IL-2 sutures was determined (Fig. 4A), whereas control, IFNγ, and IL-2 sutures generated minimal IL-12 amounts. A reduction of IL-12 over the second stimulation cycle (days 7–12) was noted for anti-CD3/CD28, anti-CD3/CD28 + IFNγ, and anti-CD3/CD28 + IL-2 sutures (Fig. 4A). During the third cycle (days 13–18), the amount of IL-12 increased for anti-CD3/CD28, anti-CD3/CD28 + IFNγ, and anti-CD3/CD28 + IL-2 sutures (Fig. 4A). By the fourth cycle (days 19–24), there was a reduction in IL-12 levels to one half of the values measured during the first cycle of stimulation for anti-CD3/CD28, anti-CD3/CD28 + IFNγ, and anti-CD3/CD28 + IL-2 sutures (Fig. 4A). The progressive decrease in IL-12 over three cycles of stimulation was also seen with the PBMCs of one HNSCC patients stimulated over an 18-day period and is shown in Fig. 4D. Quantities of IL-12 generated by controls and HNSCC patient were similar. To further confirm the effectiveness of anti-CD3/CD28 suture to stimulate IL-12 expression, were stimulated 12 HNSCC patients’ PBMCs in vitro over a 24-day period. IL-12 values were measured after the first 18 days of stimulation (Fig. 5A).

Similarly for IFNγ, maximum quantities generated during the first cycle for control, IFNγ, IL-2, anti-CD3/CD28, anti-CD3/CD28 + IFNγ, and anti-CD3/CD28 + IL-2 sutures were measured for three normal controls (Fig. 4B) and one HNSCC patient (Fig. 4E). A reduction in more than one half of the initial IFNγ levels was identified during the second cycle for IFNγ and IL-2 sutures for the normal donors (Fig. 4B), whereas IFNγ levels remained elevated for anti-CD3/CD28, anti-CD3/CD28 + IFNγ, and anti-CD3/CD28 + IL-2 sutures for the normal donors (Fig. 4B) and HNSCC patient (Fig. 4E). By the third cycle, IFNγ was reduced by one half or nearly one half compared with first cycle values for anti-CD3/CD28, anti-CD3/CD28 + IFNγ, and anti-CD3/CD28 + IL-2 sutures (Fig. 4B and E). Three sutures (anti-CD3/CD28, anti-CD3/CD28 + IFNγ, anti-CD3/CD28 + IL-2) generated strong Th1 immune responses with elevated levels of IFNγ and IL-12 for a 18-day period, whereas control, IFNγ, and IL-2 sutures produced minimal cytokines. To further confirm the effectiveness of anti-CD3/CD28 suture to stimulation of IFNγ expression, we stimulated 12 HNSCC patients’ PBMCs in vitro over a 24-day period. IFNγ values remained elevated over the first 18 days of stimulation (Fig. 5B). IL-5 was elevated for anti-CD3/CD28, anti-CD3/CD28 + IFNγ, and anti-CD3/CD28 + IL-2 sutures during the first 6 days of stimulation but demonstrated minimal levels during the remaining cycles for normal donors (Fig. 4C) and decreased by day 12 for the HNSCC patients (Fig. 4F).

Phenotypic Analysis. To identify the T lymphocytes expanded during the immune response generated, cells were characterized for viability and expression of CD3, CD4, CD8, the ratio of CD4 to CD8, CD28, and CD45R0 over four cycles; the first cycle ended on day 6, the second cycle on day 12, the third cycle on day 18, and the fourth cycle on day 24 (Fig. 6A–D). Unstimulated control, IFNγ, or IL-2 sutures did not significantly change the distribution of T cells over the four cycles (Fig. 6A–D). For statistical comparisons done between pairs of stimulants, the range of unadjusted P values was from 0.24 to 0.996. Stimulation with anti-CD3/CD28, anti-CD3/CD28 + IFNγ, or anti-CD3/CD28 + IL-2 sutures significantly changed the T-cell phenotypic distribution of CD4, CD8, CD4-to-CD8 ratio, and CD45R0 cells over the four cycles compared with suture controls (range of unadjusted P values for paired comparison, 0.000 to 0.002).

Anti-CD3/CD28 sutures significantly decreased the percentage of CD4 cells (P = 0.002), increased the percentage of CD8 cells (P = 0.000), decreased the CD4-to-CD8 ratio (P < 0.001), and increased the percentage of CD45R0 cells (P = 0.000) over the four cycles compared with suture controls. Similarly, anti-CD3/CD28 + IFNγ sutures significantly decreased the percentage of CD4 cells (P = 0.002), increased the percentage of CD8 cells (P = 0.000), decreased the CD4-to-CD8 ratio (P < 0.001), and increased the percentage of CD45R0 cells (P = 0.000) over the four cycles compared with suture controls. Anti-CD3/CD28 + IL-2 sutures significantly decreased the percentage of CD4 cells (P < 0.001), increased the percentage of CD8 cells (P = 0.000), decreased the CD4-to-CD8 ratio (P = 0.000), and increased the percentage of CD45R0 cells (P = 0.000) over the four cycles compared with suture controls.

Flu-Specific T-Cell Expansion. To measure the effect of bioactive sutures to generate an antigen-specific immune response, we tested flu-peptide as a recall antigen. One HLA-A2+ donor and two HLA-A2+ cancer patients with sufficient numbers of PBMCs and LNMCs were studied. Lymphocytes were stimulated for 3 days in vitro under four conditions: (a) unstimulated suture control (three stitches per well), (b) flu-peptide (50 μg/mL), (c) anti-CD3/CD28 suture (three stitches per well), and (d) anti-CD3/CD28 (three stitches per well) + flu-peptide (50 μg/mL). Next, cells were washed and rested in fresh medium for 3 days, then were pulsed with flu-peptide (50 μg/mL) for 24 hours and were analyzed for intracellular expression of IFNγ. Flu-specific T cells were stained double positive for CD3/IFNγ, CD4/IFNγ, or CD8/IFNγ and were counted (Fig. 7). From control PBMCs, the greatest numbers of
Fig. 4 Quantity of IL-12 p40 (A and D), IFNγ (B and E), or IL-5 (C and F) secreted by normal donor PBMCs (n = 3, A–C) or HNSCC patient PBMCs (n = 1, D–F, respectively) over 18–24 days for unstimulated control, IFNγ, IL-2, anti-CD3/CD28, anti-CD3/CD28 + IFNγ, or anti-CD3/CD28 + IL-2 suture.
flu-specific T cells were expanded by anti-CD3/CD28 suture, followed by anti-CD23/CD28 suture and flu-peptide, followed by flu-peptide; and the least numbers of flu-specific T cells were expanded by control suture (Fig. 7A). From the LNMCs of HNSCC patient 25, the greatest number of flu-specific T cells were expanded by anti-CD3/CD28 suture, followed by anti-CD23/CD28 suture and flu-peptide, followed by flu-peptide; the least numbers were expanded by control suture (Fig. 7B). From the PBMCs of HNSCC patient 25, the greatest number of flu-specific T cells were expanded by anti-CD3/CD28 suture, followed by anti-CD23/CD28 suture, followed by flu-peptide and by control suture (Fig. 7C). From the PBMCs of HNSCC patient 15, the greatest number of flu-specific T cells were expanded by anti-CD23/CD28 suture and flu-peptide, followed by flu-peptide and by control suture (Fig. 7D). From the LNMCs of HNSCC patient 15, there was significant cell death under all stimulating conditions, and inadequate numbers of T cells were available to count (data not shown). The overall numbers of flu-specific T cells expanded from patient 15 were less than those of patient 25 or of control (Fig. 7D). The cause for this cell death was not identified.

DISCUSSION

Strategies for stimulating in vivo the immune system of cancer patients has taken on many forms such as intravenous infusion, local injections, adoptive cellular infusions, and gene therapy. However, the ideal delivery devise that carries a variety of stimulants, has an extended half-life, is inexpensive, and is retrievable has yet to be developed. In hopes of developing such a devise, we studied the development of surgical suture into a bioactive compound. By using suture as a carrier rather than for its classic purpose of wound closure, it can be used to manipulate in vivo the immune system. Sutures can be placed in vivo with a modified-Seldinger technique, whereby after placement, the stitch is kept long and attached to the skin surface, similar to a surgical drain. At any point after placement, it may be retrieved or replaced from its localized position in the body.

Previous studies from our laboratory (University of California Irvine Medical Center), one of the key cytokines in driving a cytotoxic or T helper 1 response was observed to be consistently elevated (Fig. 1). Interestingly, anti-CD3/CD28 suture is primarily a T-lymphocyte stimulating strategy, whereas IL-12 is primarily secreted from antigen-presenting cells. To examine whether T lymphocytes were responsible for elevated IL-12 levels, stimulations were done with whole PBMCs or isolated T cells (Table 1). Stimulations done on negatively selected, >95%-pure T cells with anti-CD3/CD28, anti-CD3/CD28 + IFNγ, or anti-CD3/CD28 + IL-2 did not generate any IL-12 (Table 1), although there were elevated levels of IFNγ generated (data not shown). Only in the presence of whole PBMCs was there any IL-12 present, demonstrating that cells other than T lymphocytes were responsible for IL-12 levels. To determine whether anti-CD3/CD28-costimulated T cells could be stimulating APCs to secrete IL-12, we examined the expression of CD40L on suture-stimulated T cells over a 6-day period. CD40L expression was found to be expressed on >10.2 to 15.6% of activated T lymphocytes within 72 hours of stimulation. To further confirm that CD40L was responsible for elevated IL-12 levels, we preformed suture stimulation in the presence of MG-132. MG-132 inhibits the translocation of NF-κB p65 subunit intranuclear and blocks its effect as a key transcription factor required for T-cell expression of CD40L. Therefore, suture stimulations in the presence of MG-132 would be expected to inhibit CD40L expression and result in reduced levels of IL-12. This was confirmed when stimulation of PBMCs in the presence of MG-132 inhibited T-cell expression of CD40L (Fig. 2G–I; Table 2), reduced the levels of IL-12 (Table 1), and reduced the intranuclear presence of NF-κB (Fig. 3).
Our strategy of using anti-CD3/CD28, anti-CD3/CD28 + IFNγ, or anti-CD3/CD28 + IL-2 sutures not only stimulated T lymphocytes but also indirectly stimulated other effector cell populations, such as APCs via the up-regulation of CD40L on activated T cells. Generating a prolonged Th1 response in the setting of many cancers can be a very difficult task because of the immunosuppressive environment that tumors frequently induce. An immune-stimulating strategy that generates a Th1 immune response in not only T lymphocytes but also other effector cells, such as APCs, should be considered a positive step in the fight against multiple tumors.

In addition, we tested whether our suture-stimulating strategy could expand antigen-specific T cells by measuring the number of flu-specific T cells expanded from one donor and two cancer patients after suture stimulation. The total number of flu-specific T cells was increased after stimulating with anti-CD3/CD28 suture alone or with anti-CD3/CD28 suture + flu-peptide from the PBMCs of a normal donor (Fig. 7A) and from the LNMCs and PBMCs of one HNSCC patient (Fig. 7B and C). From another HNSCC patient who was tested, only a modest number of flu-specific PBMCs could be expanded (Fig. 7D), and the LNMCs from this individual died in culture. The cause for this death was not identified. The finding that HNSCC patients’ T lymphocytes are susceptible to apoptosis and death has been clearly recognized by others (29–31). From our previous studies, we have been able to identify a group of HNSCC patients with very poor CD3 receptor function with whom there is a much higher incidence of recurrent or metastatic cancer (32). Unfortunately our bioactive suture may be effective in only a subgroup of HNSCC patients, but not effective in all HNSCC patients. A method for identifying which individuals may respond to bioactive sutures, and those who may not, is needed. Perhaps future testing for the frequency of T regulator cells (CD4+/CD25+) cells, tumor expression of Fas-L, T-cell intracellular expression CD3 chain, or other possible studies may be helpful in identifying which cancer patients might be better candidates for our stimulating strategy.

Our bioactive suture-stimulating strategy has been consistently effective in normal donors at stimulating a Th1 immune response. The stimulation of HNSCC patients’ PBMCs has been fairly consistent in generating elevated levels of IL-12 and IFNγ. This has been confirmed by the stimulating of 12 HNSCC patients’ PBMCs with anti-CD3/CD28 for up to 24-days in vitro (Fig. 5). The response of HNSCC LNMCs may not be as strong as PBMCs, as suggested by Fig. 1D–I. Fortunately, in several HNSCC patients, bioactive sutures have generated antigen-specific immune responses (Fig. 7B–D) and Th1 immune responses (Fig. 5).

To define the effective half-life of sutures, we stimulated

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**Fig. 6** T-lymphocyte phenotype after stimulation for cycle 1 (A, day 6, n = 3 normal donors PBMCs, n = 1 HNSCC patient’s PBMCs), cycle 2 (B, day 12, n = 3 normal donors’ PBMCs, n = 1 HNSCC patient’s PBMCs), cycle 3 (C, day 18, n = 3 normal donors PBMC, 1 HNSCC patient PBMC), cycle 4 (D, day 24, n = 3 normal donors’ PBMCs). Stimulating conditions of control, IFNγ, IL-2, anti-CD3/CD28, anti-CD3/CD28 + IFNγ, or anti-CD3/CD28 + IL-2 sutures.
PBMCs from three normal donors and one cancer patient for 6 days in vitro. On the 6th day, sutures were re-used to stimulate fresh PBMCs from the same donor in fresh medium. This cycle was repeated every 6 days for a total of four cycles to simulate the conditions in vivo in which cells would traffic in and out of the area where sutures had been placed. If the sutures were not transferred to fresh medium, the cell cultures would be exhausted of serum because of the high cellular activation that occurs during suture stimulation. Sutures were found to have a stimulating half-life of 18 days by maintaining elevated levels of IFNγ/H9253 and IL-12 (Figs. 4A, B, D, E, and 5A, B). A decrease in CD4+ cells, an increase in CD8+ cells, a decrease in the ratio of CD4-to-CD8 cells, and an increase in CD45RO cells, compared with suture controls, were noted with anti-CD3/CD28 sutures over all four cycles (Fig. 6A–D). Previously, anti-CD3/CD28-coated beads have been used to stimulate and expand lymphocytes ex vivo for use in adoptive cellular therapy trials for the treatment of HIV (33–36) and cancer (36). Several trials have specifically focused on the expansion of CD4+ cells and demonstrated that, after re-infusion into patients, cells remained present in patients for more than 1 year (37). Other trials have expanded PBMCs with beads and have demonstrated the expansion of both CD4+ and CD8+ cell populations (21, 38). Our anti-CD3/CD28 suture based-strategy (with or without IFNγ or IL-2 sutures) expanded a larger percentage of CD8+ cells (Fig. 6), compared with bead-based strategies. We did not experience as large a percentage of CD4+ cell expansion (33–36, 38). The enhanced expansion of CD8+ cells within a Th1 immune environment should be considered a very positive step toward generating a cytolytic immune response. Recently, bead-based strategies have also been used to create artificial APCs (39, 40) and to generate antigen-specific immune responses. The binding of such stimulants onto surgical sutures may be a future option as well.

Unique to this stimulating strategy is the fact that the vehicle (suture) may be substituted, removed, or changed at any time. Because the stimulus is attached to the suture, it may be localized to a specific area, thereby reducing its diffusion away from the desired location. Binding stimulants to the suture has also extended their half-life and may overcome the immunosuppressive environment often identified in head and neck cancer patients (3, 32). These stimulants may also be added to other existing immunotherapeutic strategies (peptide vaccines, dendritic cell vaccines, and so forth) by helping to booster the site of vaccination. Presently, all monoclonal antibodies, cytokines, and sutures used in this study are FDA approved for use in humans. Additionally, a wide variety of sutures, cytokines, and antibodies may be purchased at relatively inexpensive prices. Future innovations in immunotherapy should investigate the development of novel delivery techniques for enhancing the immune system of cancer patients, while keeping the cost for such strategies at a minimum.
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Bioactive Suture: A Novel Immunotherapy for Head and Neck Cancer

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