

Induction of Antitumor Immunity via Intratumoral Tetra-Costimulator Protein Transfer¹

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ABSTRACT

Our group recently described a novel two-step Fc_γ1 fusion protein transfer method, which entails the docking of Fc_γ1 fusion proteins onto cells precoated with chemically palmitated protein A (pal-prot A). In the present study, we have adapted this protein transfer method, originally used in an *ex vivo* context, for *in situ* tumor cell engineering, and in so doing, we have evaluated its utility for the induction of antitumor immunity via combinatorial costimulator protein transfer on to tumor cell surfaces. The feasibility of “painting” cells with preformed conjugates of a murine B7–1 costimulator derivative, B7–1·Fc_γ1, and pal-prot A in a single step was first established *ex vivo*. Next, B7–1·Fc_γ1:pal-prot A transfer was accomplished *in vivo* by directly injecting the preformed conjugates into highly aggressive L5178Y-R lymphomas grown intradermally in syngeneic mice. The presence of cell surface-associated B7–1 epitopes on cells of the injected tumors was documented by flow cytometric analysis of cells recovered subsequently from the injected tumors. B7–1·Fc_γ1, along with Fc_γ1 fusion protein derivatives of three additional costimulators (Fc_γ1·4–1BBL, CD48·Fc_γ1, and Fc_γ1·CD40L) geared toward a variety of immune effectors, were together preconjugated with pal-prot A and injected directly into tumor beds. Significantly, this “tetra-costimulator” combination, delivered intratumorally, induced complete tumor regression in ~45% of treated mice, whereas control injections of pal-prot A alone had no therapeutic effect. Furthermore, there was evidence for systemic antitumor immunity in that tumor-specific CTLs were detected in spleens recovered from cured mice, and these mice were uniformly protected against tumor rechallenge at distant tumor sites. Hence, combinatorial costimulator transfer, coupled to intratumoral delivery, may have special advantages for the induction of antitumor immunity.

INTRODUCTION

One cancer vaccination strategy of interest entails the administration of “immunogenic” tumor cells to elicit systemic antitumor immunity. Such immunogenic tumor cells can be engineered in a number of ways, including via the neoexpression of immune costimulators on their surfaces (reviewed in Ref. 1). Enforced expression of surface T-cell-directed costimulators, such as B7–1 (CD80; Ref. 2), or dendritic cell activators, such as CD40L (3, 4), confers on certain tumor cell lines the capacity to elicit systemic antitumor immune responses. However, in the face of idiosyncratic results obtained for different tumor cell lines when costimulators are used individually (5), we (6) and others (7–10) have advocated the use of more than one costimulator in combination to additionally augment the immunogenic potential of engineered tumor cells. Nonetheless, to date, only a limited number of costimulator combinations have been explored. Moreover, whereas costimulator combinations have been selected with their synergistic and/or additive effects in mind, the opportunity remains

for rationally designing costimulator combinations that would simultaneously invoke multiple antitumor immune cell effectors.

In most studies to date, enforced expression of costimulators on tumor cell surfaces has been achieved through gene transfer. However, gene transfer has limitations in this context, both experimentally and clinically. For one, it does not enable fine control of protein product expression, thus precluding the titration of costimulator mixtures at optimal levels. A second and more fundamental limitation of gene transfer is that it is poorly suited for the combinatorial expression of multiple gene products in the same cell, even when viral vectors are invoked. The challenge increases with the number of gene products to be expressed. Protein transfer offers advantages over gene transfer in both regards, because protein transfer is well suited for the simultaneous delivery of well-defined amounts of multiple proteins onto individual cells.

Previously, we (11, 12) and others (13) reported the engineering of immunogenic tumor cells by painting on their surfaces GPI³-modified derivatives of the B7–1 (CD80) costimulator. More recently, we reported an even simpler two-step protein transfer method that uses precoated pal-prot A as artificial docking sites for Fc_γ1 fusion proteins, such as B7–1·Fc_γ1 (14). The present study builds on this latter protein transfer method in two ways. First, protein transfer is invoked here for the first time to express complex costimulator arrays, with up to four costimulator·Fc_γ1 derivatives (B7–1·Fc_γ1, Fc_γ1·4–1BBL, CD48·Fc_γ1, and Fc_γ1·CD40L) now being used in combination to simultaneously drive more than one antitumor immune effector pathway. Second, protein transfer is accomplished here for the first time *in situ*, rather than *ex vivo*, via intratumoral injection of costimulator·Fc_γ1:pal-prot A conjugates. The data point to the efficacy in an animal tumor model for this unique “tetra-costimulator, intratumoral” protein transfer strategy.

MATERIALS AND METHODS

Mice. Female DBA/2J mice, 6–8 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and used experimentally under protocols approved by the University of Pennsylvania Laboratory Animal Regulatory Committee.

Cell Lines. The murine cell lines Ag104A (sarcoma) and T50 (bladder carcinoma) were gifts from Drs. Lieping Chen (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) and Abraham Hochberg (Hebrew University, Jerusalem, Israel), respectively. The murine mastocytoma cell line P815 and the T-lymphoma cell line L5178Y-R were purchased from the American Type Culture Collection (Manassas, VA). The Ag104A, T50, and P815 cell lines were maintained in DMEM supplemented with 10% FCS, whereas the L5178Y-R cell line was maintained in DMEM supplemented with 10% FCS, 0.11 g/liter sodium pyruvate, and 1.125 g/liter sodium bicarbonate. For *in vivo* studies, an L5178Y-R cell bank was generated from which cell cultures were freshly started and maintained for <3 weeks before inoculation into mice.

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³ The abbreviations used are: GPI, glycosyl-phosphatidylinositol; pal-prot A, palmitated protein A; mAb, monoclonal antibody; DAB, 3,3'-diaminobenzidine; MFI, mean fluorescence indices; NK, natural killer; APC, antigen-presenting cell; ID, intradermal; IP, intraperitoneal.

mAb. FITC-mouse antihuman CD40L mAb (TRAP1), rat antimouse B7-1 (1G10), and FITC-goat antirat IgG were purchased from BD PharMingen (San Diego, CA). Rat antimouse CD48 was purchased from Serotec Ltd. (Oxford, United Kingdom).

Costimulator-Fc γ ₁ Fusion Proteins. The strategy for assembling chimeric expression cassettes encoding murine B7-1-Fc γ ₁ and CD48-Fc γ ₁ mirrored that reported previously for human B7-1-Fc γ ₁ (14). The coding sequences for the extracellular domains of murine B7-1 (V38-T247; Swiss-prot accession number: Q00609) and murine CD48 (F23-R261; Swiss-prot accession number: P18181) were linked in-frame to a coding sequence for the Fc γ ₁ domain of human IgG1 within our expression constructs pmB7-1-Fc γ ₁/REP7 and pmCD48-Fc γ ₁/EE14, respectively. Because 4-1BBL and CD40L are type II membrane proteins, the Fc γ ₁ domain was instead appended to their NH₂ termini. Hence, in the pFc γ ₁m4-1BBL/EE14 and pFc γ ₁mCD40L/EE14 expression constructs, the coding sequence for the Fc γ ₁ domain of human IgG1 was linked in-frame to the extracellular domains of mouse 4-1BBL (R104-E309; Swiss-prot accession number: P41274) and human CD40L (H47-L261; Swiss-prot accession number: P29965), respectively. The fusion proteins were produced in 293 (for B7-1-Fc γ ₁) or Chinese hamster ovary (CD48-Fc γ ₁; Fc γ ₁4-1BBL; and Fc γ ₁CD40L) cell transfectants and purified by protein A-agarose chromatography, as described previously (14). Purified recombinant proteins were analyzed by SDS-PAGE on precast 3-8% NuPAGE gels, as per the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA).

Protein Transfer *ex Vivo*. Procedures for generating pal-prot A (15) and using it for protein transfer (14) have been detailed previously. However, whereas the earlier studies called for the sequential (two-step) addition of pal-prot A followed by Fc γ ₁ fusion proteins, protein transfer is accomplished here in one step by using preformed conjugates of pal-prot A and each of the Fc γ ₁ fusion proteins (or control human IgG1). These preformed conjugates were generated by combining the components at a 1:1 ratio (w/w) in PBS/0.1% BSA (transfer buffer) at ambient temperature for 30 min. P815 and L5178Y-R tumor cells, grown in suspension, were suspended in transfer buffer after three washes in the same buffer. Ag104A and T50 tumor cells, grown as adherent cells, were detached with 5 mM EDTA in PBS before being suspended in the transfer buffer. Protein transfer was accomplished by coincubating 2×10^6 cells with 12 μ g of each of the preformed pal-prot A:Fc γ ₁ fusion protein (or IgG1) conjugates at 37°C for 1 h in 0.2 ml of the transfer buffer, supplemented with 0.1% sodium azide, 10 mM 2-deoxyglucose (to prevent endocytosis), 1 mM phenylmethylsulfonyl fluoride, 1 μ M leupeptin, and 1 μ M pepstatin (to prevent proteolysis). For simultaneous transfer of more than one Fc γ ₁ fusion protein, each costimulator-Fc γ ₁ fusion protein was conjugated with pal-prot A separately, as described above, and the resultant conjugates were then combined at equal mass ratios and transferred to the cells under the same conditions described above.

Cells coated with control FITC-human IgG were analyzed without additional immunostaining by flow cytometric methods described previously (14). To detect cell surface-associated murine B7-1-Fc γ ₁ or CD48-Fc γ ₁, cells were immunostained with rat antimouse B7-1 mAb (1G10; BD PharMingen) or rat antimouse CD48 mAb (Serotec) as primary antibody, respectively, and FITC-goat antirat IgG as secondary antibody (BD PharMingen). Of note, rat and goat antibodies were chosen here because neither of them binds to protein A. Because neither rat or goat antibodies are available for the detection of 4-1BBL or CD40L, biotinylated derivatives of their corresponding Fc γ ₁ fusion proteins were used in experiments aimed at monitoring protein transfer efficiencies. Fc γ ₁4-1BBL and Fc γ ₁CD40L were biotinylated with EZ-Link Sulfo-NHS-LC-Biotin, as per the manufacturer's protocol (Pierce, Rockford, IL) and were then detected with FITC-avidin (BD PharMingen).

Protein Transfer *in Vivo*. For experiments monitoring the distribution of pal-prot A injected intratumorally, pal-protein A was biotinylated and injected in a PBS solution containing 80 μ g/ml of the biotinylated protein at 50 μ l/tumor. The tumors were excised 1 or 18 h later, cross-sectioned, stained with avidin-peroxidase and DAB, and counter-stained with H&E.

For experiments monitoring *in vivo* protein transfer efficiency of B7-1-Fc γ ₁: pal-prot A conjugates, each of the two protein elements were combined at 1:1 (w/w) ratio in PBS to a total protein concentration of 160 μ g/ml, without the other components included in the conjugate mixtures described above for the *ex vivo* protein transfer experiments. The conjugates were injected into L5178Y-R tumors grown intradermally at 50 μ l/tumor. Cells were recovered from excised tumors 1 h after injection of the conjugates, treated with ACK

lysing buffer (BioWhittaker, Walkersville, MD) to remove blood cells, stained with rat antimouse B7-1 mAb and FITC-goat antirat IgG, and analyzed by flow cytometry.

Costimulator Protein Transfer Therapy. Pal-prot A conjugates of B7-1-Fc γ ₁, CD48-Fc γ ₁, Fc γ ₁4-1BBL, and Fc γ ₁CD40L were generated separately by combining each of the two components at a 1:1 ratio (w/w) in PBS, to a total protein concentration of 160 μ g/ml. To constitute the tri- and tetra-costimulator solutions, equal volumes of the component Fc γ ₁ fusion protein:pal-prot A conjugates were combined. The pal-prot A control solution contained pal-prot A alone and was prepared in PBS at 80 μ g/ml.

L5178Y-R tumors in syngeneic DBA/2J mice (Jackson Laboratory) were treated as follows. Mice (identified individually by earmarks) were injected intradermally (via a 26-gauge needle) with 5×10^5 L5178Y-R tumor cells on the right flank (day 0). Starting at day 4, when the L5178Y-R tumors generally reached a size of 25-50 mm², the tumors were injected (again via a 26-gauge needle) with one of the conjugate-containing solutions (50 μ l/tumor) once daily for each of 4 consecutive days. Cured mice were rechallenged either intradermally (5×10^5 L5178Y-R tumor cells) on the opposite (left) flank or i.p. (10^4 L5178Y-R tumor cells) at least 5 weeks after the initial tumor inoculation. All of the mice were monitored daily, and tumor sizes were measured three times per week with a caliper. Mice were euthanized when they became moribund or when their tumors exceeded 400 mm² in size.

Cytotoxicity Assay. Bulk splenocytes, prepared 3-6 weeks after tumor rechallenge, were cultured in 24-well plates (10^7 cell/2 ml/well) with mitomycin C-treated L5178Y-R tumor cells (2×10^5 /well) as stimulators in RPMI 1640 containing 10% FCS, 15 mM HEPES, and 50 μ M β -mercaptoethanol. After 5 days, viable cells were harvested and used as effectors in a standard JAM assay (16), with [³H]labeled L5178Y-R and (syngeneic) P815 tumor cells as the specific and nonspecific tumor targets, respectively.

RESULTS

Single-step Protein Transfer with IgG:pal-prot A Conjugates.

Pal-prot A-based Fc γ ₁ fusion protein transfer was performed previously as a two-step procedure, with the pal-prot A being precoated onto cells before the addition of the second, Fc γ ₁-containing fusion protein component (14). To simplify the procedure and adapt it for direct *in vivo* use, we preassembled the pal-prot A and Fc γ ₁-containing components to enable one-step protein transfer. To this end, pal-prot A and FITC-human IgG were pre-conjugated and then combined with each of four different tumor cell lines, two adherent (Ag104A and T50) and two nonadherent (L5178Y-R and P815). This one-step protein transfer approach yielded efficient membrane incorporation in all of the cases (Fig. 1), albeit with differences among the lines that did not correlate with adherent *versus* nonadherent growth properties. As expected, conjugates containing native (nonpalmitated) protein A did not attach to cells (Fig. 1), confirming that the membrane incorporation is lipidation-dependent.

Next, the stability of incorporated conjugates at the cell surface was determined. L5178Y-R and Ag104A cells, coated with FITC-human IgG:pal-prot A conjugates, were subsequently incubated at 37°C for a 6-day period and serially monitored by flow cytometry. Conjugates were remarkably stable at cell surfaces, with only a marginal decrement in MFI over the 6-day period (Fig. 2). Of note, because the FITC-labeled human IgG alone did not bind to these metabolically blocked cells (Figs. 1 and 2), the cell-associated fluorescent signal being detected by flow cytometry is indicative of human IgG that has been tethered to cell membranes via pal-prot A. Taken together, these data established that efficient, reproducible, and stable membrane incorporation is achievable by the modified one-step method.

Costimulator-Fc γ ₁ Protein Transfer *ex Vivo*. Moving beyond IgG:pal-prot A test conjugates, we next applied the one-step method to costimulator protein transfer *per se*. Fc γ ₁ fusion protein derivatives of four costimulators, directed at lymphoid (B7-1, CD48, and 4-1BBL) and APC (CD40L) effectors, were produced (Table 1). The

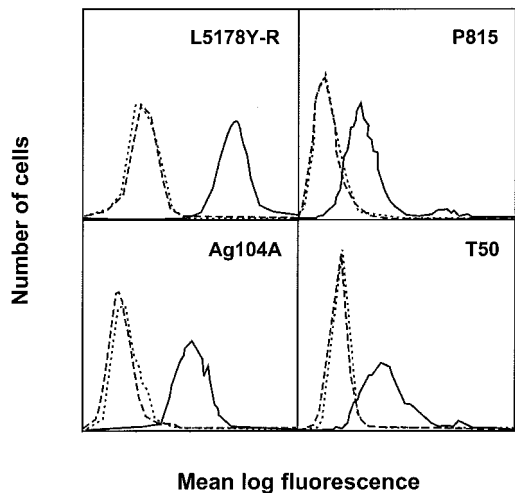


Fig. 1. Protein transfer of preassembled FITC-labeled human IgG:pal-prot A conjugates. Cell lines, as specified, were combined for 1 h at 37°C with either FITC-labeled human IgG:pal-prot A conjugates (—), FITC-labeled IgG conjugated to native (nonlipidated) protein A (---), or FITC-labeled human IgG alone (----). Cell-associated fluorescence was assessed by flow cytometry, as described in “Materials and Methods.” Events were gated on live cells.

Fc γ ₁ component was positioned at the COOH termini of those fusion proteins corresponding to type I membrane proteins (B7-1 and CD48), whereas it was placed at the NH₂ termini of those fusion proteins corresponding to type II membrane proteins (4-1BBL and CD40L). This variable positioning of the Fc γ ₁ component serves to distance it from the functional regions of the appended domains. The chimeric expression cassettes were incorporated into either the pREP7 β EBV episomal expression vector (for B7-1·Fc γ ₁) or the pEE14 amplification/expression vector (for CD48·Fc γ ₁; Fc γ ₁·4-1BBL; and Fc γ ₁·CD40L), which in turn, enabled quantitative expression in 293 and Chinese hamster ovary cell transfectants, respectively. The fusion proteins were affinity-purified to near homogeneity by protein A chromatography, as visualized by SDS-PAGE under reducing conditions (Fig. 3A). Of note, the native molecular masses for B7-1 (17), CD48 (18), CD40L (19), 4-1BBL (20), and human IgG1 Fc (21) have all been reported previously to be larger than expected because of extensive glycosylation. The SDS-PAGE masses of the four Fc γ ₁ fusion proteins generated here (Fig. 3A) correspond well to what would be predicted based on the reported native masses of their component parts.

To document that the costimulator·Fc γ ₁ proteins can be transferred to cells by the one-step method, pal-prot A conjugates were formed individually with each fusion protein and subsequently transferred to L5178Y-R tumor cells, either individually (not shown) or simultaneously (Fig. 3B). In both instances, efficient membrane incorporation of proteins was detected by flow cytometry. Significantly, the tetra-costimulator combination yielded a uniform population of cells that are all coexpressing each of the four costimulators (Fig. 3B), thus establishing the feasibility of combinatorial protein transfer.

The functionality of these four costimulator Fc γ ₁ fusion proteins was formally demonstrated in appropriate bioassays. To test the activity of B7-1·Fc γ ₁, CD48·Fc γ ₁, and Fc γ ₁·4-1BBL, the costimulator proteins were “painted” on mitomycin C-inactivated L5178Y-R cells, which in turn costimulated proliferation of purified syngeneic splenic T cells (not shown). In the case of Fc γ ₁·CD40L, the soluble protein stimulated the proliferation of purified human B cells in the presence of human IL-4; this stimulation could be blocked with TRAP1, an anti-CD40L mAb (not shown).

Intratumoral Costimulator·Fc γ ₁ Protein Transfer. As a first step toward applying the protein transfer method to tumor therapy, we determined the feasibility of intratumoral costimulator·Fc γ ₁ protein transfer. The murine T lymphoma line L5178Y-R was chosen for these studies, because it readily forms tumors when injected either intradermally or i.p., is highly metastatic (generally killing mice in <2 weeks with liver metastases), and expresses levels of MHC class I (H-2K^d) that are comparable with those on splenocytes from syngeneic DBA/2J mice (data not shown). The L5178Y-R cells do not express native B7-1, B7-2, or CD40L, and express only low levels of CD48 (data not shown).

On intradermal seeding, L5178Y-R cells form well-circumscribed tumors that are amenable to intratumoral injection. Biotinylated pal-prot A and a nonlipidated control, biotinylated protein A, were injected separately into intradermally seeded L5178Y-R tumors, and the injected tumors were excised 1 h later, sectioned transversely across the needle track, stained with H&E, and counter-stained with avidin-peroxidase with DAB as chromogen to detect immobilized biotin (Fig. 4A). H&E sections showed lymphoid neoplasm infiltrating the dermis and subcutis. A band-like necrotic zone was present at the base of tumors right above the deep fascial plane in both injected and noninjected tumors. The needle track was identified in injected tumors. Necrosis around the needle tract was noted, although there was no significant difference in necrosis around the needle track between tumors injected with biotin-pal-prot A or biotin-protein A. The avidin-biotin complex product was visualized as brown staining under the light microscope. In noninjected tumors as well as tumors injected with the biotin-protein A, only RBCs showed light brownish color because of nonspecific reactions with DAB (Fig. 4, A–F).

In marked contrast to these controls, tumors injected with biotin-pal-prot A additionally displayed intense staining of tumor cells, extending to a 400 × 600 μ m² section across the needle track (Fig. 4, G–I). Significant levels of tumor cell staining were still observed even 18 h after injection with biotin-pal-prot A (not shown). These histo-

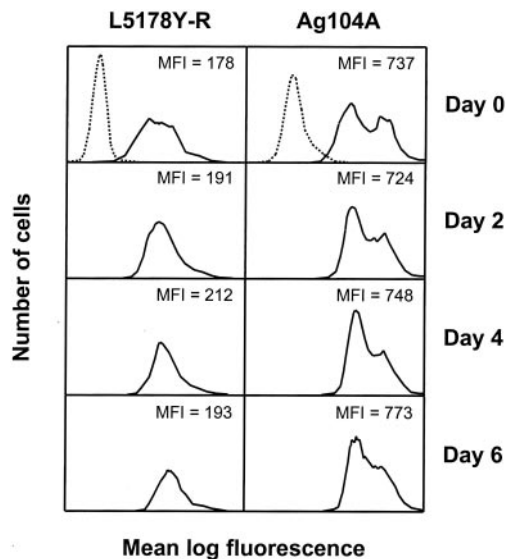


Fig. 2. Stability of FITC-labeled human IgG:pal-prot A conjugates at cell surfaces. L5178Y-R (left panel) and Ag104A (right panel) cells were metabolically inhibited with 0.1% sodium azide and 10 mM 2-deoxyglucose (to prevent endocytosis), and coated with FITC-labeled human IgG:pal-prot A conjugates for 1 h at 37°C, as detailed in “Materials and Methods.” After washing to remove free conjugates, the coated cells were incubated for 6 days at 37°C in medium (DMEM supplemented with 10% FCS). Cell aliquots sampled at 2-day intervals were preserved at 4°C and then were all analyzed together (—) by flow cytometry at the end of the 6-day period. Cells treated with FITC-labeled human IgG alone served as the negative control (----). Events were gated on intact cells.

Table 1 *Fc γ_1 fusion protein derivatives of immune costimulators*

Name	Structure	Costimulator domain	Molecular weight	
			Predicted	SDS-PAGE (M_r) ^a
B7-1·Fc γ_1	○● ^b	mouse B7-1 (V38-T247)	49962	85
CD48·Fc γ_1	○●	mouse CD48 (F23-R216)	48236	76
Fc γ_1 ·4-1BBL	●○	mouse 4-1BBL (R104-E309)	48574	76
Fc γ_1 ·CD40L	●○	human CD40L (H47-L261)	49753	56

^a The apparent size determined by SDS-PAGE is expected to be larger than the predicted size because of glycosylation.

^b ○, costimulator; ●, human Fc γ_1 .

chemical data indicate that intratumorally injected pal-prot A coats tumor cells *in situ*, penetrates to a significant depth beyond the needle track, and persists on tumor cells many hours after injection.

Next, B7-1·Fc γ_1 was combined with pal-prot A, and the resulting conjugates were injected into intradermal L5178Y-R tumors. After injection (1 h), cells were recovered from an excised (~1 mm) strip of tumor tissue encompassing the needle track, and these suspended cells were analyzed for B7-1 epitope expression by immunofluorescence and flow cytometry. As shown in Fig. 4B, 33% of the cells from B7-1·Fc γ_1 :pal-prot A-injected but not noninjected or negative control B7-1·Fc γ_1 :prot A-injected tumors were B7-1⁺ cells. In addition to validating the intratumoral protein transfer strategy, these immunofluorescence data confirmed that the transferred proteins are accessible for molecular recognition (in this case, via mAbs).

Induction of Antitumor Immunity by Intratumoral Costimulator Transfer. With the intratumoral, “multicostimulator” protein transfer strategy well in hand, we next investigated its antitumor therapeutic utility. To start, we established that pal-prot A, when injected into the tumor bed on its own (4 μ g within a 50- μ l injection volume in intradermal L5178Y-R tumors), did not influence tumor growth or animal survival (data not shown). With this as baseline data, we moved on to assess the therapeutic efficacy of various combinations of costimulator·Fc γ_1 :pal-prot A conjugates, with up to four costimulators in the mix. Of note, the previous experience with costimulator combinations is limited (with only one report of more than two costimulators; Ref. 10), and it is largely confined to *ex vivo* manipulation of cancer vaccine cells by gene transfer. Here we incorporated up to four costimulators, leveraging protein transfer and intratumoral delivery.

In a first set of tumor cure experiments performed in the L5178Y-R T lymphoma model, we evaluated the antitumor effects of three different costimulator combinations: a tetra-costimulator combination encompassing activators of T cells, NK cells, and dendritic cells (B7-1·Fc γ_1 , CD48·Fc γ_1 , Fc γ_1 ·4-BBL, and Fc γ_1 ·CD40L); a tri-costimulator combination directed at T cells and NK cells only (B7-1·Fc γ_1 , CD48·Fc γ_1 , and Fc γ_1 ·4-BBL); and the dendritic cell activator Fc γ_1 ·CD40L alone. Of note, the human form of CD40L is active in mice (22). Each of these costimulator permutations (injected as mixtures of pal-prot A conjugates) yielded beneficial effects with respect to tumor growth (Fig. 5A) and animal survival (Fig. 5B), but their relative efficacies varied. In a typical experiment, 6 of 12 mice injected with the tetra-costimulator combination showed complete tumor regression as compared with 4 of 12 mice for each of the other two groups (Fig. 5A). Animal survival data mirrored these differences. Hence, 6 of 12 tetra-costimulator-injected animals were alive at day 35 in contrast with 4 of 12 for each of the other two groups (Fig. 5B). In contrast with the treated animals, control animals injected with pal-prot A alone all died with metastatic tumor by day 33.

Three additional tumor cure experiments were performed focusing on tetra-costimulator, intratumoral protein transfer. The aggregated survival data, as summarized in Table 2, showed that 45% (18 of 40)

of tetra-costimulator-treated animals were cured as opposed to 6% (2 of 34) of control animals (either untreated or treated with pal-prot A alone).

Systemic Antitumor Immunity Induced by Intratumorally Injected Costimulator Conjugates. Having documented local tumor regression in a substantial percentage of tetra-costimulator-injected tumors, we next looked for evidence of systemic antitumor immunity. To this end, cured mice were rechallenged with L5178Y-R tumor cells ranging from 5 to 8 weeks after the first inoculation of the tumor cells. For this rechallenge, tumor cells were administered at sites distant from the original tumor, either intradermal or i.p. As summarized in Table 3, all (11 of 11) of the animals resisted rechallenge with the tumor cells. It is especially notable that 6 of 8 animals subjected to i.p. tumor rechallenge 6–9 months after the initial tumor cell inoculation were resistant to tumor. These rechallenge experiments point to a persistent, systemic antitumor immunity that is evoked by intratumoral costimulator inoculation.

To additionally support the existence of systemic antitumor immunity, we recovered splenocytes from tetra-costimulator-treated mice 3 weeks after tumor rechallenge and looked for recall responses against the L5178Y-R tumor cells after 5 days of *in vitro* restimulation with tumor cells (Fig. 6). The bulk splenocytes from cured, tetra-costimulator-treated mice showed strong CTL activity and efficiently lysed the tumor cells in a standard JAM assay. The CTL activity was L5178Y-R-specific, with significantly less lysis of another syngeneic tumor line, P815. Furthermore, naïve mice failed to develop any

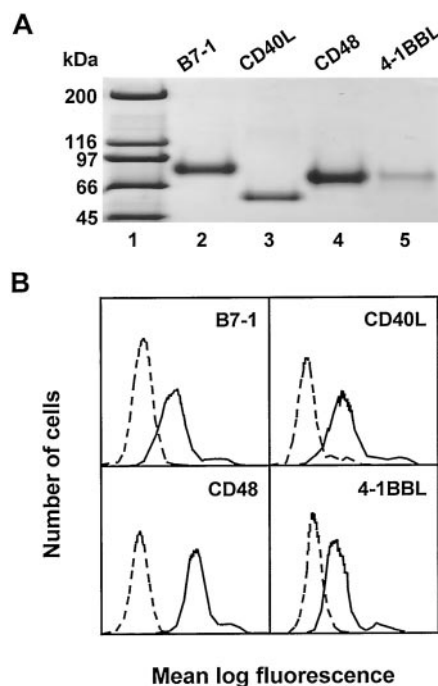


Fig. 3. Combinatorial protein transfer with a mixture of costimulator·Fc γ_1 :pal-prot A conjugates. A, Fc γ_1 derivatives of the four costimulators shown were size-fractionated on a precast 3–8% NuPAGE gel after purification by protein A-agarose affinity chromatography. B, L5178Y-R tumor cells were combined with a mixture of the Fc γ_1 derivatives of B7-1, CD48, CD40L, and 4-1BBL (biotinylated), each preincubated with pal-prot A (mix A). Same cells were alternatively combined with a similar mixture (mix B) where Fc γ_1 ·CD40L was biotinylated instead. After incubation for 1 h at 37°C, cells were divided and stained separately for B7-1 or CD48 epitopes with rat antimouse B7-1 mAb or rat antimouse CD48 mAb, and FITC-labeled goat antirat immunoglobulin. FITC-labeled avidin was used to stain for biotinylated 4-1BBL or biotinylated CD40L in cells combined with mix A or mix B, respectively. The stained cells were analyzed by flow cytometry for the presence of each of the four costimulators, designated in the upper right quadrant of each box (—), with staining-matched, nontransferred L5178Y-R cells as control (---). Events were gated on live cells.

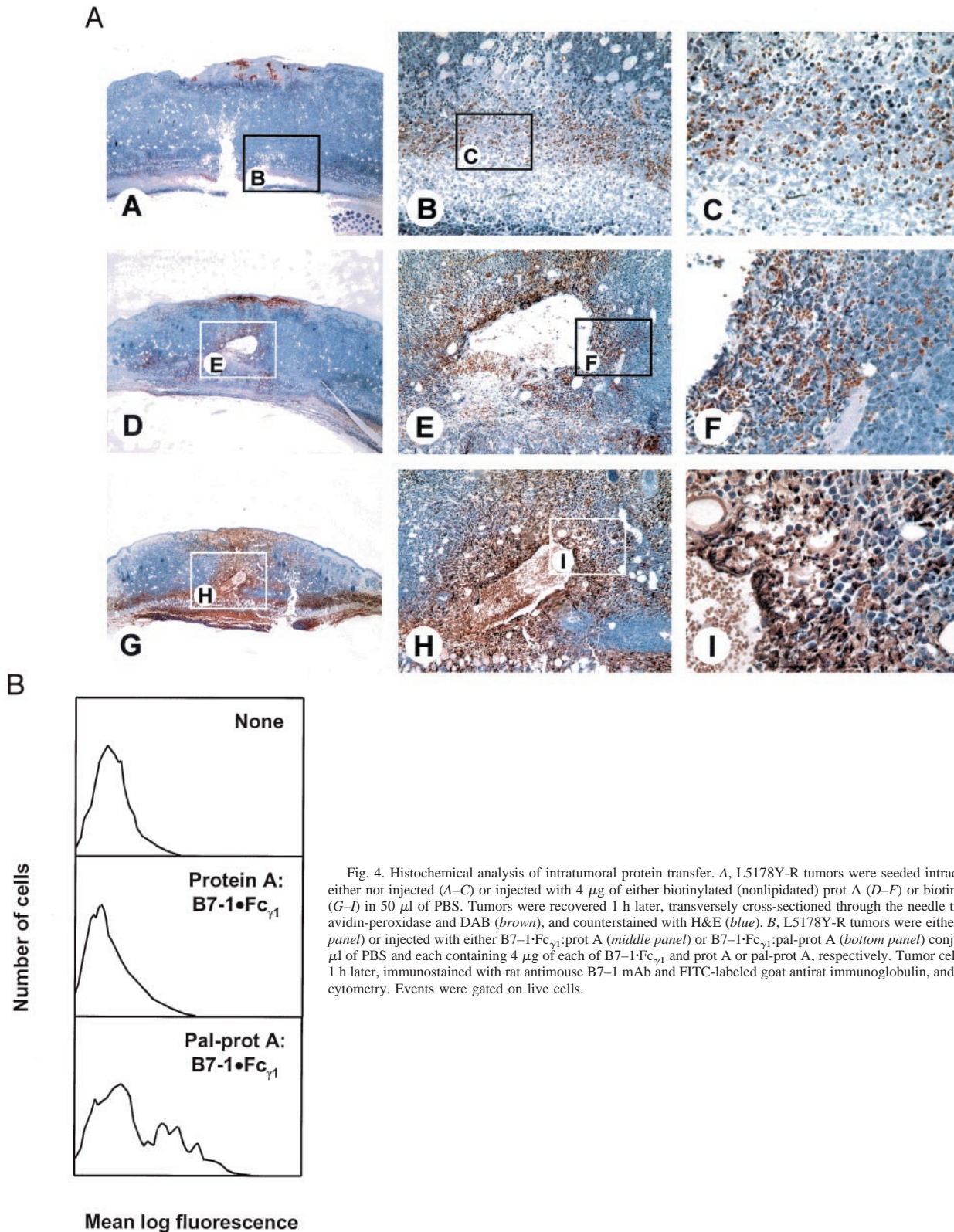


Fig. 4. Histochemical analysis of intratumoral protein transfer. *A*, L5178Y-R tumors were seeded intradermally and then either not injected (*A–C*) or injected with 4 μ g of either biotinylated (nonlipidated) prot A (*D–F*) or biotinylated pal-prot A (*G–I*) in 50 μ l of PBS. Tumors were recovered 1 h later, transversely cross-sectioned through the needle track, stained with avidin-peroxidase and DAB (brown), and counterstained with H&E (blue). *B*, L5178Y-R tumors were either not injected (*top panel*) or injected with either B7-1•Fc γ 1:prot A (*middle panel*) or B7-1•Fc γ 1:pal-prot A (*bottom panel*) conjugates, each in 50 μ l of PBS and each containing 4 μ g of each of B7-1•Fc γ 1 and prot A or pal-prot A, respectively. Tumor cells were recovered 1 h later, immunostained with rat antimouse B7-1 mAb and FITC-labeled goat antirat immunoglobulin, and analyzed by flow cytometry. Events were gated on live cells.

cytolytic activity against the L5178Y-R tumor cells during the *in vitro* restimulation phase, indicating that this response is dependent on an immunizing effect resulting from previous treatment of the tumor. Thus, tumor-specific CTL can be recovered from a secondary lymphoid compartment that is by definition distal from the costimulator-coated tumor cells at the injection site.

DISCUSSION

The present study documents the utility of intratumoral, tetra-costimulator protein transfer for cancer vaccination. This constitutes the first example of intratumoral protein transfer, which is accomplished here via the intratumoral inoculation of preformed molecular

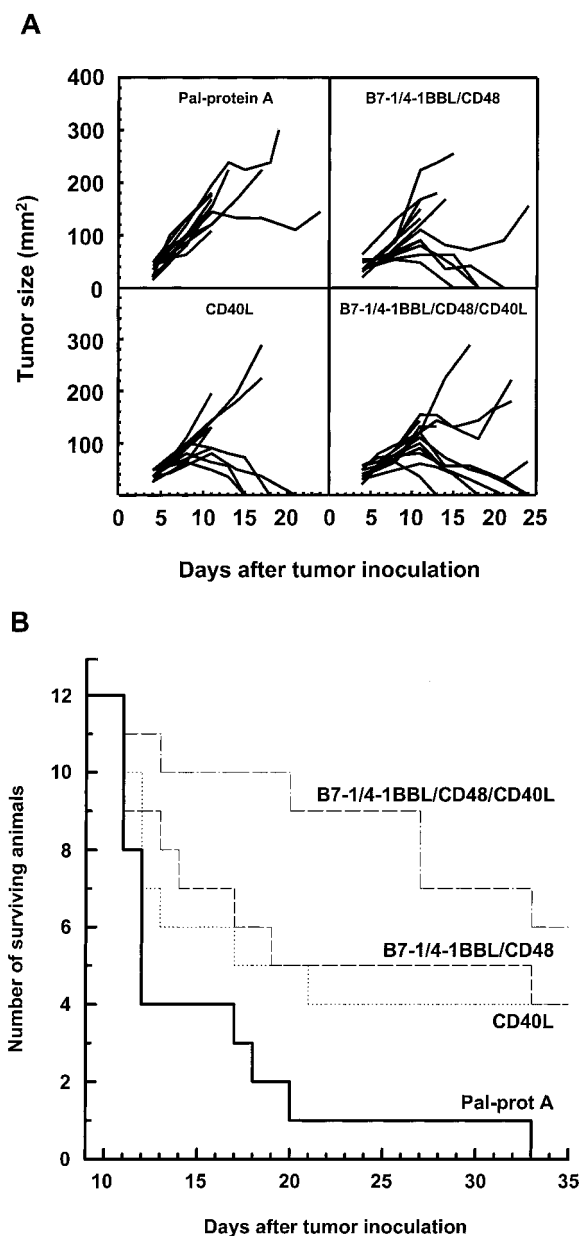


Fig. 5. Antitumor effects of intratumorally inoculated costimulator-Fc γ_1 :pal-prot A conjugates. Mice were seeded with intradermal L5178Y-R tumors and were injected with one of the costimulator-Fc γ_1 :pal-prot A conjugate combinations shown or pal-prot A alone (control) at 8 μ g of total conjugates/tumor, once daily for each of 4 consecutive days. *A*, tumor size is graphed for individual, ear-marked animals as a function of days after tumor inoculation. Lines ending at intermediate time points correspond to animals that died of tumor during the monitoring period. *B*, survival curve is for the same tumor-bearing mice out to 35 days of observation. The animals were injected with either pal-prot A alone (—), Fc γ_1 ·CD40L:pal-prot A (---), a tri-costimulator combination of B7-1·Fc γ_1 :pal-prot A/Fc γ_1 ·4-1BBL γ_1 :pal-prot A/CD48·Fc γ_1 :pal-prot A (----), or the tetra-costimulator combination of B7-1·Fc γ_1 :pal-prot A/Fc γ_1 ·4-1BBL γ_1 :pal-prot A/CD48·Fc γ_1 :pal-prot A/Fc γ_1 ·CD40L:pal-prot A (----). The results were compiled from two independent experiments, each with six earmarked individuals per group.

conjugates consisting of Fc γ_1 -chimerized costimulators linked to membrane-anchoring, pal-prot A. Remarkably, murine L5178Y-R T lymphomas painted with immunostimulatory proteins *in situ* by this method elicit not only local tumor regression but also systemic tumor-specific immunity.

One distinguishing feature of this study relates to the number of costimulators being used. The preponderance of preclinical studies, as well as clinical trials to date, have dealt with enforced expression of individual costimulators. The more limited multicostimulator litera-

ture has been mostly confined to no more than two costimulators at a time (7–9), with one group recently invoking up to three (10). Here we have used four costimulators, leveraging the unique advantages of protein transfer for achieving combinatorial surface protein expression. Even more significantly, costimulators were chosen here not simply for their synergistic action in activating singular effectors but additionally for their collective capacity to invoke multiple antitumor immune effectors. The capacity to deliver complex arrays of immunostimulatory proteins to tumors via protein transfer opens the door to more elaborate combinatorial surface protein transfer schema in the pursuit of ever more effective cancer vaccination strategies.

Whereas costimulator-expressing tumor vaccine cells are intended to function as nonprofessional “tumor APC” (1, 23), some have argued the efficacy of such vaccine cells can be attributed, at least in part, to their triggering of cross-priming of tumor antigens by bystander professional APC (24). The immune-activating proteins in this study were chosen with both of these activation pathways in mind. Hence, whereas the triad of B7-1, 4-1BBL, and CD48 amplify the ability of tumor cells to directly prime lymphoid cells, CD40L was added to the mix to promote the activation of, and cross-priming by, professional APC, particularly dendritic cells (25–30). Significantly, whereas the lymphoid-directed costimulator triad and CD40L each showed therapeutic efficacy on its own, the combination yielded optimal antitumor effects. This finding implies that both direct priming and cross-priming mechanisms may be operative in this context.

Within the costimulator triad, the choice of costimulators was prompted by their reported synergies and subset selectivity. Pairwise combinations of B7-1/4-1BBL and B7-1/CD48 synergistically activate T cells (7, 9, 31). Moreover, these three costimulators may preferentially influence different lymphoid subsets. Thus, B7-1 and 4-1BBL are preferentially linked to the CD4⁺ and CD8⁺ T-cell subsets, respectively (32, 33). Furthermore, murine CD48 additionally activates NK cells (34, 35). Given the higher order complexity of tetra-costimulator combinations, sorting out the precise contributions of individual costimulators within the mix to the activation of various antitumor effectors will require elaborate additional experimentation.

The preponderance of cellular cancer vaccine literature deals with tumor or dendritic cells that have been engineered *ex vivo*, often by approaches that that would be hard to implement clinically. Intratu-

Table 2 Tetra-costimulator treatment of L5178Y-R tumors

Experiment	Fraction of mice cured per treatment group		
	Control		
	None	Pal-prot A	Tetra-costimulator
1		0/12	6/12
2		1/8	4/8
3	0/8		3/8
4		1/6	5/12
Total		2/34	18/40
Percentage		6	45

Table 3 Tumor rechallenge via various routes

Experiment	Route	Fraction of mice per group resisting tumor rechallenge		
		Naive	Cured	
			Short-term ^a	Long-term ^b
1	ID	0/3	6/6	
2	IP	0/4		2/3
3	IP	0/4	5/5	4/5
Total		0/11	11/11	6/8
Percentage		0	100	75

^a 5–8 weeks after initial tumor inoculation.

^b 6–9 months after initial tumor inoculation.

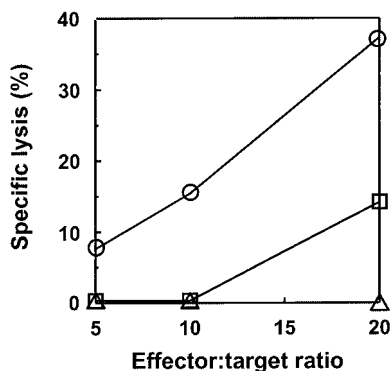


Fig. 6. Specific cytotoxicity against L5178Y-R tumor cells in mice treated with a tetra-costimulator mix. Bulk splenocytes were obtained from mice cured of their intradermal L5178Y-R tumors via intratumoral inoculation of a tetra-costimulator mix (B7-1-Fc γ_1 :pal-prot A/Fc γ_1 · 4-1BBL:pal-prot A/CD48-Fc γ_1 :pal-prot A/Fc γ_1 · CD40L γ_1 :pal-prot A). Tumor-specific cytolytic activity was assessed via the JAM assay with L5178Y-R (○) and P815 tumor cells (□) as specific and nonspecific tumor target cells, respectively. Splenocytes from naive mice (△) were used as control effector, with L5178Y-R as target.

moral delivery methods offer clinical advantages, at least for those tumors that can be readily accessed, because these methods bypass cumbersome *ex vivo* cellular manipulation. Whereas intratumoral transfer of cytokine genes has been evaluated by others (36), the intratumoral delivery of costimulator proteins has not. The observed efficacy of this latter therapeutic approach is encouraging and now provides motivation for undertaking additional studies directed at mechanistic questions, including the identification of the site(s) where the triggering of lymphoid and APC effectors occurs.

Early on, our group advocated protein transfer as a tool for engineering both tumor cell and dendritic cell vaccines (1). Subsequent studies by us (11, 12, 37, 38) and others (13) validating this concept centered around the use of GPI-modified costimulator and MHC derivatives as “protein paints.” However, GPI proteins have some limitations. Because the GPI moiety is appended to the COOH termini of proteins, this post-translational modification cannot be conferred to type II membrane proteins, a class of proteins that includes important costimulators of the tumor necrosis factor family such as 4-1BBL and CD40L. This constraint, along with the poor yields obtained when scaling-up the production of certain GPI derivatives, prompted our recent development of a two-component protein transfer method that combines pal-prot A and Fc γ_1 -derivatized proteins (14). This approach not only enables the transfer of type II membrane proteins, as demonstrated here, but it invokes components that can each be produced in quantity as recombinant soluble proteins. Of note, whereas the original report (14) applied the two components to cells in two distinct steps, the present study simplifies their use by preparing them as molecular conjugates, which can be applied in a single step. Moreover, when it comes to combinatorial protein transfer, there is no need to produce each protein as a lipidated derivative, which is the case with GPI proteins.

The use of a membrane-anchoring “bridge” protein (in this case, protein A) that can be palmitated and used subsequently with multiple costimulator-Fc γ_1 fusion protein partners, is preferable to the use of costimulator-Fc γ_1 fusion proteins that have been directly palmitated. There are several reasons. First, excess palmitation can interfere with costimulator protein activity. Consequently, the palmitation reaction would have to be titrated for each new costimulator protein (because of variability in the number and distribution of target lysine residues within these proteins) and even for different preparations of the same protein. In contrast, protein A can be palmitated in bulk reactions, and each large preparation can be evaluated afterward for membrane-binding and Fc-binding activities. Second, because directly palmitated

costimulator proteins would have palmitate residues attached randomly, the membrane topology of these proteins would vary considerably once anchored to membranes. Third, because protein A has five potential Fc-binding sites per molecule, it is likely that more than one costimulator-Fc γ_1 fusion protein is engaged by each membrane-anchored, pal-prot A molecule. Hence, it is possible that our protein A-based method results in costimulator aggregates, thereby increasing the “functional valency” of bound costimulators. This may be especially significant here, because our Fc γ_1 fusion protein derivatives of B7-1 and 4-1BBL lack their native homodimerization and trimerization motifs, respectively, and were detected as monomers by nonreducing SDS-PAGE.⁴ In contrast, directly palmitated costimulator-Fc γ_1 fusion proteins would not have the added valency conferred by protein A.

The set of protein transfer tools continues to expand. Another two-component protein transfer method has been reported, which uses nitrilotriacetic acid di-tetradecylamine and polyhistidine-tagged costimulators in combination (39). Nonlipidation-based methods, such as the use of a transmembrane protein sequence to directly insert transmembrane-derivatized fusion proteins to tumor cells, have also been explored (40). Together, these various protein transfer methods, along with the expanding array of known costimulators, now offer a growing set of options for tumor cell engineering, both *ex vivo* and *in vivo*.

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⁴ Unpublished observations.

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