Educational Resources

Spectral Flow Cytometry Webinar Series

Watch our webinar series and learn how the ID7000™ system builds on Sony's experience with spectral analysis and simplifies many operations to advance the field of flow cytometry.

Watch Now

SONY

Models for Lymphoma

Most human B cell lymphomas express a unique cell surface immunoglobulin (Ig) molecule. The idiotype (Id) of this Ig is considered a unique tumor marker and can be used as an immunogen to induce anti-tumor immunity (Lynch et al., 1972). However, Id is usually a poor immunogen by itself, and immunologic adjuvants or genetic or chemical conjugation to immunogenic carriers, such as keyhole limpet hemocyanin (KLH), are required to render it immunogenic. Most murine B cell tumors are weakly immunogenic and lethal in syngeneic mice (Table 20.6.1). These tumor models have been shown to closely mimic different types of human B cell malignancies—from mature, nonsecreting B cell lymphomas to Ig-secreting myelomas. These include BCL1 and A31 B cell splenic lymphomas, both of which express surface IgM, 5T33M myeloma, which predominantly secretes IgG2b, and MOPC315 plasmacytoma, which secretes IgA.

The authors' experience is mostly with the A20 and 38C-13 lymphomas. A20 is a BALB/c B cell lymphoma line derived from spontaneous reticulum cell neoplasm. These cells are IgG^+, Ig^+ (with polyvalent anti-Ig), Ia^+, Fc^+, IgM^-, IgA^-, and complement-receptor negative. A20 cells are tumorgenic in mice and have a generation time of 18 hr. When grown in Click's medium, these cells originally expressed very little surface IgG. However, when A20 cells are grown in RPMI 1640, they express a high level of surface Ig. T cell factors and mitogens can induce these tumor cells to secrete IgG extracellularly (Kim et al, 1979). Their pattern of spread in vivo mimics those of human lymphomas (Levitsky et al., 1996).

38C-13 is a carcinogen-induced lymphoid tumor originally isolated from a T cell–depleted mouse of the C3H/eB strain. 38C-13 cells have features of the transformed counterpart of small B lymphocytes and grow well in culture using RPMI with 10% FBS and 0.05 mM 2-mercaptoethanol. 38C-13 cells express surface IgM(κ) (little-to-no secreted IgM), are $Thy\text{-}1^-$, Ia^-, Fc^+, IgA^-, and are complement-receptor negative (Bergman and Haimovich, 1977).

A number of different approaches have been utilized as therapy, such as passive serotherapy with monoclonal anti-idiotype antibodies (Weiner et al., 1990), active immunization with Ig-KLH protein (Dyke et al., 1991; Campbell et al., 1990), and protein or genetic immunizations with lymphoma idiotype or Fv fragments (See review by Biragyn and Kwak, 1999; Hakim et al., 1996; Biragyn et al., 1999). In addition, successful therapy can be elicited by treatment with anti-CD40 MAb (French et al., 1999) or by immunization with modified cellular vaccines expressing various cytokines (Levitsky et al., 1996). Herein, we describe experimental procedures for development of therapeutic vaccines, particularly "second-generation" recombinant vaccines. A brief outline of the topics covered is shown in Figure 20.6.1. Specifically, a general procedure for handling and culturing lymphoma cell lines in vitro and their subsequent challenge into syngeneic mice is described (see Basic Protocol 1). Basic Protocols 2, 3, and 4 describe the production of various Id-based or cellular lymphoma vaccine formulations. In particular, the novel approach of rendering nonimmunogenic lymphoma–derived scFv or Id immunogenic by fusing it with a chemokine moiety is described (see Basic Protocol 2). As an alternative, a protocol for expression and purification of these chemokine-fusion proteins from *E. coli* is included (see Basic Protocol 3). Finally, although procedures for using cellular tumor vaccines, modified to express various cytokines, are described in other units (e.g., *UNIT 20.1*), a similar approach can be used for these B cell lymphoma models. Thus, a general procedure is included for cloning of cytokine genes, for example, murine GM-CSF, in A20 lymphoma cells (see Basic Protocol 4). The isolation of intact Ig protein from B cell

Contributed by Arya Biragyn and Larry W. Kwak
Current Protocols in Immunology (2001) 20.6.1-20.6.30

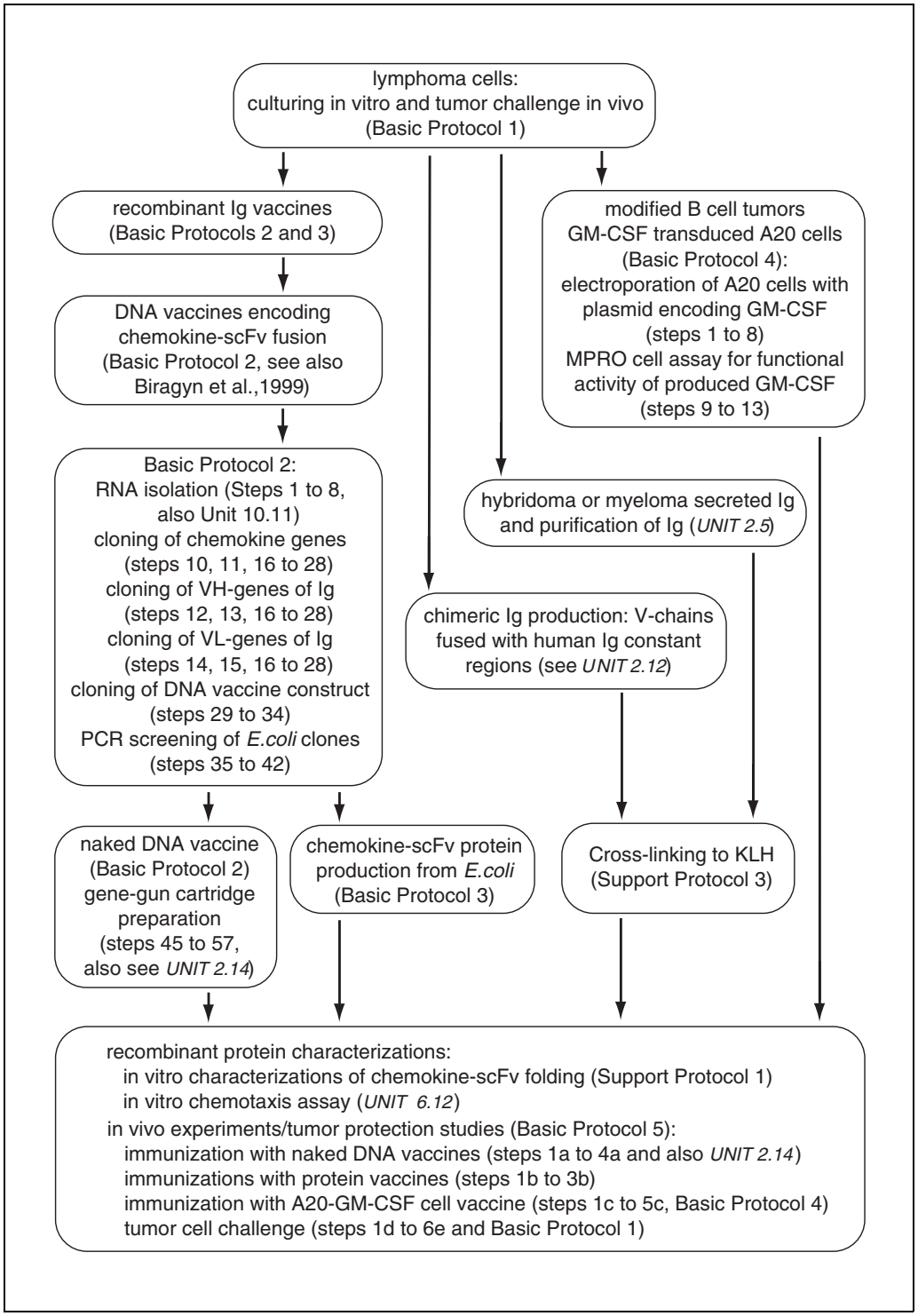

Figure 20.6.1 Flow chart outlining strategies used for vaccine production for murine B cell lymphomas

tumors is not described, since it is similar to Ig production from hybridomas, extensively covered in other units (*UNIT 2.5*) and previous publications (see Literature Cited). However, short protocols are presented for chemically conjugating intact Ig protein with KLH to produce a prototype protein vaccine (Support Protocol 3) and using the Helios Gene Gun System to immunize mice with recombinant DNA tumor vaccines (Support Protocol 4).

Table 20.6.1 Commonly Used Murine B Cell Lymphomas[a]

Tumor cell Line	Mouse strain	Id expression	Anti-Id MAbs available	Vaccine carriers used	Major references
38C-13 B cell lymphoma (Bergman and Haimovich, 1997)	C3H/HeN	IgM,*k* surface expressed and secreted	SIC5 (Maloney, et al., 1985) and MS11G6 (Weiner and Kaminski, 1990)	*Chemokine:* IP-10, MCP-3 *Cytokine:* GM-CSF, IL-2, IL-4 *Peptide:* Human IL-1β, human or Mouse Ig Fc *Viral epitope:* PreS2, DomA of HBsAg	Biragyn et al., 1999; Syrengelas et al., 1996; Chen et al., 1994; Hakim et al., 1996
A20 and A20.2J B cell lymphoma (Kim et al., 1979)	BALB/c	IgG2a,*k* surface expressed and secreted	See Bartnes et al., 1997; Sato et al., 1998	*Chemokine:* IP-10, MCP-3 *Viral epitope:* PoreS2, DomA of HBsAg	Biragyn et al., 1999
A31 B-Cell Splenic Lymphoma (Dyke et al., 1991)	(CBA/H × C57B1/6) F1	IgM,*k* surface expressed and secreted	See Tutt et al., 1998	Fragment C of tetanus toxin	King et al., 1998
BCL1 B-Cell Lymphoma (Krolick et al., 1979)	BALB/c	IgM, lambda surface expressed and secreted	See Krolick et al, 1979; Manetti et al., 1997	—	Hawkins et al., 1993

[a]The tumor cell lines, syngeneic mouse strains, and availability of anti-Id monoclonal antibody reagents are listed. References for chemokine, cytokine, and peptide carriers used to render scFv immunogenic are shown in the last column (Adapted from Biragyn and Kwak, 1999).

MOUSE MODELS OF INTRAPERITONEAL OR SUBCUTANEOUS LYMPHOMAS

BASIC PROTOCOL 1

38C-13 is a rapidly growing lymphoma cell line in vivo. Syngeneic (C3H) mice injected intraperitoneally (i.p.) or subcutaneously (s.c.) with 10^3 to 10^4 tumor cells have a median survival of about 14 and 21 days, respectively. On the other hand, A20 lymphoma is slower-growing in vivo, with a median survival of ~40 days for syngeneic (BALB/c) mice after i.p. challenge with 2×10^5 cells (minimal lethal dose). For each tumor model, the authors recommend freezing down standard stocks of cells and removing aliquots as needed. All in vivo experiments should be repeated with 10 mice per experimental group.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% CO 2 incubator unless otherwise specified.

Materials

A20 B cell lymphoma (ATCC #TIB-208; store cell stocks in 1- to 2-ml aliquots in liquid N_2)

38C-13 B cell lymphoma (Bergman and Haimovich, 1977; available from authors; store 1–5 × 10^6 cell/ml stock in 1- to 2-ml aliquots in liquid N_2)

RPMI 1640 medium (e.g., Life Technologies)

Complete RPMI medium (*APPENDIX 2A*) with and without 10% heat-inactivated FBS

Dulbecco's phosphate-buffered saline (DPBS, without Ca^{2+} and Mg^{2+}; Life Technologies)
6- to 12-week-old female C3H/HeN and BALB/c mice

50-ml conical centrifuge tubes
Centrifuge with Sorvall H-1000B swinging-bucket rotor, or equivalent
1-ml disposable syringes and 27.5-G needles

Additional reagents and equipment for counting cells in a hematocytometer and determining cell viability by trypan blue exclusion (*APPENDIX 3B*), ear tagging (*UNIT 1.5*), and subcutaneous and intraperitoneal injection of mice (*UNIT 1.6*)

Prepare 38C-13 and A20 cells

1. Thaw frozen cells rapidly at 37°C and dilute contents of each aliquot into 10 ml of plain RPMI medium (without FBS antibiotics or L-glutamine) in a 50-ml conical centrifuge tube.

2. Centrifuge 5 min at 500 × *g* (1500 rpm in Sorvall H-1000B rotor), room temperature. Discard supernatant.

3. Resuspend 1–6 × 10^6 cells gently in 10 ml complete RPMI medium (with 50 µM 2-ME and 10% FBS) and transfer to 25-cm^2 culture flask. Return cells to incubator and allow to grow for 2 days.

4. Split cells 1:10 in fresh complete RPMI with 2-ME and 10% FBS and continue incubation overnight.

   Upon completion of this incubation the cells should be in logarithmic growth phase.

5. Transfer cells to a 50-ml centrifuge tube and centrifuge 5 min at 500 × *g* (1500 rpm in Sorvall H-1000B rotor), 4°C. Rinse the cell pellet twice, each time by resuspending in 50 ml DPBS and centrifuging again at 500 × *g*. Discard supernatant.

6. Resuspend cell pellet in 10 ml of 1× DPBS and count cells using a hematocytometer and trypan blue exclusion (*APPENDIX 3B*).

   Viability of cells should be greater than 95% as judged by trypan blue exclusion.

Challenge mice with tumor cells

7. Adjust cell concentration in 1× DPBS to 4 × 10^3 cells/ml (for i.p. injection of 38C-13 tumor), 5 × 10^4 cells/ml (for s.c. injection of 38C-13), or 1 × 10^6 cells/ml (A20 tumor); keep cells on ice.

8. Identify mice in all experimental and control groups by ear tagging (*UNIT 1.5*). Inject 0.5 ml (2 × 10^3 cells/mouse) i.p. or 0.2 ml (10^4 cells/mouse) s.c. of 38C-13, or 0.2 ml (2 × 10^5 cells/mouse) i.p. of A20 tumor cells, using 1-ml disposable syringes with 27.5-G needles.

   Techniques for intraperitoneal (i.p.) and subcutaneous (s.c.) injection are described in UNIT 1.6.

9. Begin checking mice for tumor growth and survival 10 to 12 days after challenge with 38C-13 cells and 25 days after challenge with A20 cells.

   38C-13 tumor cells usually grow rapidly as ascites, and mice may die within 2 to 3 days after the first signs of tumor growth. Both 38C-13 and A20 grown s.c. often develop large tumor nodules, which may become necrotic and begin to ulcerate or bleed; it is advisable to sacrifice the mice before this point.

CLONING OF LYMPHOMA-DERIVED Ig VARIABLE REGION GENES (V_H AND V_L) AND CONSTRUCTION OF SINGLE-CHAIN Fv FUSIONS FOR USE AS NAKED DNA VACCINES

Lymphoma is clonally restricted to express a unique Ig, and its idiotype is considered a tumor marker and potential immunogen to elicit anti-idiotypic anti-tumor responses. Heavy and light chain variable region genes of Ig can be expressed as a genetically linked protein, designated single chain Fv (scFv), which usually retains the conformation of the parental idiotype. However, syngeneic mice immunized with lymphoma-derived Ig or scFv alone from 38C-13 and A20 do not elicit anti-Id responses. Rather, chemical or genetic fusion of Ig or scFv with carriers or cytokines is required for immunogenicity (Campbell et al., 1990). Previously, the authors of this unit reported that scFv can also be rendered immunogenic by targeting them to APC and DC using chemokine carriers (Biragyn et al., 1999). Herein, we describe a novel and simple approach which produces immunogenic lymphoma-derived scFv and can be used for both protein- and DNA-based vaccinations.

Specifically, a complete protocol is described below, starting from cloning genes for lymphoma scFv and genes for chemokines MCP-3 and IP-10 and ending with immunization of mice with DNA vaccines. The specific primer sequences for cloning of scFv, IP-10, and MCP-3 chemokine genes are shown in Tables 20.6.2 and 20.6.3. The specific primer sequences for cloning of scFv alone in a mammalian expression vector is shown in Table 20.6.4. Chemokine genes are cloned from LPS-induced murine monocyte cell lines and fused in-frame with scFv as a chemokine-scFv in a mammalian expression vector. Almost any commercially available mammalian expression vector can be used. The mammalian expression vector plasmid pcDNA3.1(+) (Invitrogen) was modified in the authors' laboratory, where the scFv was cloned under the control of promoter and enhancer elements of the early gene of CMV and the transcription termination signal sequence from BGH gene V (Fig. 20.6.2). If desired, a 5′-UTR (Kozak sequence) can be inserted by PCR with specific primers encoding the 5′-CCACC**ATG**G sequence, where the optimal Kozak sequence CCACC precedes the **ATG** codon. Use of the following restriction enzyme sites is recommended: *Nco*I (to use as the first amino acid ATG of its recognition sequence CCATGG), *Dra*I (to utilize the TAA-codon as an ochre stop signal), *Eco*RI, *Hin*dIII, *Sma*I or *Xma*I, *Xho*I, *Sal*I, *Bam*HI, and *Bgl*II.

NOTE: The 5′-untranslated region (UTR) of the scFv gene should be modified to be close to "Kozak's consensus sequence"; the 3′-UTR transcription termination region (from SV40 or BGH polyA region) should be used if high levels of expression are required. It is important that chemokine genes be cloned with their own signal leader sequences (SL) to secrete a resulting fusion protein from mammalian cells. However, SL should be omitted if recombinant protein is to be produced in bacteria. Human proinflammatory chemokines are usually active in mice and can also be used.

Materials

DEPC-treated (RNase-free) H_2O (*UNIT 10.11*)
ANA-1 murine monocyte cell line (Blasi et al., 1985; gift of Luigi Varesio, Instituto G. Gaslini, Genoa, Italy) or LPS-induced murine monocytes
Solution D (see recipe; also see *UNIT 10.11* for general discussion of guanidinium extraction of RNA)
2 M sodium acetate, pH 4.0 (*UNIT 10.11*)
Phenol, water-saturated (*UNIT 10.11*)
49:1 (v/v) chloroform/isoamyl alcohol
Isopropanol
70% and 100% (absolute) ethanol
25 mM $MgCl_2$

10× PCR buffer II (Perkin-Elmer)
2.5 mM 4dNTP mix: 2.5 mM each dNTP in water; store at −20 °C
RNAsin (human placental ribonuclease inhibitor; Promega)
Random hexamer primer mixture (e.g., Pharmacia Biotech)
200 U/µl AMV reverse transcriptase
30 µM forward and reverse PCR primers: to clone V_H and V_L from 38C-13 and A20 murine B cell lymphomas (Table 20.6.2) and IP-10 and MCP-3 chemokines (Table 20.6.3)
AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems)
Klenow fragment of DNA polymerase
10× loading buffer (*UNIT 10.4*)
Low-melting-temperature agarose (Type XI, Sigma; also see *UNIT 10.4*)
Ethidium bromide solution (*UNIT 10.4*)
TAE electrophoresis buffer (*UNIT 10.4*)
DNA size ladder: e.g., λ phage DNA cut with *Hin*dIII
5 M NaCl (*APPENDIX 2*)
Phenol, buffer-saturated (*UNIT 10.1*)
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 10.1*)
TE buffer, pH 7.5 (*APPENDIX 2*)
10 U/µl T4 polynucleotide kinase and 10× polynucleotide kinase buffer
10 mM ATP
T4 DNA ligase and 10× DNA ligase buffer
Restriction endonucleases: *Nco*I, *Eco*RI, *Hin*dIII, *Sma*I or *Xma*I, *Dra*I, *Xho*I, *Sal*I, *Bam*HI, and *Bgl*II
3 M sodium acetate (*APPENDIX 2*)
myc-his-pcDNA3.1 plasmid: pcDNA3.1(+) (Invitrogen) modified by inserting c-myc and His-tags (Fig. 20.6.2; for plasmid modification, see Chapter 10 of this manual and Ausubel et al. 2001)
Competent *E. coli* cells (*APPENDIX 3N*) or XL1 Blue competent cells (Stratagene)
LB medium or other bacterial complete medium without antibiotics
LB medium (*UNIT 10.3*) and LB plates (*UNIT 10.19*) with 100 µg/ml ampicillin (Amp)
GTE solution (optional; *UNIT 10.3*)
0.2 N NaOH/1% (w/v) SDS (prepare just before use)
3 M potassium acetate, pH 4.8 (*UNIT 10.3*)
AmpliTaq DNA polymerase (Perkin-Elmer)
Agarose (for routine use, Sigma; not LMP)
Qiagen P-2500 column plasmid purification kit

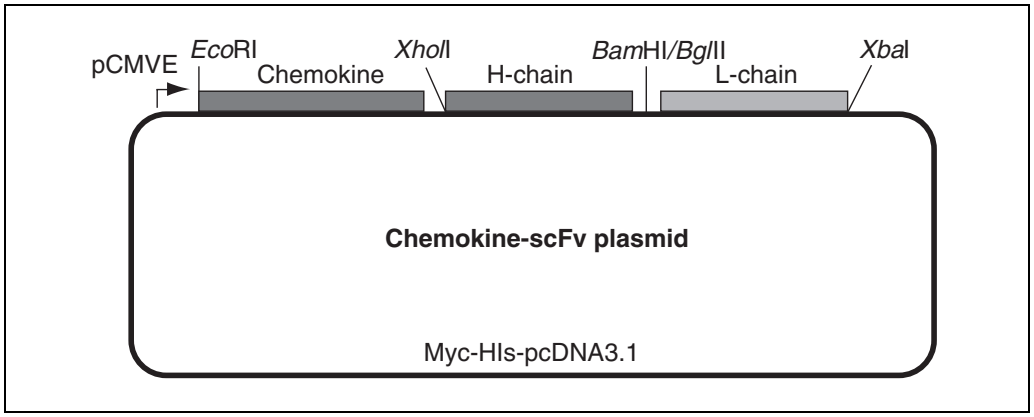

Figure 20.6.2 Schema of cloning chemokine-scFv. The expression vector plasmid, modified pcDNA3.1(+) from Invitrogen with c-myc-tag and six His-tag introduced, was opened by *Eco*RI and *Xba*I sites, and fragments were cloned using restriction sites shown. Note that the *Sal*I site was used at the 5′-end for A20 V_H , instead of *Xho*I.

Table 20.6.2 Primers for PCR Amplification of H and L Chain V Regions from 38C-13 and A20 Lymphomas

Primer	Primer sequence
38C-13 lymphoma:	
PRV_H38S-5′[a]	ACTCGAGGTGAAGCTGGTGGAGTCTGGA
PRV_H38FB-3′[b]	*AGGATCCGCCGCCGCCAGAACCACCACCACCAG*AGGAGACTGTGAGAGTGGTGCCTT
PRV_L38FB-5′[c]	*AAGATCTGGTGGCGGTGGGAGC*GACATCCAGATGACACAGTCTCCA
PRV_L38S-3′[d]	ATCTAGATTTTATTTCCAGCTTGGTCCCCCCTCCGAA
A20 lymphoma:	
PRV_H20AS-5′[e]	AGTCGACGTCCAACTGCAGCAGTCAGGGCCTGACCTT
PRV_H20AFB-3′[b]	*AGGATCCGCCGCCGCCAGAACCACCACCACC*TGAGGAGACTGTGAGTTCGGTGCCTTG
PRV_L-20AFB-5′[c]	*AAGATCTGGTGGCGGTGGGAGC*GATGTTGTGATGACGCAGACTCCACTC
PRV_L20AS-3′[D]	ATCTAGATTTGACTTCCAGCTTTGTGCCTCCA

[a]*Xho*I restriction site underlined.
[b]*Bam*HI restriction site underlined; linker sequences for $(Gly_4S)_3$ in italics
[c]*Bgl*II restriction site underlined; linker sequences for $(Gly_4S)_3$ in italics
[d]*Xba*I restriction site underlined.
[e]*Sal*I restriction site underlined.

Table 20.6.3 Primers for PCR Amplification of Murine IP-10 and MCP-3

Chemokine and primer	Primer sequence
IP-10:	
PRIP10S-5′[a]	AGAATTCCACC**ATG**AACCCAAGTGCTGCCGTCATTTTC
PRIP10S-3′[b]	*ACTCGAGATCACTCTTCGGCGCCTGAGCGTCGTT*AGGAGCCCTTTTAGACCTTTTTTG
PRIP10M-5′[c]	CACC**ATG**GCCATCCCTCTCGCAAGGACGGTCCGCT
MCP-3:	
PRMMCP3S-5′[a]	AGAATTCACCATGAGGATCTCTGCCACGCTTCTGTGC
PRMMCP3S-3′[b]	*ACTCGAGATCACTCTTCGGCGCCTGAGCGTCGTT*AGGCTTTGGAGTTGGGGTTTTCAT
PRMMCP3M-5′[c]	CACC**ATG**GCCCAACCAGATGGGCCCAATGCA

[a]*Eco*RI restriction site underlined; first chemokine codon, Met, in bold.
[b]*Xho*I restriction site underlined; sequence of spacer fragment in italics.
[c]*Nco*I restriction site underlined; first chemokine codon, Met, in bold.

Table 20.6.4 Sequences of Signal Leaders and PCR Primers to be used for H and L Chain V Regions to Enable Secretion

Primer/oligonucleotide	DNA sequence
Signal leader sequences of 38C-13 V_H and V_L genes:	
SLH38[a]	**ATG**AAGTTGTGGCTGAACTGGATTTTCCTTGTAACACTTTTAAATGGTATCCAGTGT
SLK38[b]	**ATG**GAGACGTCTATTCAGTTCCTGGGGCTCTTGTTGTTCTGGCTTCATGGTGCTCAGTGT
PCR primers to amplify H-chain with SL:	
PRV_H38Ld-5′[c]	GATATCCACC**ATG**GAGTTGTGGCTGAACTGG
$PRV_H38Ld20AV_H$-3′[d]	**TGACTGCTGCAGTTGGAC**ACACTGGATACCATTTAAAAG
$PR20AV_H$ls-3′[g]	GTCGACGCTAGCTGAGGAGACTGTGAGTTCGGT
PCR primers to amplify L-chain with SL:	
PRV_L38Ld-5′[e]	GATATCCACC**ATG**GAGACGTCTATTCAGTTCC
PRV_L38Ld-3[f]	**CTGCGTCATCACAACATC**ACACTGAGCACCATGAAG
$PR20AV_L$ls-3′[g]	GTCGACTTACGTTTGATTTGACTTCCAGCTTTGTGCCT

[a]Signal leader sequence of heavy (SLH38) chain is adapted from Carroll et al. (1988). Initiator (Met) codon in bold.
[b]Signal leader sequence of light (SLK38) chain is adapted from Campbell et al. (1987). Initiator (Met) codon in bold.
[c]*Eco*RV site is underlined.
[d]The 5′-end sequence of A20 H-chain is bold and underlined.
[e]*Eco*RV and *Nco*I sites underlined.
[f]5′-end of L-chain of A20 is bold and underlined.
[g]*Sal*I site is underlined.

Gold particles (0.8 to 1 μm; Degussa; also available at higher price from Bio-Rad)
0.1 M spermidine (store frozen in single-use aliquots)
2.5 M $CaCl_2$
Ultrapure nitrogen gas

Heating block
Thermal cycler (see *UNIT 10.20*)
Water bath sonicator
Tefzel tubing (0.104-in. i.d. × 0.192-in. o.d.; Bio-Rad)
Tubing Prep Station (Bio-Rad; provided with Helios Gene Gun System)

Additional reagents and equipment for RNA isolation by the guanidinium thiocyanate method (see *UNITS 2.12 & 10.11*), PCR amplification of DNA (*UNIT 10.20*), agarose gel electrophoresis (*UNIT 10.4*), alkaline lysis to isolate DNA from bacteria (*UNIT 10.3*), and transient transfection (*UNITS 10.14 & 10.15*) and immunoblotting (*UNIT 8.10*)

NOTE: Use endotoxin-free ultra-pure distilled water, tissue culture grade (Advanced Biotechnologies) at all points where water is called for.

Isolate total RNA from lymphoma and monocyte cells

Any available method for RNA isolation is acceptable, particularly the approach detailed here, which is based on Chomczynski and Sacchi's guanidinium thiocyanate method (Chomczynski and Sacchi, 1987; also see *UNITS 2.12 & 10.11*). All RNA isolation procedures must be performed on ice or at 4°C, unless otherwise specified. Use DEPC-treated water (*UNIT 10.11*) to prepare all reagents and solutions used in steps 1 to 9.

1. Place 1×10^6 ANA-1 or LPS-induced murine monocytes in exponential growth phase in a 1.5-ml microcentrifuge. Microcentrifuge for a few minutes at 2000 rpm, 4°C.
2. Discard supernatant and mix pellet by vortexing briefly.
3. To the pellet add 500 μl solution D, 50 μl 2 M sodium acetate, pH 4.0, 500 μl water-saturated phenol, and 100 μl 49:1 chloroform/isoamyl alcohol. Mix gently by inverting tubes. Incubate on ice for 15 min.
4. Microcentrifuge 30 min at maximum speed, 4°C. Transfer ~400 μl of upper aqueous phase into a new microcentrifuge tube. Add an equal volume (400 μl) of chloroform/isoamyl alcohol. Mix by vortexing, microcentrifuge 5 min at maximum speed, 4°C, then transfer ~400 μl of the resulting upper aqueous phase into a new microcentrifuge tube.
5. Add an equal volume (400 μl) of isopropanol and place on dry ice for 5 min (or at −20°C for 30 min). Recover precipitate by centrifuging for 10 min at maximum speed, 4°C. Discard supernatant.
6. Dissolve the pellet in 200 μl of solution D and repeat step 5.

   The pellet should be clearly visible.
7. Rinse the pellet with 100 μl of 70% ethanol, microcentrifuge briefly, discard supernatant, and air dry.
8. Resuspend RNA (pellet) in 30 μl of RNase-free water.

Synthesize cDNA

9. Set up cDNA synthesis mixture (20 µl total reaction volume) by mixing:

   1 µl resuspended RNA (from step 8)
   2 µl 25 mM $MgCl_2$
   2 µl 10× PCR buffer II
   2 µl 2.5 mM 4dNTP mix
   1 µl RNAsin
   2 µl random hexamer primer mixture
   9.5 µl RNase-free (DEPC-treated) water
   0.5 µl (100 U) AMV reverse transcriptase

   Incubate for 10 min at room temperature, then for 40 min at 42°C.

   The resulting cDNA may be stored at −20°C.

Amplify IP-10 and MCP-3 chemokine genes by PCR

10. Add 5 µl of cDNA (from step 9) to a new microcentrifuge tube along with:

    9.5 µl 25 mM $MgCl_2$

    9.5 µl 10 × PCR buffer II

    0.3 µl 2.5 mM 4dNTP mix

    1 µl 30 µ forward primer (e.g., PRIP10S-5′ for IP-10, or PRMMCP3S-5′ for mMCP-3; see Table 20.6.3)

    1 µl 30 µM reverse primer (e.g., PRIP10S-3′ for IP-10, or PRMMCP3S-3′ for mMCP-3; see Table 20.6.3)

    73.2 µl H_2O

    0.5 to 1 µl (2 U) AmpliTaq Gold DNA polymerase

    No mineral oil overlay is added if PCR thermocycler has a heated cover

11. Carry out PCR using the following amplification cycles:

1 cycle:	9 min	94°C	(preheating/denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	60°C	(annealing)
	30 sec	72°C	(extension)
1 cycle:	5 min	72°C	(extension)

Amplify heavy chain variable region DNA fragments from 38C-13 and A20 by PCR

12. Mix new PCR reaction as described in step 10, but using 1 µl of 30 µM forward primer (PRV_H38S-5′ for 38C-13 and PRV_H20AS-5′ for A20 lymphomas, respectively; see Table 20.6.3) and 1 µl of 30 µM reverse primer (PRV_H38FB-3′ for 38C13 and PRV_H20AFB-3′ for A20 lymphomas, respectively; see Table 20.6.2).

13. Carry out PCR as described in step 11.

Amplify light chain variable region DNA fragments from 38C-13 and A20 by PCR

14. Mix new PCR reaction as described in step 10, but using 1 µl of 30 µM forward primer (PRV_L38FB-5′ for 38C-13 and PRV_L20AFB-5′ for A20 lymphomas, respectively; see Table 20.6.2) and 1 µl of 30 µM reverse primer (PRV_L38S-3′ for 38C13 and PRV_L20AS-3′ for A20 lymphomas, respectively; see Table 20.6.2).

15. Carry out PCR as described in step 11.

16. When PCR reaction is completed, add 0.5 µl Klenow fragment (5 U) to the each tube from steps 11, 13 and 15. Incubate 10 min at room temperature, then stop the reaction by mixing with 10 µl of 10× loading buffer.

17. Separate PCR fragments by electrophoresing on a 1.4% low-melting-temperature agarose gel (LMG) with ethidium bromide in TAE buffer (*UNIT 10.4*). Run in parallel λ phage DNA cut with *Hin*dIII (or any other ladder to judge DNA fragment size).

18. Visualize the DNA fragment (~250 to 400 bp) under UV light, cut out the fragment of the gel containing it, and transfer it into a new microcentrifuge tube containing 370 µl water and 34 µl 5 M NaCl.

    Cut out a piece of gel with as small a fragment as possible to reduce the sample volume. The chemokine genes and V-region fragments of lymphoma migrate at about 250 to 400 bp.

19. Heat each tube for 10 min at 65° to 70°C and extract DNA by vortexing in 500 µl of buffer-saturated phenol.

    No chloroform is used at this stage.

20. Microcentrifuge each tube for 10 min at maximum speed, room temperature.

21. Transfer aqueous phase into a new tube and extract it with 500 µl 25:24:1 phenol/chloroform/isoamyl alcohol. Microcentrifuge 5 min at maximum speed, room temperature.

22. Transfer the aqueous phase (400 µl) into a new tube and mix with 1 ml 100% ethanol. Chill on dry ice for 5 min (or 30 min in a –20°C freezer if no dry ice is available) and precipitate the DNA fragment by microcentrifuging 10 min at maximum speed, room temperature (DNA should be visible as a tiny white pellet). Discard the supernatant and rinse the pellet by adding 100 µl 70% ethanol and briefly microcentrifuging. Gently remove the supernatant and dry each pellet. Resuspend each pellet in 8 µl of TE buffer, pH 7.5.

Modify the ends of chemokine and heavy and light chain variable region DNA fragments to enable their cloning

23. Prepare the following reaction mix in a separate new tube:

    7 µl PCR fragment (from step 22)
    2 µl 10× T4 polynucleotide kinase buffer
    2 µl 10 mM ATP
    8 µl H_2O
    1 µl (10 U) T4 polynucleotide kinase

24. Incubate 30 min at 37°C.

25. Add to the reaction mixture:

    17 µl water
    2 µl 10× T4 DNA ligase buffer
    1 µl (10 U) T4 DNA ligase

    Incubate 30 to 60 min at room temperature.

26. Heat-inactivate ligase for 10 to 15 min at 70°C.

    Steps 23 to 26 are needed for complete restriction enzyme digestion of end sites; it can be omitted if high efficiency of cloning is not needed

27. Add 0.8 µl 5 M NaCl (to bring the final concentration of Na^+ to 100 mM) and 1 µl (5 to 10 U) of each of the appropriate restriction enzymes as described below. Incubate 1 hr at 37°C:

    *Eco*RI and *Xho*I: for cutting chemokine fragments
    *Xho*I and *Bam*HI: for cutting V_H-chain fragment of 38C-13
    *Sal*I and *Bam*HI: for V_H-chain fragment of A20
    *Bgl*II and *Xba*I: for cutting V_L-chain fragments of 38C-13 and A20.

28. Purify restriction fragments as follows:

    a. Add 160 µl water to each tube.
    b. Extract digested fragments with 200 µl 25:24:1 phenol/chloroform/isoamyl alcohol (see step 21).
    c. Precipitate DNA fragments by adding 20 µl 3 M sodium acetate and 2.5 vol of 100% ethanol (see step 22).
    d. Microcentrifuge 5 min at maximum speed.
    e. Rinse pellet with 70% ice-cold ethanol.
    f. Resuspend in 8 µl TE buffer, pH 8.0.

    The DNA fragments are ready now for cloning into an expression vector plasmid; e.g., myc-his-pcDNA3.1, digested with EcoRI and XbaI enzymes and purified from LMG, as described in steps 17 to 22.

Clone fragments into an expression vector plasmid

29. Combine the following mixture in a new tube:

    2 µl (100 ng) of sample 1: MCP-3 or IP-10 chemokine
    1.5 µl (100 ng) of sample 2: H-chain fragment from 38C-13 or A20
    1.5 µl (100 ng) of sample 3: L-chain fragment from 38C-13 or A20
    0.5 µl (10 ng) expression vector plasmid myc-his-pcDNA3.1
    12.5 µl water
    2 µl 10× T4 DNA ligase buffer
    1 µl (10 U) T4 DNA ligase

    The myc-his-pcDNA3.1 plasmid is pcDNA3.1(+) (Invitrogen) modified by inserting c-myc and His-tags (Fig.20.6.2).

30. Incubate above mixture for 1 to 2 hr at 16°C or leave overnight at 16°C.

    The three-to-four fragment cloning procedure described here is usually works quite efficiently (Fig. 20.6.2). However, each fragment can also be cloned separately in intermediate plasmids and then assembled.

31. Transform 80 µl of a 10^8 cell/ml suspension of competent *E. coli* by mixing with 8 µl of ligation mixture (from step 30). Incubate 30 min on ice.

32. Heat shock transformation mixture at 42°C for 60 sec.

33. Add 500 µl LB or any bacterial complete medium without antibiotics and incubate 30 min at 37°C.

34. Microcentrifuge 3 to 5 min at 3000 rpm, discard most of supernatant, and resuspend pellet in 50 to 100 µl of the growth medium used in step 33. Plate *E. coli* on LB plates containing 100 µg/ml ampicillin. Incubate plates at 37°C overnight until visible *E. coli* colonies appear.

Screening of transformed E. coli

PCR-based screening of *E. coli* colonies is simple and reliable. *E. coli* colonies can be sampled individually by growing them in liquid medium.

35. Pick individual *E. coli* colonies using individual toothpicks and grow for 1 to 2 hr at 37°C in 1 ml of Amp-LB medium. Precipitate bacteria by microcentrifuging 10 sec at maximum speed. Discard supernatant.

    The pellet size will be very small, and sometimes not obviously visible

36. Perform brief alkaline lysis (also *UNIT 10.3*) by resuspending bacterial pellet in 50 µl GTE solution or water and 100 µl 0.2 N NaOH/1% SDS. Incubate at room temperature for 3 min.

37. Add 100 µl 3 M potassium acetate solution and incubate 3 min at room temperature.

38. Add 200 µl 25:24:1 phenol/chloroform/isoamyl alcohol, vortex 10 sec, then microcentrifuge 3 min at maximum speed.

39. Transfer 200 µl of the top (aqueous) phase into a new tube and precipitate DNA by adding 160 µl isopropanol and microcentrifuging for 10 min at maximum speed.

40. Rinse pellet with 50 µl of 70% ethanol and dry.

41. Resuspend pellet in 20 µl water and use 1 µl for PCR screening.

42. For each sample, prepare the following (25-µl) PCR reaction mix:

    1 µl DNA (from step 41)
    2.5 µl 10× PCR buffer II
    2.5 µl $MgCl_2$

    0.5 µl forward primer (Table 20.6.2 and 20.6.3)
    0.5 µl reverse primer (Table 20.6.2 and 20.6.3)
    0.5 µl 2.5 mM 4dNTP mix
    16.5 µl H_2O
    1 U AmpliTaq DNA polymerase (not AmpliTaq Gold)

43. Perform PCR using the amplification cycles described in step 11, but omit 9 min 94°C initial preheating/denaturation cycle.

44. Analyze PCR products by electrophoresis on a 1.2% agarose (routine use, not LMP) gel in TAE buffer (*UNIT 10.4*).

Verify sequence of the entire gene by DNA sequencing to make sure that PCR cloning did not introduce mutations

45. Purify large quantities of plasmids of interest using the Qiagen P-2500 column kit, following the manufacturer's instructions.

46. Test plasmids for expression of recombinant protein by transiently transfecting mammalian cell lines, such as COS-7 or 293 cells (see *UNITS 10.14 & 10.15*); test for the protein of interest by immunoblotting using 9E10 MAb (*UNIT 8.10*).

Prepare gene gun cartridges

There are several ways to deliver DNA in vivo (*UNIT 2.14*). The Helios Gene-Gun System (Bio-Rad) is very simple and efficient and requires much lower amounts of DNA than other methods. Plasmid DNA is precipitated onto gold particles using 2.5 M $CaCl_2$. The authors generally use 0.89 to 1 µm gold particles (Degussa), although others have reported the potential importance of using larger gold particles. Also important for the quality of

gene-gun cartridges is the quality of tubing used. Here a method is described that was originally adapted from the manual for the Accell Gene Delivery Device (Agracetus).

47. Weigh 90 to 95 mg gold in a 2 ml Eppendorf tube and mix it with 300 µl 0.1 M spermidine. Sonicate gold and spermidine mixture 10 sec using a water bath sonicator to break up gold clumps into single bead suspension.

    90 to 95 mg of gold makes about 160 to 200 cartridges.

48. Add 200 µg plasmid (a 1 µg/µl solution in water) into the gold-spermidine mixture. Mix by vortexing.

49. Add 600 µl 2.5 M $CaCl_2$ dropwise into the DNA gold mixture while gently vortexing. Incubate mixture for 5 to10 min at room temperature.

50. Microcentrifuge 10 sec at maximum speed to bring down the gold. Discard supernatant.

51. Wash the pellet by adding 1 ml 100% ethanol (room temperature), vortexing, and microcentrifuging for 10 sec at maximum speed. Discard supernatant. Repeat washing three more times.

52. Resuspend gold particles in 100% ethanol at a ratio of 7 ml ethanol per mg gold.

At this step the mixture can be stored at −20°C in a tightly closed and sealed container. The DNA-gold mixture is now coated on Tefzel tubing using the Tubing Prep Station according to the manufacturer's instructions (Bio-Rad).

53. Prior to use, dry the Tefzel tubing by purging it for at least 10 min with ultrapure nitrogen gas. Using a syringe attached to one end of the tube, fill it with the DNA-gold suspension prepared in step 52, and insert it into the Tubing Prep Station. Let sit for 7 min.

    Gold should be precipitated at the bottom of the tubing after 5 to 7 min.

54. Remove ethanol using a peristaltic pump at the rate 1 in. per sec.

55. Detach peristaltic pump when all of the ethanol is drawn off. Rotate on a tube turner at 20 rpm for 3 min, allowing gold to smear in the tube.

56. Open valve on the flow meter to 0.3 to 0.4 liters per minute to dry tube for 3 min.

    Tubing should be dried completely.

57. Close nitrogen valve, stop rotation, and remove the tube from the Tubing Prep Station. Cut gold-coated tube into 0.5-in. pieces (each of which is a Gene Gun cartridge). Store cartridges at 4°C with dessicant pellets in 50-ml polypropylene centrifuge tubes, tightly sealed with Parafilm for 6 months or longer.

    For immunization procedure using these cartridges with the Helios Gene Gun, see Support Protocol 4.

BASIC PROTOCOL 3

PRODUCTION OF scFv FUSION PROTEINS FROM BACTERIA

The efficiency and yield of scFv protein production may vary drastically depending on the nature of the particular scFv and strategy for expression. This feature of scFv proteins is also true for chemokine-scFv fusions. For example, production of 38C-13 lymphoma–derived scFv is not efficient when it is expressed as a soluble and secretable protein in *E. coli*. However, large quantities of scFv can be produced in *E. coli* when it is expressed as insoluble inclusion bodies. Herein we describe a procedure for production of chemokine-scFv fusion proteins expressed in bacterial inclusion bodies, using a method adapted from the original report by Buchner et al. (1992). The procedure involves purification of insoluble bacterial inclusion bodies, which are solubilized by 6 M guanidinium·HCl solution and subsequently refolded at low protein concentrations. The resulting fusion proteins can be used either for analytical characterization or as immunogens in vivo.

The use of bacterial expression plasmids with tight regulatory elements, such as the T7 polymerase promoter (pET11, Stratagene), are advised for expression of toxic Fv-fragments. This is a two-step process. First, DNA cloning is performed in strains of *E. coli* such as XL1 Blue or TG1, which do not allow protein expression from the T7 promoter. Then, protein is produced by transforming a specific bacterial strain which expresses T7 DNA polymerase—Bl21 (DE3) from Stratagene.

NOTE: The scFv and its fusions are expressed using the pET11d vector, which has unique *Nco*I and *Bam*HI sites. The cloning procedure is similar to that described in Basic Protocol 2 (steps 10 to 42) with only a few modifications, such as cloning chemokine genes without their secretion leader sequences. The DNA fragments for mature proteins of IP-10 and MCP-3 are cloned utilizing different forward (5′-) primers (PRIP10M-5′ or PRMMCP3M-5′, respectively; Table 20.6.3) and the same 3′-primers. Therefore, the final expression cassette is very similar to the one shown in Fig. 20.6.2, used for DNA vaccinations, except it does not contain a mammalian signal leader sequence. The bacterial expression plasmid is constructed by ligating three DNA fragments: fragment 1, consisting of *Nco*I and *Xho*I restriction enzyme–cut PCR fragment for IP-10 or MCP-3 chemokines (amplified with primers PRIP10M-5′ or PRMMCP3M-5′and PRIP10S-3′ or PRMMCP3S-3′; Table 20.6.3); fragment 2, consisting of DNA fragment for scFv cut with *Xho*I and *Xba*I blunt end enzymes (removed from the naked DNA expression cassette; Fig. 20.6.2); and fragment 3, consisting of pET 11 plasmid cut with *Nco*I and *Bam*HI blunt end enzymes.

Materials

100 ng/µl pET11 bacterial expression plasmid, containing the T7 promoter element (Stratagene)
Competent *E. coli* cells: XL1 Blue and BL21(DE3) (Stratagene)
LB medium (*UNIT 10.3*)
LB plates (*UNIT 10.19*) with 100 µg/ml ampicillin (Amp)
Glycerol growth medium (see recipe)
20% (v/v) glycerol
Induction medium (see recipe)
20% (w/v) sucrose solution in H_2O, ice cold
TE buffer for proteins (see recipe)
50 mg/ml lysozyme (store frozen)
5 M NaCl (*APPENDIX 2*)
25% (v/v) Triton X-100
6 M guanidinium solution (see recipe)
BSA Protein Assay Kit (Pierce) or equivalent
Dithioerythritol (DTE)
Refolding solution (see recipe), prechilled to 10°C

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2*) containing 100 mM ultrapure urea
Heparin Sepharose CL-6B (Pharmacia Biotech)
20 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
Glycerol column buffers with 50 mM, 300 mM, and 1 M NaCl (see recipe)
Triton X-100
Phosphate-buffered saline (*APPENDIX 2*) containing an additional 300 mM NaCl (~450 mM total) with or without 5% to 10% (v/v) glycerol

Heating block
2-liter Ehrlenmeyer flask
30°C incubator with shaker
Beckman Avanti J-251 centrifuge with JLA-10.5 and JA-17 rotors (or equivalent)
Tissue-Tearor Homogenizer (Fisher)
10°C water bath
Dialysis membrane (Spectra/Por, MWCO 6000 to 8000, 50-mm width; Spectrum)
2-liter plastic container
Chromatography column: Poly-Prep, 0.8 × 4–cm (Bio-Rad)
Additional reagents and equipment for dialysis (*APPENDIX 3H*), polyacrylamide gel electrophoresis (Ausubel et al., 2001), determination of proper folding of scFv (Support Protocol 1), and in vitro chemotaxis assay (*UNIT 6.12*)

Express chemokine-scFv protein in E. coli

1. Perform a simplified transformation procedure by mixing 1 µl (100 ng) plasmid DNA with 20 µl BL21(DE3) competent cells. Incubate on ice for 1 to 10 min.

   At this stage, there is no need for high-efficiency transformation. This simplified method should yield 10 to 300 colonies per plate.

2. Heat the mixture for 1 min at 42°C.
3. Add 100 µl LB medium to the mixture, then plate on LB plates containing 100 µg/ml ampicillin. Incubate plates overnight at 37°C.
4. The next day, add 6 ml of LB medium onto the top of the agar surface of each plate containing bacterial colonies and resuspend all colonies by washing with the medium using a 5-ml pipet.
5. Inoculate 50 ml of glycerol growth medium with 0.5 ml bacterial suspension in a 2-liter flask. Store the rest of the bacterial suspension in 1-ml aliquots in 20% glycerol at −70°C.
6. Grow bacteria for 6 to 8 hr at 30°C with vigorous shaking.
7. Add 50 ml fresh glycerol growth medium and continue shaking for 3 to 5 hr.
8. Dilute the culture with 300 ml induction medium and continue growing overnight in 2-liter flasks.

Prepare bacteria spheroplasts and inclusion bodies

9. Centrifuge at 9000 × *g* (5000 rpm in a JLA-10.5 rotor), 4°C.
10. Resuspend cell pellet in 60 ml of ice-cold 20% sucrose solution.
11. Incubate on ice for 10 min.
12. Centrifuge as in step 9.
13. Resuspend cell pellet in 100 ml ice-cold distilled water.
14. Incubate on ice for 10 min.
15. Pellet cells by centifuging 20 min at 20,000 × *g* (12, 000 in a JA-17 rotor), 4°C, and discard supernatant.

The pellet can be stored frozen.

16. Resuspend pellet in 30 ml ice cold TE buffer for proteins. Homogenize pellet completely using Tissue-Tearor homogenizer.
17. Add 25 µl of 50 mg/ml lysozyme and mix well.
18. Incubate at room temperature for 30 to 60 min.
19. Add 6 ml 5 M NaCl and 5.2 ml 25% Triton X-100 solution. Mix well.
20. Continue incubation at room temperature for 30 min.

    Cell lysis causes increased viscosity of the mixture due to unfolded chromosomal DNA.
21. Homogenize mixture well using Tissue Tearor homogenizer, to completely shear chromosomal DNA.
22. Centrifuge for 45 min at 27,000 × *g* (14,000 rpm in a JA-17 rotor), 4°C.
23. Homogenize the pellet completely in 30 to 35 ml of ice cold TE buffer for proteins.
24. Repeat step 22.
25. Repeat steps 22 and 23 four more times.

    The pellet can be stored frozen after any centrifugation step
26. Solubilize inclusion bodies (pellet) by homogenizing in 5 ml 6 M guanidine solution using Tissue Tearor homogenizer. Collect supernatant with solubilized proteins by centrifuging as in step 22. Determine approximate protein concentration using BSA Protein Assay Kit or Bradford assay (*UNIT 3.4*).

    Keep the total volume 8 to 10 ml.
27. Add dithioerythritol powder to a final concentration of 0.3 M and dissolve by shaking.
28. Incubate at room temperature for at least 30 min.

    The sample can be stored at −20°C at this step.

Allow for protein refolding

29. Add 1 to 8 ml of sample dropwise to 1000 ml of prechilled (10°C) refolding solution while vigorously mixing with a magnetic stirrer. Incubate the mixture for 3 to 5 days in a 10°C water bath, without stirring.

    The final protein concentration should not exceed 80 µg/ml.
30. Dialyze sample overnight (*APPENDIX 3H*) at 4°C against 40 liters of 20 mM Tris·Cl, pH 7.5/100 mM urea.

    Usually a single dialysis in 40 liters of dialysis buffer is sufficient for up to 4 liters of refolding mixture.
31. Centrifuge dialyzed sample 15 min at 9000 × *g* (5000 rpm in JLA-10.5 rotor), 4°C.
32. Gently remove supernatant into a clean 2-liter plastic container.

    Supernatant can be filtered through a 0.45-µm filter, if needed. Do not discard the pellet; it can be stored frozen and reused, starting from step 26.

Perform heparin-Sepharose column purification

33. Equilibrate 2 ml heparin Sepharose CL-6B in 20 mM Tris·Cl, pH 7.5, in a 0.8 × 4–cm Poly-Prep column.
34. Apply the dialyzed and renatured protein to the heparin-Sepharose column and perform chromatography at 4°C.

35. When all sample material has passed through the column, wash with glycerol column buffer containing 50 mM NaCl and 0.1% Triton X-100, until flowthrough reaches OD_{280} <0.05.
36. Elute bound proteins successively with glycerol column buffer containing 300 mM and 1 M NaCl. Collect 2-ml fractions.
37. Measure OD_{280} of each fraction.

    Chemokine-scFv protein will usually be retained in the second and third fractions.
38. Characterize purified fusion proteins by reducing PAGE and, if necessary, immunoblot hybridization using 9E10 anti–c-myc MAb (*UNIT 8.10*).

    A variety of PAGE protocols are presented in Chapter 10 of Ausubel et al. (2001).
39. Dialyze samples in PBS containing an additional 300 mM NaCL, with or without 5% to 10% glycerol. Keep samples at 4° or deep freeze for longer storage.

    Chemokine-scFv tends to precipitate when dialyzed and stored frozen at high concentrations.
40. Test folding of scFv by its ability to inhibit binding of polyclonal or monoclonal anti-iodiotypic antibody to its idiotype (see Support Protocol 1). Perform in vitro chemotaxis assays using cells expressing the respective chemokine receptor (See *UNIT 6.12*).

TUMOR PROTECTION USING GENETICALLY MODIFIED WHOLE TUMOR CELL VACCINES (GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR-TRANSDUCED A20 LYMPHOMA)

BASIC PROTOCOL 4

Additional lymphoma antigens have not been defined. Therefore, one general approach to active immunotherapy is to utilize genetically modified autologous tumor cells as a source of Ag. Expression of various cytokine genes in tumor cells may enhance the immunogenicity and potency of cell-based vaccines. Tumor cells are modified/transduced in vitro to express immuno- modulatory molecules such as cytokines (GM-CSF, IL-2, and IL-4), chemokines (IP-10), or costimulatory molecules (B7.1 and B7.2). Although transiently transfected cells can be used, stable transfectant tumor cells are selected using selective markers, such as the neomycin analog G418 or hygromycin. Stable transfectants are characterized by flow cytometry (*UNIT 5.4*) and ELISA (*UNIT 10.17*). Herein we describe a brief strategy for producing and testing stably transduced A20 lymphoma cells expressing murine GM-CSF which can elicit T cell–dependent protective and therapeutic immunity against early pre-established tumors (Levitsky et al., 1996).

Materials

1 mg/ml mammalian expression plasmid, encoding murine GM-CSF, dissolved in TE buffer, pH 8.0 (linearized by single restriction cut, purified by phenol/chloroform extraction and ethanol precipitation; see Ausubel et al., 2001, and Chapter 10 in this manual)
A20 cells (ATCC #TIB-208) growing in exponential phase
RPMI 1640 medium
Complete RPMI medium with 10% FBS (*APPENDIX 2A*)
MPRO cells (ATCC # CRL-11422; Tsai and Collins, 1993) growing in exponential phase
Complete DMEM medium with 10% FBS (*APPENDIX 2A*)
Hanks' balanced salt solution (*APPENDIX 2A*) without Ca^{2+} and Mg^{2+}
6- to 12-week-old BALB/c female mice

Gene Pulser II System for electroporation (Bio-Rad)

Sterile electroporation cuvettes (0.4-cm electrode gap; Bio-Rad)
96-well flat-bottom microtiter plates (Costar)
Sorvall centrifuge with H-1000B swinging-bucket rotor (or equivalent)
50-ml conical polypropylene centrifuge tubes (e.g., Falcon)

Additional reagents and equipment for culturing cells cells (see *APPENDIX 3*), ELISA (*UNIT 2.1*), immunoblotting (*UNIT 8.10*), labeling cells for determination of [^{3}H]thymidine uptake (*APPENDIX 3D* and *UNIT 3.12*), and injection of mice (*UNIT 1.6*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2, 5% O_2 incubator unless otherwise indicated.

Electroporate A20 cells with GM-CSF plasmid DNA

1. Mix 20 µg of plasmid DNA and 800 µl (1×10^6) of A20 cells from an exponentially growing culture in RPMI medium in an ice-cooled electroporation cuvette (0.4-cm electrode gap).
2. Incubate on ice 10 min.
3. Electroporate cells at 250 V and 960 mF using Bio-Rad Gene Pulser II System.
4. Incubate cells on ice for 10 min.
5. Wash cells by mixing in 10 ml cold complete RPMI/10% FBS medium and centrifuging 5 min at $200 \times g$ (1000 rpm in an H-1000B rotor), 4°C. Discard supernatant.
6. Resuspend cells in 2 ml complete RPMI/10% FBS and plate 100 µl (2×10^4 cells per well of a 96-well flat bottom plate). Incubate 2 days in tissue culture incubator.
7. Add 100 µl of corresponding selection medium (e.g., 0.25 µg/ml mycophenolic acid, 5 mM histidinol, or 500 µg/ml G418; see *UNIT 10.17A*), and incubate 1 day.
8. Gently remove the culture medium from wells and replace with 200 µl fresh selective medium. Change the medium once a week until clones begin to grow.

Test culture media and cell lysates by ELISA (UNIT 2.1) and by immunoblot hybridization (UNIT 8.10) using anti-GM-CSF mAb. Test functional activity of GM-CSF on MPRO cell, which is a GM-CSF dependent murine promyelocyte cell line (Tsai and Collins, 1993).

Assay for functional activity of GM-CSF produced by A20-transduced cells

9. Pipet 50 µl conditioned medium from GM-CSF-transduced cells growing in step 8 into triplicate wells of a 96-well tissue culture plate and dilute to 1:2 using complete DMEM/10% FBS. Dilute in parallel control conditioned medium from untransfected A20 cells and soluble GM-CSF starting from 20 ng/ml in the control conditioned medium.
10. Add into each well 50 µl (1×10^4) MPRO cells in DMEM/10% FBS and incubate for 48 hr at 37°C.
11. Pulse cells with 1 µCi [^{3}H]thymidine (*APPENDIX 3D*).
12. Harvest cells or store plates at −20°C overnight.
13. Measure thymidine incorporation on a β scintillation counter (*UNIT 3.12* and *APPENDIX 3D*).

IN VIVO TUMOR PROTECTION AND IMMUNOTHERAPY EXPERIMENTS

In general, vaccine formulations can be tested either for their ability to elicit anti-tumor protection or for eradication of established tumor. Although, the latter is the more challenging task, both assays are feasible using 38c-13 and A20 lymphomas. However, use of the slower-growing lymphoma, A20, is recommended for immunotherapy experiments. Both types of in vivo experiments, tumor protection and therapy of established tumor, are described in this protocol. Moreover, the protocol starts with description of immunization strategies for tumor protection experiments using DNA ("a" steps), protein ("b" steps), and cytokine-transduced vaccines ("c" steps), followed by tumor challenge protocols, including a tumor challenge protocol for protection studies ("d" step; same as tumor challenge steps in Basic Protocol 1) and a detailed experiment for immunotherapy of established A20 lymphoma ("e" steps). All in vivo experiments should be repeated at least three times with 10 mice per experimental group (6- to 12-week-old syngeneic female mice). Ig-KLH, whole-cell, or chemokine-sFv fusion protein vaccines require immunization once or twice every two weeks, while DNA vaccines require three or more immunizations every two weeks. Sera (200 µl) are drawn from the retro-orbital spaces of five randomly selected mice per group before each boost and tested for the presence of anti-idiotypic antibodies by ELISA (Support Protocol 2), to monitor immunization, prior to tumor challenge.

Materials

6- to 12-week-old female C3H/HeN mice (for experiments with 38C13 lymphoma) or BALB/c mice (for experiments with A20 lymphoma)
Gene Gun cartridges coated with plasmid DNA vaccines (see Basic Protocol 2)
Protein vaccines: Ig-KLH (for positive control; see Support Protocol 3) and chemokine fusion scFv formulations (see Basic Protocol 3)
Dulbecco's phosphate-buffered saline (DPBS, without Ca^{2+} and Mg^{2+}; Life Technologies)
Tumor cells (also see Basic Protocol 1):
  A20 B cell lymphoma cells (ATCC)
  38C-13 B cell lymphoma (Bergman and Haimovich, 1977; gift of Ronald Levy, Stanford Univ.)
  Cytokine (e.g., GM-CSF) -transduced or modified A20 cells
Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS; *APPENDIX 2*), ice-cold

Electric clippers
Helios Gene Gun (Bio-Rad)
Compressed Ultra Pure Helium gas; maximum pressure, 2600 psi – with high-pressure helium tank gas regulator
Hearing protection (e.g., ear plugs)
1-ml disposable syringes and 27.5-G needles
γ irradiator (e.g., Nordion Gammacell)
Sorvall centrifuge with H1000B swinging-bucket rotor, or equivalent

Additional reagents and equipment for immunization using Gene Gun (*UNIT 2.14*), handling and restraint (*UNIT 1.3*) and bleeding (*UNIT 1.7*) of mice, ELISA to measure anti-idiotypic antibody production (Support Protocol 2), injection of mice (*UNIT 1.6*), growth of tumor cells and tumor cell challenge (see Basic Protocol 1), counting viable cells using trypan blue exclusion (*APPENDIX 3B*)

NOTE: The gene gun and tank regulator are supplied as part of the complete Helios Gene Gun System (Bio-Rad) or can be purchased separately from Bio-Rad.

Immunization using gene gun delivery system

1a. Shave abdomens of mice with electric clippers prior to the immunizations.

2a. Assemble the Gene Gun and the cartridges with DNA-coated gold and deliver immunizations using helium pressure at 400 psi as described in *UNIT 2.14*. Deliver four shots for every mouse per immunization. Use hearing protection, e.g., ear plugs, while operating Gene Gun.

3a. Administer booster immunization to the mice two or three more times by repeating steps 1a and 2a after 2 weeks, and if desired 2 weeks after that.

4a. Collect sera at days 28 and 42 from the retro-orbital space (*UNIT 1.7*) and perform ELISA to assess anti-Id antibody production (Support Protocol *2*).

Immunization with protein vaccines

1b. Adjust chemokine-scFv protein concentration to 0.5 mg/ml and Ig-KLH (positive control) to 0.25 mg/ml in ice-cold DPBS.

2b. Inject each mouse (*UNIT 1.6*) with 200 µl intraperitoneally (i.p.) or subcutaneously (s.c.) with the vaccine or control preparation using a 1-ml disposable syringe and 27.5-G needle. Perform booster immunization after 2 weeks.

Optimal immune response is elicited after four immunizations of chemokine-scFv, while a single immunization is sufficient for Ig-KLH.

3b. Collect sera on days 28, 42, and 56 from the retro-orbital space (*UNIT 1.7*) and perform ELISA to assess anti-Id antibody production (Support Protocol 2).

Immunization with cytokine transduced or modified A20 cells

1c. Grow A20 lymphoma cells transduced with cytokine gene such as GM-CSF (see Basic Protocol 1, steps 1 to 4). Ensure that are in the logarithmic growth phase when harvesting for injection, i.e., that flasks are <50% confluent.

2c. Centrifuge cells 5 min at 200 × *g* (1000 rpm in H-1000B rotor), 4°C, in 50-ml Falcon tubes. Aspirate supernatant and resuspend pellet in 40 ml ice-cold HBSS (without Ca^{2+} and Mg^{2+}). Repeat the wash a second time.

3c. Resuspend cell pellet in 10 ml ice-cold HBSS and count viable cells using trypan blue exclusion (*APPENDIX 3B*).

Viability should be >90%.

4c. Adjust cell concentration to 1×10^7/ml in ice-cold HBSS. Irradiate at 5000 rad using a γ irradiator.

Keep cells on ice.

5c. Inject each mouse s.c. with 100 µl of 1×10^6 irradiated cells using a 1-ml disposable syringe and 27.5-G needle.

Tumor challenge for protection studies

1d. At 3 days prior to challenge, thaw frozen cells and begin growing them (see Basic Protocol 1, steps 1 to 4). When incubation is completed, prepare cell suspension (see Basic Protocol 1, steps 5 to 7) and inject mice (see Basic Protocol 1, step 8). Begin checking mice for tumor growth and survival 10 to 12 days after challenge with 38C-13 and 25 days after challenge with A20 cells.

The minimal lethal dose of tumor should be calibrated for each stock of tumor cells. As few as 100 38C-13 cells can kill a syngeneic mouse. A20 tumor cell dose can be increased to

1×10^6, particularly for i.v. challenge. 38C-13 tumor cells usually grow rapidly as ascites, and mice may die within 2 to 3 days after the first signs of tumor growth. Both 38C-13 and A20 injected s.c. often develop large tumor nodules, which may become necrotic and begin to ulcerate or bleed; it is advisable to sacrifice the mice before this point. Data from at least three independent experiments, with 10 mice per group, are analyzed by Kaplan-Meier statistics (BMDP Statistical Software).

Immunotherapy of established A20 lymphoma

1e. At 3 days prior to challenge, thaw frozen A20 cells and begin growing them (see Basic Protocol 1, steps 1 to 4).

2e. On day 0, prepare cell suspension (see Basic Protocol 1, steps 5 to 7) and inject 2.5 $\times 10^5$ A20 cells per mouse i.p. using 1-ml disposable syringes with 27.5-G needles (also see Basic Protocol 1, step 8).

3e. Between day 1 and 4, begin immunizing mice as described above in steps 1a to 4a, 1b to 3b, or 1c to 5c.

4e. Perform booster immunizations, repeating step 3e on days 4, 8 and 18.

5e. Collect sera on day 28 from the retro-orbital spaces (*UNIT 1.7*), and perform ELISA to assess anti-Id antibody production (Support Protocol 2).

6e. Begin checking mice for tumor growth and survival 25 days after challenge with A20 cells.

A20 cells grown in mice often develop large tumor nodules, which may become necrotic and begin to ulcerate or bleed; it is advisable to sacrifice the mice before this point. Animals are killed upon development of tumor (increasing abdominal girth and palpable abdominal mass). Data from at least three independent experiments, with 10 mice per group, are analyzed by Kaplan-Meier statistics.

ELISA TO DETERMINE PROPER FOLDING OF scFv PROTEIN

SUPPORT PROTOCOL 1

Folding of scFv is assessed by ability of scFv to compete for binding of anti-Id monoclonal antibody (S1C5, anti-38C-13 Id) to intact Ig protein. Sera from mice immunized with prototype Ig-KLH protein vaccines are a good source of polyclonal anti-Id antibodies, if monoclonal antibodies not available. Anti-idiotypic sera are obtained from mice immunized intraperitoneally or subcutaneously with 50 µg of lymphoma derived Ig cross-linked with KLH (Campbell et al., 1990; also see Support Protocol 3).

Materials

10 µg/ml lymphoma-derived IgM (for 38C-13) or IgG2a (for A20): available from Kwak Laboratory (*kwak@mail.ncifcrf.gov*)
Polyclonal anti-idiotypic sera or monoclonal 38C-13 Id-specific antibody S1C5
Coating buffer, pH 9.6 (*UNIT 7.12*)
ELISA wash buffer (see recipe)
5% (w/v) fat-free powdered milk in PBS (see *APPENDIX 2* for PBS)
Diluent buffer: 2% (w/v) BSA in PBS
scFv protein (see Basic Protocol 3)
Horseradish peroxidase (HRPO)–conjugated goat anti-mouse IgG or IgG_1 antibody (Caltag)
ABTS peroxidase substrate (Kirkegaard & Perry)

96-well flat-bottom ELISA plates (e.g., Immunol 4, Dynatech) and plate sealers
96-well U-bottom tissue culture plates (Costar)
Multiwell scanning spectrophotometer

Additional reagents and equipment for ELISA (*UNIT 2.1*)

1. Coat 96-well flat bottom plates with 10 µg/ml (50 µl per well) of 38C-13 IgM or A20 IgG2a in coating buffer for 2 hr at 37°C or overnight at 4°C.

   General instructions on coating, washing, and other routine ELISA procedures are found in UNIT 2.1.

2. Wash plates 3 to 4 times with ELISA wash buffer.
3. Block wells with 100 µl 5% dry milk in PBS for 60 min at room temperature.
4. In a separate 96-well U-bottom plate incubate 50 µl of corresponding anti-Id mAb or polyclonal anti-idiotypic serum (diluted 1:500) mixed with serially diluted amounts of scFv protein (starting from 50 µg/ml) in diluent buffer for 20 min.
5. Discard the blocking solution from the 96-well flat-bottom plate in step 3.
6. Transfer 50 µl/well of the scFv/anti-Id Ab mixture from the U-bottom plate in step 4 into the corresponding wells of the flat-bottom plate in step 5.
7. Incubate for 40 min at room temperature.
8. Wash plate as in step 2.
9. To each well, add 50 µl HRPO-conjugated anti-mouse goat IgG antibody at a 1:4000 dilution in diluent buffer.

   It is important to use highly specific secondary antibody which does not bind to IgM or IgG2a, respectively, coating the plates.

10. Incubate 40 min at room temperature.
11. Wash plate as in step 2.
12. Add 70 µl of ABTS peroxidase substrate solution.
13. Measure OD at 405 nm upon color development.

    SIC5 monoclonal antibody or polyclonal anti-Id sera from IgM immunized mice binds properly folded idiotype of 38C13 IgM. Therefore, if the folding of scFv is correct, it should compete with binding of SIC5 to IgM and decrease the optical density at 405 nm in a dose-dependent manner. In contrast, the optical density at 405 nm would remain unchanged in the case of incorrectly folded scFv or irrelevant Id.

SUPPORT PROTOCOL 2

ELISA TO MEASURE ANTI-IDIOTYPIC ANTIBODY PRODUCTION IN IMMUNIZED MICE

This protocol is used to confirm the presence of and to qualitatively measure anti-idiotypic antibody in the serum of 38C-13- or A20-immunized mice.

Additional Materials *(also see Support Protocol 1)*

Serum from 38C-13 or A20-immunized mice

1. Coat, wash, and block ELISA plate (see Support Protocol 1, steps 1 to 3).
2. Discard blocking solution. Perform serial dilutions of serum in the wells of the plate, starting with 1:3 dilutions of serum.
3. Incubate for 1 hr at room temperature.
4. Wash plate as in step 2 of Support Protocol 1.

5. Add 50 µl of appropriate secondary HRPO-conjugated anti-mouse IgG antibody in diluent buffer.

   It is important to use highly specific secondary antibody which does not bind to IgM or IgG2a, respectively, coating the plates.

6. Incubate 40 min at room temperature.
7. Wash plate as in step 2 of Support Protocol 1.
8. Add 70 µl of ABTS peroxidase substrate solution.
9. Measure OD at 405 nm upon color development.

   Efficacy of the vaccine is judged by its ability to elicit polyclonal antibodies that recognize parental-tumor-derived Ig, but not irrelevant isotype-matched immunoglobulin. Therefore, it is recommended that highly pure Ig be used for coating of ELISA plates, to avoid a nonspecific cross-reaction.

CHEMICAL CROSS-LINKING OF KLH WITH LYMPHOMA-DERIVED Ig

SUPPORT PROTOCOL 3

Intact lymphoma Ig protein can be rendered immunogenic by conjugation with keyhole limpet hemocyanin (KLH). Ig-KLH vaccination (50 µg Ig/mouse) can provide a positive control for in vivo protection experiments (see Support Protocol 4).

Materials

KLH in saturated ammonium sulfate slurry (Calbiochem-Novabiochem)
IgM derived from 38C-13 lymphoma (Eshar et al., 1979; Maloney et al., 1985) or IgG2a from A20 (hybridoma is available from the Kwak laboratory; *kwak@mail.ncifcrf.gov*)
Glutaraldehyde (Sigma)
Dulbecco's PBS (without Ca^{2+} and Mg^{2+}; Life Technologies; also see *APPENDIX 2A*)
Dialysis membrane, MWCO 10,000 (Spectra/Por; Spectrum)

Additional reagents and equipment for dialysis (*APPENDIX 3H*)

1. Separately dialyze solutions of 5 mg/ml Ig and 5 mg/ml KLH against 1 liter of Dulbecco's PBS overnight at 4°C using a 10,000 MWCO dialysis membrane.
2. Dilute each solution to 1 mg/ml with cold Dulbecco's PBS.
3. Mix equal volumes of KLH and Ig.
4. Add glutaraldehyde to give a final concentration of 0.1% (w/v).
5. Rock or stir slowly at room temperature for 4 hr.
6. Dialyze extensively (3 times) against 1 liter of Dulbecco's PBS.
7. Divide Ig-KLH into 2-ml aliquots and store frozen up to several years at −20°C.

   The mixture may turn yellowish, and precipitation may occur upon dialysis or freezing and thawing.

REAGENTS AND SOLUTIONS

Use endotoxin-free ultra-pure distilled water, tissue culture grade (Advanced Biotechnologies) in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***APPENDIX 5****.*

ELISA wash buffer, 10×

108 g NaCl
6 ml Triton X-100
Add H_2O to 1.2 liters
Dilute 1:10 before use
Store up to 1 year at room temperature

Glycerol column buffers, with 50 mM, 300 mM, and 1 M NaCl

5% (v/v) glycerol
50 mM, 300 mM, or 1 M NaCl
20 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
Store up to 1 month at 4°C

Glycerol growth medium

Superbroth (see recipe) supplemented with:
1% (v/v) glycerol
100 µg/ml carbenicillin
50 µg/ml ampicillin
Store up to 1 month at 4°C

Guanidinium solution, 6 M

6 M guanidinium·HCl
100 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)
2 mM EDTA, pH 8.0 (*APPENDIX 2*)
Store up to 6 months at room temperature

Induction medium

Superbroth (see recipe) supplemented with:
0.8 mM isopropyl β-D-thiogalactopyranoside (IPTG)
100 µg/ml carbenicillin
50 µg/ml ampicillin
Prepare fresh

Refolding solution

100 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)
0.5 M L-arginine-HCl
4 mM oxidized glutathione (GSSG; Boehringer Mannheim)
2 mM EDTA, pH 8.0 (*APPENDIX 2*)
Prepare fresh

Solution D

4 M guanidinium thiocyanate
25 mM sodium citrate
0.5% Sarkosyl (*N*-laurylsarcosine)
0.1 M 2-mercaptoethanol
Store up to 6 months at room temperature

Superbroth

Per liter:
32 g tryptone
20 g yeast extract
5 g NaCl
5 ml 1 N NaOH
Store up to 1 year at 4°C

TE buffer for proteins

50 mM Tris·Cl, pH 7.4 (*APPENDIX 2*)
20 mM EDTA
Store up to 6 months at 4°C

COMMENTARY

Background Information

Murine transplantable lymphomas generally serve well as models for human lymphomas, based on their mature B cell immunophenotype and widespread patterns of dissemination. However, almost all of the available tumor lines display aggressive growth characteristics in vivo. Therefore, they resemble the aggressive, rather than indolent, human B cell lymphomas. Indeed, it has been difficult to demonstrate therapeutic efficacy of either passive or active immunotherapy against pre-existing tumor burdens, because these tumors grow very quickly (mean doubling time in vitro 12 hr for 38C-13). However, idiotype vaccination, combined with non-curative doses of chemotherapy designed to slow tumor growth, has proven successful in some cases (Campbell et al., 1990, Kwak et al., 1996).

Lynch and Eisen (Lynch et al., 1972; Sirisinha and Eisen, 1971) first demonstrated the phenomenon of idiotype-specific tumor resistance against MOPC plasmacytomas. Successful immunization required formulation of idiotype protein with complete Freund's adjuvant. The underlying cellular mechanism of idiotype-specific tumor resistance in the MOPC plasmocytoma models appeared to be primarily T cell mediated. Support for this conclusion came from the studies of Jorgensen et al. (1980), who reported a correlation of tumor resistance with proliferation of splenocytes in vitro after immunization with light chain protein alone, without apparent antibody production. Subsequently, $CD4^+$ T cells from such immunized mice were successfully cloned and demonstrated to be sufficient to mediate tumor resistance by adoptive transfer experiments (Bogen and Weiss, 1993).

The phenomenon of idiotype-specific tumor resistance was subsequently reproduced in a number of leukemia and lymphoma models (see Biragyn and Kwak, 1999 for a review). However, while idiotype specific protective anti tumor immunity appears to be primarily dependent upon T cell effector mechanisms in the MOPC and other murine myeloma models, the opposite appears to be the case for most murine lymphomas. Specifically, the preponderance of evidence indicates that idiotype-specific tumor resistance in the 38C-13 lymphoma model is dependent upon antibody production. Adoptive transfer of immune serum, but not immune splenocytes, can provide tumor protection in this model (Campbell et al., 1990). Moreover, induction of anti-idiotypic antibodies often correlates with tumor protection in 38C-13 model, although the magnitude of protection does not strictly depend on the absolute levels of antibodies produced. A broad range of serum antibody levels can be observed, depending on the immunogen, from less than 1 μg/ml to greater than 500 μg/ml. Although the role of CTL capable of lysing 38C-13 tumor cells is still unclear, $CD8^+$ effector T cells induced by Id-KLH plus GM-CSF or by genetic vaccinations are required in the 38C-13 model, as demonstrated by in vivo T cell–subset depletion experiments (Campbell et al., 1990, Kwak et al., 1996, Biragyn et al., 1999). The mechanism of protective immunity in the A20 model elicited by idiotype vaccination remains to be elucidated, but preliminary data indicate that dependence on T cell immunity is predominant. For example, generation of serum anti-idiotypic antibodies does not correlate with tumor protection in the A20 model (Biragyn et al., 1999). Finally, idiotype protein vaccines combined with GM-CSF elicited $CD8^+$ CTL in human patients (Bendandi et al., 1999).

The authors of this unit have emphasized a novel vaccine approach which is based on use

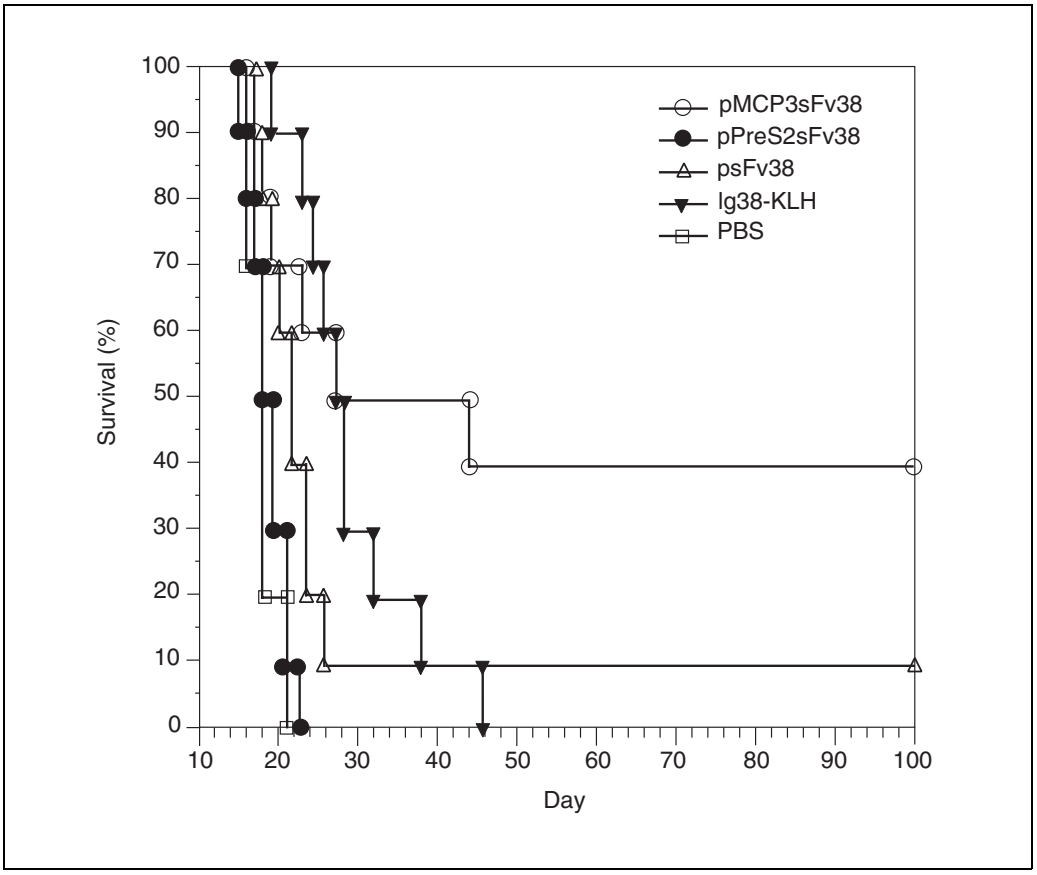

Figure 20.6.3 Immunization with DNA vaccines encoding chemokine-fused sFv elicit protective anti-tumor immunity (a representative experiment). Ten syngeneic C3H/HeN mice per group were immunized three times biweekly by gene gun with 2 μg DNA constructs encoding MCP-3 fused with sFv38 (pMCP3sFv38), or, as negative controls, sFv38 fused with PreS2 domain of env HBV gene (pPreS2sFv38), sFv38 alone (psFv38), or PBS. As a positive control, one group of mice was immunized i.p. with 50 μg Ig-KLH. Two weeks after the last immunization, mice were challenged i.p. with 2000 38C-13 lymphoma cells from a single preparation of tumor and followed for survival.

of chemokine moiety to deliver antigens to APC, which differentially express chemokine receptors. However, many other approaches have been reported which also rendered nonimmunogenic idiotype immunogenic, such as chemical conjugation of idiotype protein with carriers such as KLH, human constant regions, or tetanus toxin fragment, as well as encapsulation in liposomes or genetic fusion to cytokines (Campbell et al., 1990; Kwak et al., 1998; Tao and Levy, 1993; King et al., 1998; Biragyn et al., 1999). The efficacy of the particular approach may also differ between lymphoma models. For example, A20 idiotype protein conjugated to KLH or Fv fragments expressed with human constant regions do not induce protective immunity, while chemokine-fused Fv elicited protective and therapeutic immunity in this model (Biragyn et al., 1999). For other lymphoma models, such as BCL1, and MOPC plasmocytoma models, it is not clear whether conjugation with an immunogenic carrier is required. The efficacy of cellular vaccines still needs to be tested more broadly. Although dendritic cell–based vaccines showed modest efficacy in the BCL1 model (Flamand et al., 1994), they did not work well in the 38C-13 model (Lespagnard et al., 1998).

Critical Parameters

Tumor protection studies for 38C-13 and A20 tumors are performed using six to 12-week-old syngeneic female mice (C3H/HeN and BALB/c mice, respectively). All in vivo experiments should be repeated at least three times with 10 mice per experimental group. Negative control groups should include mice vaccinated with irrelevant scFv- or Ig- vaccine formulations (see Basic Protocols 2 and 3). Ig-KLH protein vaccines (50 μg in 200 ml PBS, administered once intraperitoneally; see Support Protocol 4) are usually used as a positive

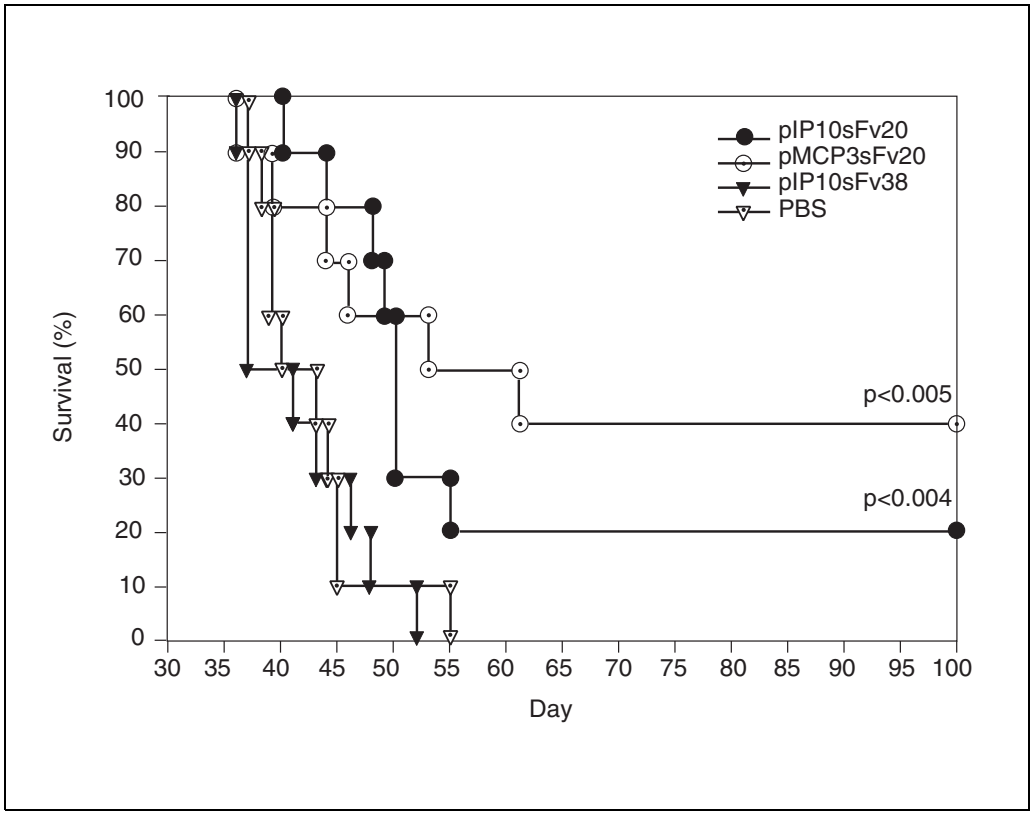

Figure 20.6.4 Immunotherapy of established A20 lymphoma with DNA vaccines encoding chemokine fused scFv. BALB/c mice were vaccinated at days 1, 4, 8 and 18 with DNA encoding MCP-3 or IP-10 fusion with A20 tumor–derived scFv (pMCP3sFv20 and pIP10sFv20, respectively) after injection of a lethal dose of A20 tumor on day 0 (see Basic Protocol 5). Controls received a DNA vaccine encoding chemokine fusion with scFv from the 38C-13 tumor (pIP10sFv38). Differences in survival between groups was determined by nonparametric logrank test (BMDP Statistical Software). P-values refer to comparison with pIP10sFv38.

control formulation. DNA vaccines might require at least three biweekly immunizations. Sera (200 µl) are drawn from the retro-orbital space of 5 randomly selected mice per group before each boost and tested for the presence of anti-idiotypic antibodies by ELISA (Support Protocol 2).

Prior to conducting in vivo experiments, it is advisable to perform a few preliminary experiments to determine the optimal lethal dose of tumor in syngeneic mice. The dose should be high enough to kill all control-treated mice, yet not too high to overcome the effects of an experimental therapy. For example, the authors routinely use 20 times the minimum lethal dose of 38C-13 tumor. Moreover, if the therapeutic target is idiotype, tumor surface Ig expression should be confirmed by flow cytometric staining (*UNIT 5.4*) and verified by RT/PCR amplification and DNA sequencing of the V genes. Tumor cells should be stored frozen in working aliquots in liquid nitrogen. Thereafter, mice are challenged each time with a fresh aliquot of cells from the same stock, grown for 3 to 4 days in culture.

Although the cloning procedures presented here are very efficient, it is advisable to verify the resulting constructs by DNA sequencing. Mutations are incorporated relatively often, particularly after PCR amplification, and additional mutations can accumulate or be selected out by *E. coli*, particularly if the gene product is toxic for bacteria. Moreover, DNA vaccine candidates should be tested by transient expression in COS-7 cells. Relatively high yields of scFv protein can be obtained by expressing and purifying them as insoluble bacterial inclusion bodies. This is a much simpler and more efficient procedure than purification of secreted soluble proteins from bacteria.

As described above, anti-lymphoma immunity may depend on efficient induction of both

anti-idiotypic humoral and cellular responses. Therefore, scFv and its fusions should retain the conformation of the parental idiotype. It is therefore advisable to verify the conformation of scFv fusion proteins—for example, by their ability to inhibit binding of parental native Ig to monoclonal or polyclonal anti-idiotypic antibodies.

Genetic vaccination is very simple, fast, and effective. However, the efficacy of gene-gun vaccinations depends on the quality of DNA cartridges used. Overall, the quality of cartridge-producing tubing is quite variable. Therefore, it is advisable to test each lot of new tubing. Finally, the cartridges should be evenly coated with DNA-gold particles.

Anticipated Results

The experimental vaccines described herein have been shown to consistently elicit protective antitumor immunity, and a brief study outline is shown in Fig. 20.6.1. B cell idiotype alone is usually nonimmunogenic in syngeneic mice, and therefore no tumor protection or survival is elicited in mice vaccinated with Ig or scFv protein or DNA constructs. However, the use of various adjuvants or carriers may render lymphoma-derived Ig or scFv immunogenic (Lynch et al., 1972). Mice vaccinated with Ig-KLH protein elicit up to 20% protection and tumor-free mice when challenged with high doses of syngeneic 38C-13 cells (Fig. 20.6.3). Moreover, vaccinations with DNA constructs encoding scFv fusions with MCP-3 (Fig. 20.6.3) or IP-10 elicit up to 40% to 60% survival and tumor-free mice. Immunotherapy in the 38C-13 model may not be effective unless it is combined with chemotherapy (e.g., Kwak et al., 1996). However, in the A20 lymphoma model, immunotherapy with scFv fusion protein or DNA vaccines encoding scFv fusion with MCP-3 or IP-10 (see Fig. 20.6.4) elicit about 40% tumor-free mice. Interestingly, the Ig-KLH vaccine does not elicit either protective or therapeutic immunity in the A20 model.

Time Considerations

Murine lymphomas grow relatively fast in syngeneic mice, with median survival times of 14 and 40 days in normal, syngeneic mice given 38C-13 and A20, respectively. The molecular design and cloning is a relatively rapid process, which takes 1 to 2 weeks. Immunization-challenge experiments require about 60 to 100 days, if mice are routinely immunized biweekly three times. Recombinant protein production and purification could require 1 to 2 months work.

Literature Cited

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) 2000. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Bartnes, K. and Hannestad, K. 1997. Engagement of the B lymphocyte antigen receptor induces presentation of intrinsic immunoglobulin peptides on major histocompatibility complex class II molecules. *Eur.J.Immunol.* 27:1124-1130.

Bendandi, M, Gocke, C.D., Kobrin, C.B., Benko, F.A., Sternas, L.A., Pennington, R, Watson, T.M., Reynolds, C.W., Gause, B.L., Duffey, P.L., Jaffe, E.S., Creekmore, S.P., Longo, D.L., and Kwak, L.W. 1999. Complete molecular remissions induced by patient-specific vaccination plus granulocyte-monocyte colony-stimulating factor against lymphoma. *Nature Med.* 5:1171-1177.

Bergman Y. and Haimovich, J. 1977. Characterization of a carcinogen-induced murine B lymphocyte cell line of C3H/eb origin. *J.Immunol.* 7:413-417.

Biragyn, A. and Kwak, L.W. 1999. B-cell malignancies as a model for cancer vaccines: From prototype protein to next generation genetic chemokine fusions. *Immunol. Reviews* 170:115-126.

Biragyn, A., Tani, K., Grimm, M.C., Weeks, S.D., and Kwak, L.W. 1999. Genetic fusion of chemokines to a self tumor antigen induces protective, T-cell dependent antitumor immunity. *Nature Biotechnol.* 17:253-258.

Blasi, E., Matthieson, B.J., and Varesio, L. 1985. Selective immortalization of murine macrophages from fresh bone marrow by a *raf/myc* recombinant murine retrovirus. *Nature* 318:667-670.

Bogen, B. and Weiss, S. 1993. Processing and presentation of idiotypes to MHC-restricted T cells. *Int.Rev.Immunol.* 10:337-355.

Buchner, J., Pastan, I., and Brinkmann, U.A. 1992. method for increasing the yield of properly folded recombinant fusion proteins: Single-chain immunotoxins from renaturation of bacterial inclusion bodies. *Anal. Biochem.* 205:263-270.

Campbell, M.J., Esserman, L., Byars, N.E., Allison, A.C., and Levy, R. 1990 Idiotypic vaccination against murine B cell lymphoma. Humoral and cellular requirements for the full expression of antitumor immunity. *J. Immunol.* 145:1029-1036.

Campbell, M.J., Carroll, W., Kon, S., Thielemans, K., Rothbard, J.B., Levy, S., and Levy, R. 1987. Idiotype vaccination against murine B cell lymphoma: Humoral and cellular responses elicited by tumor-derived immunoglobulin M and its molecular subunits. *J. Immunol.* 139:2825-2833.

Carroll, W.L., Starnes, C.O., Levy, R., and Levy, S. 1988. Alternative V kappa gene rearrangements in a murine B cell lymphoma. An explanation for idiotypic heterogeneity. *J. Exp. Med.* 168:1607-1620.

Chen, T.T., Tao, M.-H., and Levy, R. 1994. Idiotype-cytokine fusion proteins as cancer vaccines. Relative efficacy of IL-2, IL-4, and granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 153:4775-4787.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.

Dyke, R.J., McBride, H., George, A.J., Hamblin, T.J., and Stevenson, F.K. 1991. Idiotypic vaccination against B-cell lymphoma leads to dormant tumour. *Cell Immunol.* 132: 70-83.

Eshar, Z., Blatt, C., Bergman, Y., and Haimovich, J. 1979. Induction of secretion of IgM from cells of the B cell line 38C13 by somatic cell hybridization. *J. Immunol*. 122:2430.

Flamand, V., Sornasse, T., Thielemans, K., Demanet, C., Bakkus, M., Tielemans, F., Leo, O., Urbain, J., and Moser, M. 1994. Murine dendritic cells pulsed in vitro with tumor antigen induce tumor resistance in vivo. *Eur.J. Immunol.* 24:605-610.

French, R.R., Chan, H.T.C., Tutt, A.L. and Glennie, M.J. 1999. CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. *Nature Med.* 5: 548-553.

Hakim, I., Levy, S., and Levy, R. 1996. A nine-amino acid peptide from IL-1beta augments antitumor immune responses induced by protein and DNA vaccines. *J.Immunol.* 157: 5503-5511.

Hawkins, R.E., Winter, G., Hamblin, T.J., Stevenson, F.K., and Russell, S.J. 1993. A genetic approach to idiotypic vaccination. *J. Immunotherapy* 14: 273-278.

Jorgensen, T., Gaudernack, G., and Hannestad, K. 1980. Immunization with the light chain and the V_L domain of the isologous myeloma protein 315 inhibits growth of mouse plasmacytoma MOPC315. *Scand. J. Immunol.* 11:29-35.

Kim, K.J., Kanellopoulos, Langevin C., Merwin, R.M., Sachs, D.H., and Asofsky, R. 1979. Establishment and characterization of BALB/c lymphoma lines with B cell properties. *J.Immunol.* 122:549-554.

King, C.A., Spellerberg, M.B., Zhu, D., Rice, J., Sahota, S.S., Thompsett, A.R., Hamblin, T.J., Radl, J., and Stevenson, F.K. 1998. DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma [see comments]. *Nature Med.* 4:1281-1286.

Krolick, K.A., Isakson, P.C., Uhr, J.W., and Vitetta, E.S. 1979. BCL1, a murine model for chronic lymphocytic leukemia: Use of the surface immunoglobulin idiotype for the detection and treatment of tumor. *Immunol. Rev.* 48:81-106.

Kwak, L.W., Young, H.A., Pennington, R.W., and Weeks, S.D. 1996. Vaccination with syngeneic, lymphoma-derived immunoglobulin idiotype combined with granulocyte/macrophage colony-stimulating factor primes mice for a protective T-cell response. *Proc. Natl. Acad. Sci. U.S.A.* 93:10972-10977.

Kwak, L.W., Pennington, R., Boni, L., Ochoa, A.C., Robb, R.J., and Popescu, M.C. 1998. Cutting Edge: Liposomal formulation of self lymphoma antigen induces potent antitumor immunity. *J. Immunol.* 160:3637-3641.

Lespagnard, L., Mettens, P., Verheyden, A.M., Tasiaux, N., Thielemans, K., van Meirvenne, S., Geldhof, A., Baetselier, P. Urbain, J., Leo, O. and Moser, M. 1998. Dendritic cells fused with mastocytoma cells elicit therapeutic antitumor immunity. 1998; *Int. J. Cancer* 76:250-258.

Levitsky, H.I., Montgomery, J., Ahmadzadeh, M., Staveley-O'Carroll, K., Guarnieri, F., Longo, D.L., and Kwak, L.W. 1996. Immunization with granulocyte-macrophage colony-stimulating factor-transduced, but not B7-1-transduced, lymphoma cells primes idiotype specific T cells and generates potent systemic antitumor immunity. *J. Immunol.* 156: 3858-3865.

Lynch, R.G., Craff, R.G., Sirisinha, S., Simms, E.S, and Eisen, H.N. 1972. Myeloma proteins as tumor-specific transplantation antigens. *Proc. Natl. Acad. Sci. U.S.A.* 69:1540-1544.

Maloney, D.G., M.S. Kaminski, D. Burowski, J. Haimovich, and R. Levy. 1985. Monoclonal anti-idiotype antibodies against the murine B cell lymphoma 38C13: Characterization and use as probes for the biology of the tumor in vivo and in vitro. *Hybridoma* 4:191-209.

Manetti, C., Rouvier, E., Gautherot, E., Loucif, E., Barbet, J., and Le Doussal, J.M. 1997. Targeting BCL1 lymphoma with anti-idiotype antibodies: Biodistribution kinetics of directly labeled antibodies and bispecific antibody-targeted bivalent haptens. *Int. J. Cancer* 71:1000-1009.

Sato, K. and A. Ochi. 1998. Inhibition of B-cell receptor-antigen complex internalization by FcγRIIB1 signals. *Immunol. Lett.* 61:135-143.

Sirisinha, S. and Eisen, H.N. 1971. Autoimmune-like antibodies to the ligand-binding sites of myeloma proteins. *Proc. Natl. Acad. Sci. U.S.A.* 68:3130-3135.

Syrengelas, A.D., Chen, T.T., and Levy, R. 1996. DNA immunization induces protective immunity against B-cell lymphoma. *Nature Med.* 2:1038-1041.

Tao, M-H. and Levy, R. 1993. Idiotype/granulocyte-macrophage colony-stimulating factor fusion protein as a vaccine for B-cell lymphoma. *Nature* 362:755-758.

Tsai, S. and Collins, S.J. 1993. A dominant negative retinoic acid receptor blocks neutrophil differentiation at the promyelocyte stage. *Proc. Natl. Acad. Sci. U.S.A*90:7153-7157.

Tutt, A.L., French, R.R., Illidge, T.M., Honeychurch, J., McBride, H.M., Penfold, C.A., Fearon, D.T., Parkhouse, R.M., Klaus, G.G., and Glennie, M.J. 1998. Monoclonal antibody therapy of B cell lymphoma: Signaling activity on tumor cells appears more important than recruitment of effectors. *J. Immunol.* 161:3176-3185.

Weiner, G.J. and M.S. Kaminski. 1990. Anti-idiotypic antibodies recognizing stable epitopes limit the emergence of idiotype variants in a murine B cell lymphoma. *J. Immunol.* 144:2436-2445.

Contributed by Arya Biragyn and
Larry W. Kwak
National Cancer Institute
Frederick, Maryland