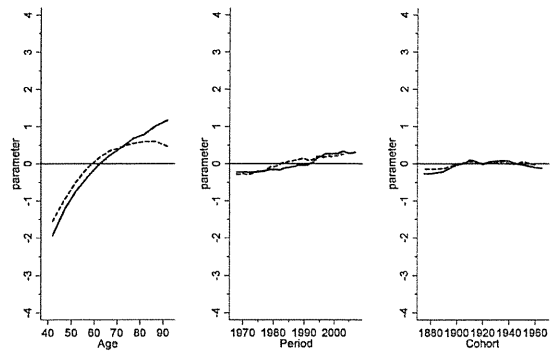

Figure 10. Age, Period and Birth Cohort Effects of Oral/ Pharynx Cancer in Both Sexes in Osaka, Japan

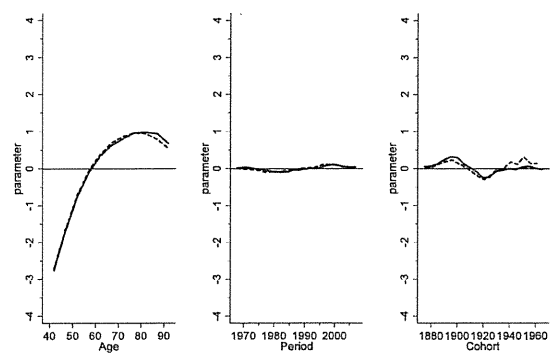

Figure 11. Age, Period and Birth Cohort Effects of Esophageal Cancer in Both Sexes in Osaka, Japan

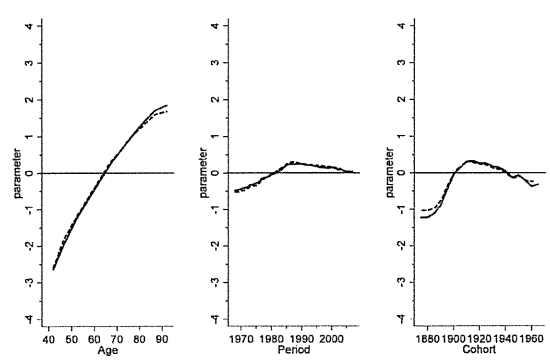

Figure 12. Age, Period and Birth Cohort Effects of Gallbladder Cancer in Both Sexes in Osaka, Japan

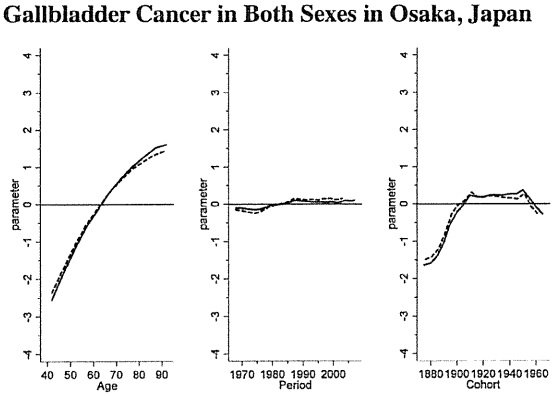

Figure 13. Age, Period and Birth Cohort Effects of Pancreas Cancer in Both Sexes in Osaka, Japan

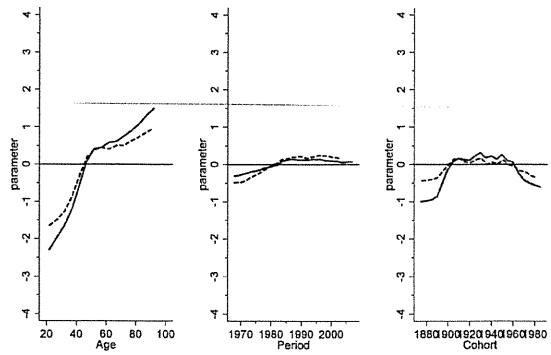

Figure 14. Age, Period and Birth Cohort Effects of Ovarian Cancer in Females in Osaka, Japan

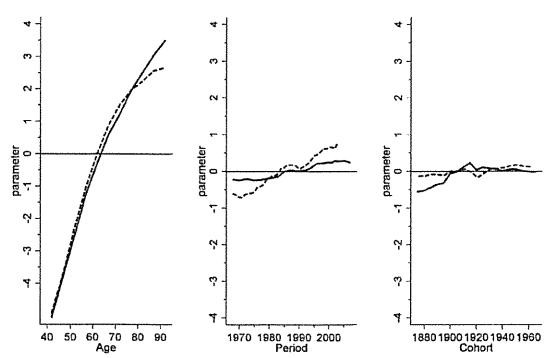

Figure 15. Age, Period and Birth Cohort Effects of Prostate Cancer in Males in Osaka, Japan

increased again after the 1950s birth cohort, while the effects for mortality still decreased. Parameters for age-cohort effects are illustrated in Figure 9.

Other sites of cancer

For oral and pharyngeal cancer in both sexes, increasing period effects were observed for both incidence and mortality (Figure 10). The cohort effects for oesophageal cancer were peaked at the cohort born in 1900s. Small dip was observed at the cohort born in the early 1920s, and then the cohort effects increased in the latest cohort (Figure 11). For gallbladder cancer, the period effects increased until the mid-1980s and then gradually decreased. The cohort effects were higher in the cohort born between 1900s and 1930s (Figure 12). For pancreas cancer, higher cohort effects were observed in the cohorts born in between 1910s and 1950s. (Figure 13)

The period effects were small for ovarian cancer. Decreased cohort effects were observed in the oldest and youngest cohorts. (Figure 14). For prostate cancer, largest aging effects were observed. Strongly increasing period effects were observed especially for incidence, while the cohort effects were small (Figure 15). Similar period effects were observed for trends in kidney cancer incidence with those in prostate cancer. The cohort effects increased until the cohort born in 1910s (Figure 16). For mortality of bladder cancer, the period effects were small. The cohort effects were peaked at the cohort

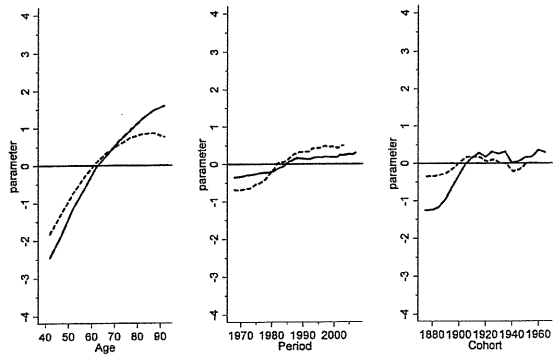

Figure 16. Age, Period and Birth Cohort Effects of Kidney Cancer in Both Sexes in Osaka, Japan

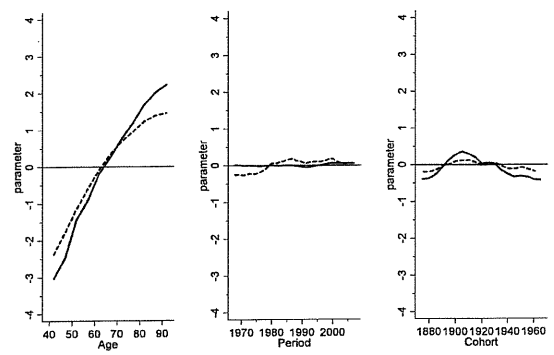

Figure 17. Age, Period and Birth Cohort Effects of Bladder Cancer in Both Sexes in Osaka, Japan

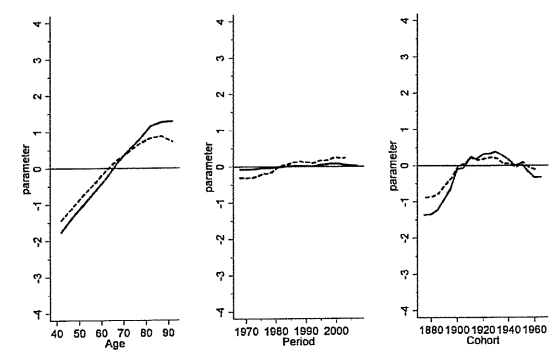

Figure 18. Age, Period and Birth Cohort Effects of Malignant Lymphoma in Both Sexes in Osaka, Japan

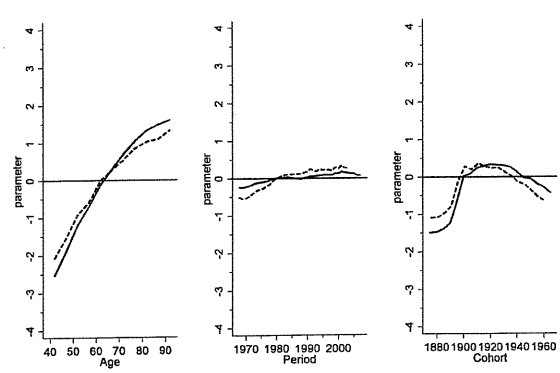

Figure 19. Age, Period and Birth Cohort Effects of Multiple Myeloma in Both Sexes in Osaka, Japan

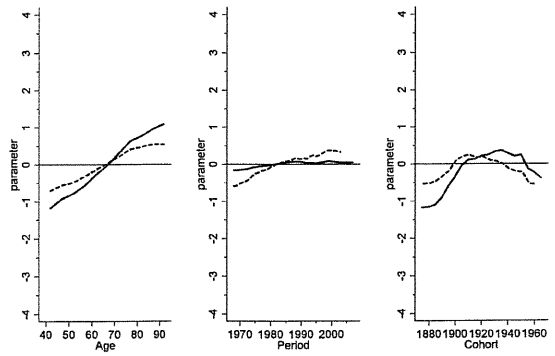

Figure 20. Age, Period and Birth Cohort Effects of Leukemia in Both Sexes in Osaka, Japan

born in 1900s, and then decreased (Figure 17).

Malignant lymphoma, multiple myeloma and leukaemia showed similar trends. Small increase in the period effects were observed for incidence trends but not for mortality. Cohort effects in the latest cohort decreased (Figure 18-20).

Discussion

Ageing effects for incidence and mortality are well-known in biological reason. For most sites of cancer, people increase the risk of growing cancer by ageing. Period effects reflect immediate effects to cancer incidence/mortality such as development of the effective treatment and screening programme. For all sites of cancer, the small period effects were observed. On the other hand, cohort effects reflect distant effects of risk factors such as smoking, dietary habits, and infectious agents. The cohort effects for all sites of cancer showed small peak at the 1950s birth cohort and decreased in the younger generation. The declining cohort effect for incidence and mortality may be mainly related with the decrease of prevalence of cancer risk factors. But the risk factors of cancer varied according to site of cancer. We need precise monitor of the trends by site, comparing with trends in the prevalence of each risk factor.

Remarkable cohort effects strongly related with decrease of the prevalence of the risk factor for stomach cancer, due to improvement of hygiene, the decrease of salt intake (Ministry of Health Labour and Walfare 1975-2007) and the prevalence of *H. pylori* infection (Haruma et al. 1997; Kobayashi et al. 2004). As a result, both age-standardised incidence and mortality rates also showed constant decrease. Small period effects indicated that there was little improvement of treatment and early detection, which should have showed immediate effect for stomach cancer.

Increasing age-standardised incidence and mortality of colorectal cancer until the mid-1990s would be explained as a result of increasing period and cohort effects. Increases of meat intake, obesity and less physical activities were risk factors of colorectal cancer. The prevalence of these risk factors is increasing in Japan, because of the change to Western lifestyle. Smoking is also one of the risk factors of colorectal

cancer. We need further investigation whether the trends of the prevalence of these risk factors corresponded with the trends in incidence and mortality. Trends of period effect indicated possibility of the immediate effect of the risk factor (Westernised lifestyle) to colorectal cancer incidence. This was confirmed at the previous study that Japanese immigrants in the US had higher incidence of colon cancer than Japanese in Japan (Haenszel and Kurihara, 1968; Shimizu et al., 1987). Improvement of diagnostic tools and treatments would be also related with the period effects.

With the liver the earlier increase in period effect could be the influence of improved diagnosis of liver cancer due to the development of diagnostic tools such as ultrasound sonography. The recent decrease was possibly caused by the effect of treatment for viral hepatitis. The influence of the prevalence of risk factors on cohort effects is clearly shown. The birth cohort born in around 1935 was suggested to show highest prevalence of HCV antibodies. The prevalence decreased in the younger generation (Tsukuma et al., 2005). As previous descriptive studies have reported, the highest cohort effect of incidence and mortality of liver cancer was observed at the cohort with the highest prevalence of HCV. The prevalence of HCV has been decreasing, so the incidence of liver cancer will continue to decrease.

Regarding the lung, the observed small period effect suggested that there was no change from immediate effects, such as development of treatment and introduction of effective screening programmes. Cohort effects reflecting change of prevalence of risk factors showed distinctive trends. The observed small dip in the middle 1930s birth cohort was consistent with the generation who had limited access to tobacco after World War II (Marugame et al., 2006). The early 1950s birth cohort peaked at the highest risk of incidence of lung cancer; they will be common age for lung cancer in the near future. Therefore the incidence will start to increase again. In some countries in Europe where tobacco control has been successful, the cohort effect of lung cancer mortality in men decreased dramatically. We need further efforts for tobacco control to decrease lung cancer in Japan (Bray and Weiderpass, 2009).

The pattern of age effect for breast cancer incidence was distinctive, which was different from the pattern in the US and western countries (Holford et al., 2006; Matsuno et al., 2007) and similar with many Asian countries(Sim et al., 2006). The pattern of age effect in mortality showed similar pattern with some other countries (Cayuela et al., 2004; Choi et al., 2006). Increasing cohort effect may be related with the recent Westernised lifestyle in Japan, in addition to dietary factor(Ministry of Health Labour and Walfare 1975-2007), reproductive factor related with the tendency to marry later and decrease of birth rate(Iwasaki et al. 2007; Ministry of Health Labour and Walfare 2010). Period effect in incidence increased in succession, while there was no effect in mortality. In some countries, decreasing period effect for mortality was observed by the improvement of treatment and effective mammography screening.(Cayuela et al., 2004; Niclis et al., 2010; Oberaigner et al., 2010).

In the cervix, decreasing cohort effect for incidence and mortality was mainly due to the improvement of the public hygiene. Since 1983, cervical cancer screening started in Japan as a nationwide public health service. But the proportion of the screening participation has been very low (about 20%). The period effect of cervical cancer incidence and mortality did not show any trend, this is because the screening programme was not successful as some countries in Europe (Quinn et al., 1999; Sasieni and Adams, 1999; Bray et al., 2005). Increase of incidence in the latest cohort possibly explained by the earlier onset of sexual activities, as the results, the prevalence of HPV also increased. Opportunistic cervical cancer screening at the gynaecological checkups also may be related with the increasing cohort effects of the younger generation in incidence.

For prostate, kidney and renal pelvis cancer, the increased period effects in incidence may be explained by the wider spread use of diagnostic tools; PSA for prostate cancer and ultrasound diagnosis for kidney cancer. These earlier detections, however, have not shown the decrease of period effect in mortality yet. For oesophageal, pancreas, kidney cancer, higher cohort effects were observed in the cohort born after 1950s. These trends suggested the possibility of increase in the incidence or mortality for these sites of cancer in future.

Although we have long-term data for both incidence and mortality in Osaka, the timeliness of incidence data is not so well at the moment. These results might not generalise to whole Japanese population, because the cancer incidence and mortality have been a little different in Osaka from other prefectures. Incidence and mortality of some sites of cancer (lung and liver) were higher than those in whole Japan.

We need to keep in mind the change in the completeness of cancer registration in Osaka when we evaluate incidence data. The percentage of under-ascertainment cases was not estimated routinely, but as an alternative index, the percentage of cases registered by death certificate only (% of DCO) was approximately 10-15% and stable in Osaka Cancer Registry during most recently two decades.(Parkin et al. 2005; Curado et al., 2007)

Among many approaches to disentangle the identification problem in the age-period-cohort model, Holford's is the most popular one in the descriptive cancer epidemiology area (Holford, 1985) and some other approaches (Yang et al., 2004; Carstensen, 2007) are still developing. Although Nakamura's method has been scarcely used in articles concerning cancer data, we adopted the method because it tackles straight on and overcome the problem in that the linear components of the three effects cannot be identified. Controlling the weighted sum of squares of first-order differences of the parameters as small as possible is a key to overcome the identification problem and Nakamura's method realizes to separate the three effects by using the framework of Bayesian approach and the minimization of the information criterion ABIC. When we compare such results, we need to pay close attention to the difference between the methods. In near future, we will need to

evaluate the difference between those methods and Nakamura's one.

In conclusion, this is the first report to show the effects of age, period and birth cohort using both incidence and mortality for various sites of cancer in Japan. Age-period-cohort model is useful approach to show these effects separately. We could evaluate cancer control activities through the results and can exploit next cancer control planning.

Acknowledgements

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研　究

肺がん化学療法時の悪心・嘔吐の実態

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松本尚也[9)※]・太田　毅[7)※]・阿部徹哉[4)※]・宮林貴大[5)※]・松山弘紀[10)※]・渡部　聡[1)※]・田中純太[1)※]
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Summary
がん化学療法時の悪心・嘔吐は患者QOLを低下させることはもとよりコンプライアンス低下を招き，治療に悪影響を与える可能性がある。今回，肺がん化学療法を受けた患者101名を対象に悪心・嘔吐の実態調査を実施した。その結果，悪心・嘔吐の「つらさ」に対して医師と患者の間での認識に乖離が認められ，遅発期の摂食障害がシスプラチン群で66%，カルボプラチン群で44%に発現することがわかった。制吐療法を行う際，遅発期まで含めた認識と対応が必要だと考えられる。

Key words：肺がん／化学療法／急性期悪心・嘔吐／遅発期悪心・嘔吐／第一世代5-HT_3受容体拮抗薬

はじめに

がん化学療法による悪心・嘔吐は副作用の中で頻度が高く，最も辛い副作用のひとつである。肺がんの化学療法では高度から中等度催吐性薬剤であるシスプラチン（以下CDDPと略す），カルボプラチン（以下CBDCAと略す）が頻用されているため，悪心・嘔吐による治療拒否や薬剤コンプライアンス低下を招く可能性がある。

化学療法による悪心・嘔吐は制吐療法の進歩により改善しているが，海外の調査では遅発性の悪心・嘔吐において医師と患者の認識の相違が報告され[1)]，また，治療レジメンによる医師と患者の悪心・嘔吐に対する認識の相違があるとの報告もあるため[2)]，患者の実態を把握することは治療を実施する上で非常に重要である。そこで，肺がん術後補助化学療法および進行・再発肺がんに対する化学療法施行患者を対象に高度及び中等度催吐性抗悪性腫瘍薬投与に起因する消化器症状（悪心・嘔吐，食欲不振）の発現状況を実態調査した。

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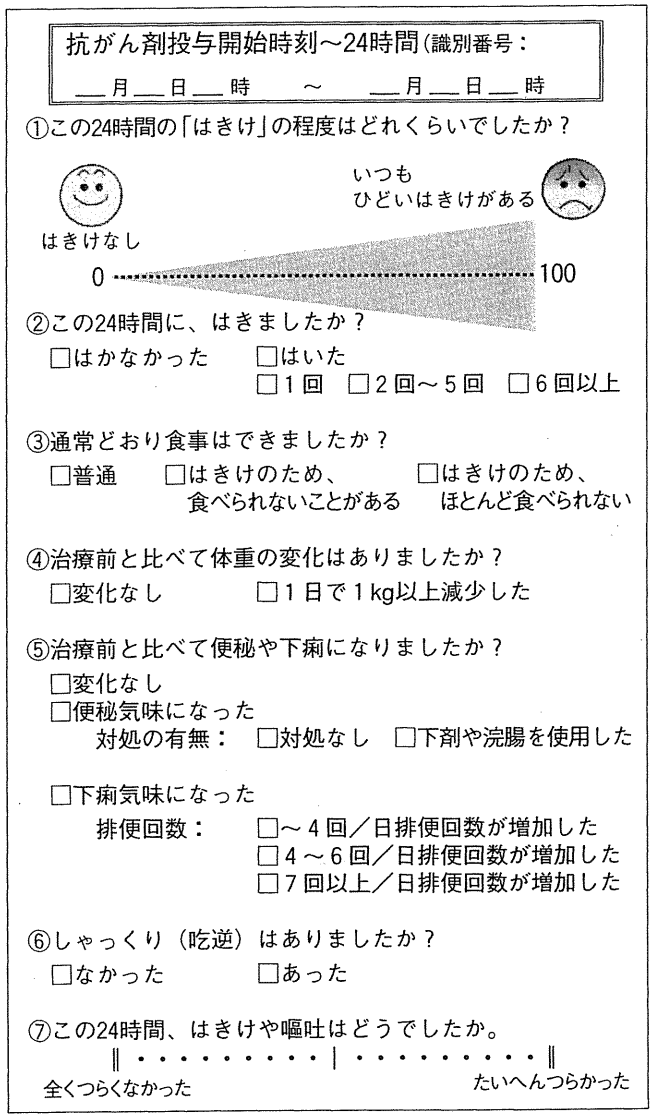
抗がん剤投与開始時刻～24時間(識別番号：
＿月＿日＿時　～　＿月＿日＿時
①この24時間の「はきけ」の程度はどれくらいでしたか？
いつも
ひどいはきけがある
はきけなし
0
100
②この24時間に、はきましたか？
□はかなかった　□はいた
□1回　□2回～5回　□6回以上
③通常どおり食事はできましたか？
□普通　□はきけのため、食べられないことがある　□はきけのため、ほとんど食べられない
④治療前と比べて体重の変化はありましたか？
□変化なし　□1日で1kg以上減少した
⑤治療前と比べて便秘や下痢になりましたか？
□変化なし
□便秘気味になった
対処の有無：□対処なし　□下剤や浣腸を使用した
□下痢気味になった
排便回数：□～4回／日排便回数が増加した
□4～6回／日排便回数が増加した
□7回以上／日排便回数が増加した
⑥しゃっくり（吃逆）はありましたか？
□なかった　□あった
⑦この24時間、はきけや嘔吐はどうでしたか。
全くつらくなかった
たいへんつらかった

図1　患者日誌

対象と方法

2009年4月～2010年1月までに新潟大学医歯学総合病院第二内科を中心とした新潟肺癌治療研究会に属する新潟県下18施設において，術後補助化学療法および進行・再発肺がんにおける化学療法に対する高度および中等度催吐性化学療法を施行した101例を対象とし，プロスペクティブに調査を行った。

本調査の適格基準は，①肺がんに対して初回化学療法を施行する患者，②高度，中等度の催吐性リスクであるCDDPもしくはCBDCAを含む化学療法が計画されている患者，③文書にて本人より本調査への同意が得られた患者とし，除外基準を①化学療法施行前24時間以内に嘔吐を経験した患者，②Day 2以降も高度，中等度催吐性の抗悪性腫瘍薬を使用する患者，③放射線療法を併用する患者とした。

5-HT_3受容体拮抗薬，ステロイドの種類や組合せ，投与期間等に関しては各施設，主治医の判断とした。

悪心・嘔吐実態調査は患者日誌（**図1**）による調査（患者側）と調査実施医師による化

表1 患者背景

特徴	患者数
年齢（年）	
男性	78
女性	23
中央値	68
年齢幅	46～82
組織型	
小細胞肺癌	23
非小細胞肺癌	78
腺癌	58
扁平上皮癌	18
扁平上皮・腺癌	1
未分化癌	1
PS	
P 0	37
P 1	61
P 2	3

Stage	患者数
Stage 分類	
IIA	1
IIIA	4
IIIB	36
IV	33
術後再発	8
Adj.	19
治療レジメン	
CDDP を含むレジメン	56
CBDCA を含むレジメン	45

表2 化学療法レジメン

	PEM	CPT-11	GEM	VNR	ETP	PAC	DOC	患者数
CDDP	12	4	8	21	5	1	5	56
CBDCA	3	0	2	5	14	21	0	45
計	15	4	10	26	19	22	5	101

PEM：ペメトレキセド ／ VNR：ビノレルビン ／ PAC：パクリタキセル ／ CPT-11：イリノテカン ／ ETP：エトポシド ／ DOC：ドセタキセル ／ GEM：ゲムシタビン

学療法レジメン投与前における催吐予測の評価および化学療法施行後の催吐事象を評価した。

患者日誌より，化学療法施行当日（Day 1）からDay 5（～120時間）まで，24時間ごとの①Visual analog scale（以下，VASと略す）を用いた悪心の程度（100mmのVASで左端が「吐き気なし」に相当する「0」，右端が「いつもひどい吐き気があった」に相当する「100」とした。悪心の有無の評価としては，5 mm未満を悪心なし，5 mm以上を悪心ありとした），②嘔吐性事象の有無，③VASを用いた悪心・嘔吐の全般的印象，満足度（左端が「全くつらくなかった」から右端が「たいへんつらかった」と印字されており，その間を20等分した）を評価した。

調査実施医師より，①化学療法投与前の治療レジメンによる催吐性に関する医師の印象（VASを用い，左端が「軽微」とそれに相当する「0」から右端が「重篤」とそれに相当する「100」が印字されており，その間を等分する「中等度」が入っているアナログスケールを用いた），②化学療法後の催吐事象の有無を評価した。

結　果

本調査の適格基準に該当した101例の患者背景を以下に示す（表1）。年齢中央値は68歳（年齢幅：46～82）であり，男/女比は78/23例，PSの0/1/2は37/61/3例であった。

表3 制吐療法レジメン

制吐療法レジメン	患者数
第一世代5-HT_3受容体拮抗薬＋ステロイド	62
第一世代5-HT_3受容体拮抗薬＋ステロイド＋メトクロプラミド	30
第一世代5-HT_3受容体拮抗薬＋ステロイド＋ドンペリドン	2
第一世代5-HT_3受容体拮抗薬＋ステロイド＋その他	2
第一世代5-HT_3受容体拮抗薬＋メトクロプラミド	1
第一世代5-HT_3受容体拮抗薬のみ	3
メトクロプラミドのみ	1

第一世代5-HT_3受容体拮抗薬の内訳（n=100）	患者数
グラニセトロン（後発品を含む）※	90
ラモセトロン※	32
アザセトロン	1

※1症例で複数薬剤使用例23例

ステロイド投与日数の内訳（n=96）	患者数
Day 1のみ投与	36
Day 2以降も投与	60

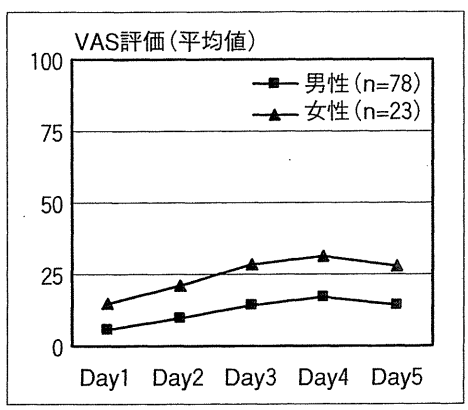

図2 性別による悪心・嘔吐の程度

肺がんの組織型では小細胞癌23例，非小細胞癌78例であった。治療レジメンはCDDPを含むレジメン56例（併用薬：ビノレルビン21例，ペメトレキセド12例等），CBDCAを含むレジメン45例（併用薬：パクリタキセル21例，エトポシド14例等）であった（表2）。制吐療法のレジメンに関しては第一世代5-HT_3受容体拮抗薬とステロイドの2剤のみの併用が62例と最も多く，次いで，これにメトクロプラミドを加えた3剤併用が30例であった（表3）。

1．性別による悪心・嘔吐の程度

悪心・嘔吐の全般的印象，満足度をVASにて評価し，性別毎に解析を行った（図2）。その結果，女性では男性と比べ悪心・嘔吐を「つらく」感じる傾向が認められた。また，男女とも抗悪性腫瘍薬投与後4日目に最も悪心・嘔吐を「つらく」感じることが多く，急性期と比べ遅発期において悪心・嘔吐を「つらく」感じることが多く認められた。

2．レジメン別による悪心・嘔吐の程度

CDDPを含むレジメンおよびCBDCAを含むレジメンでの，嘔吐発現率・摂食障害発現率を評価した。その結果，急性期の嘔吐発現率・摂食障害発現率はCDDPを含むレジメンでそれぞれ5％・16％，CBDCAを含むレジメンでそれぞれ2％・0％である一方，遅発期の嘔吐発現率・摂食障害発現率はCDDP

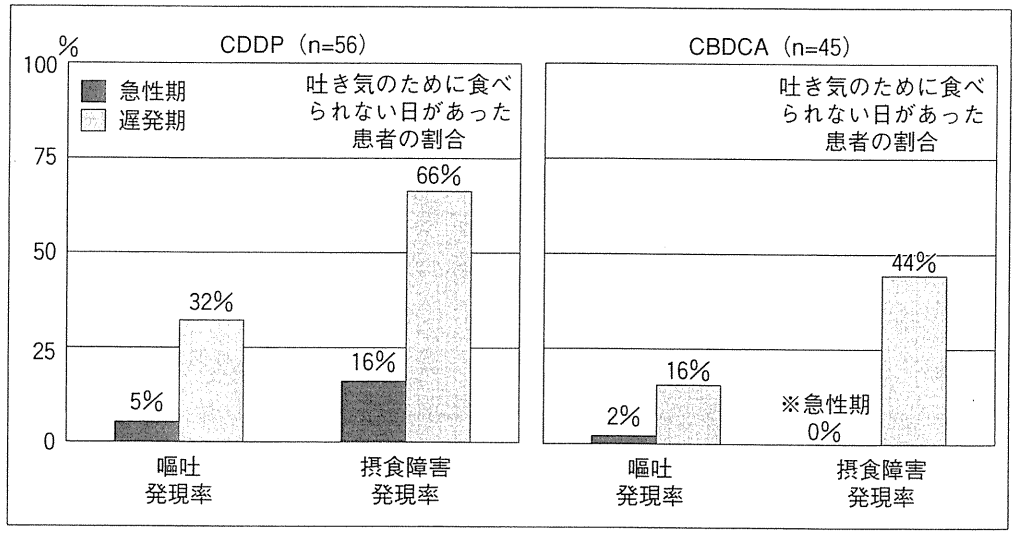

図3　レジメン別化学療法施行時の悪心・嘔吐，摂食障害発現率

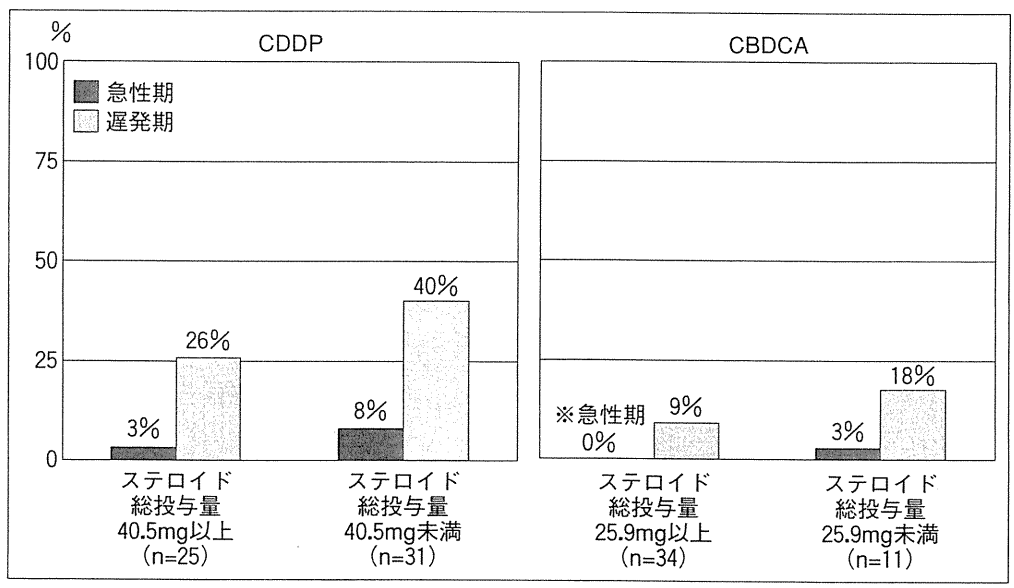

図4　ステロイド使用量と嘔吐発現の関係

を含むレジメンでそれぞれ32%・66%，CBDCAを含むレジメンでそれぞれ16%・44%であり，急性期と比較し遅発期に嘔吐や摂食障害の発現率が高い傾向がみられた（図3）。

3．制吐療法におけるステロイドの総投与量と嘔吐発現の関係

ステロイドの総投与量と嘔吐発現の関係を検討した。ステロイド総投与量のカットオフ値は日本癌治療学会より発刊されている「制吐薬適正使用ガイドライン」で推奨されている用量とした[3]。CDDPを含むレジメンでは1日目16.5mg，2～4日目8mgずつの総投与量40.5mgとし，CBDCAを含むレジメンでは1日目9.9mg，2～3日目8mgずつの総投与量25.9mgとした。その結果，CDDPを含むレジメン，CBDCAを含むレジメンいずれにおいてもガイドライン推奨用量以上のステロイド投与群は推奨用量以下の投与群と比較して嘔吐発現率は低かった。また，ガイドライン推奨用量以上のステロイド投与群においても，急性期と比較して遅発期での嘔吐発現率が高かった（図4）。

4．急性期および遅発期の悪心発現との関係

急性期および遅発期における悪心発現の関係を検討した。その結果，急性期において悪心が認められた患者のうち約96%で遅発期にも悪心が認められた。さらにレジメン別での検討では，CDDPを含むレジメンでは急性期に悪心が認められた場合，すべての患者に遅発期の悪心が認められ，CBDCAを含むレジメンにおいても急性期に悪心が認められた患者の約89%に遅発期の悪心が認められた。また，どちらのレジメンにおいても急性期に悪心が認められた患者は急性期の悪心が認められなかった患者と比較して遅発期の悪心の程度や「つらさ」が強く認められた（**図5**）。

5．医師と患者の悪心・嘔吐に対する認識の相違

各レジメンによる悪心・嘔吐に対する医師の印象・予測と，調査患者における実際の悪心・嘔吐に対する「つらさ」の認識の相違について検討した（**図6**）。その結果，CDDPを含むレジメンおよび，CBDCAを含むレジメンの両者において，急性期では医師の悪心・嘔吐の「つらさ」に対する印象と比べ，実際に投与された患者の印象は強くない傾向が認められた。

一方，遅発期では実際に投与された患者の悪心・嘔吐の「つらさ」に対する印象は医師の印象と比べ，ばらつきがあった。

考　察

今回，新潟肺癌治療研究会18施設において肺がん化学療法を実施した101例を対象に悪心・嘔吐の実態調査を実施した。その結果，医師の急性期における悪心・嘔吐の「つらさ」に対する印象はやや過剰であることが認められた。過剰であった理由として，5-HT_3受容体拮抗薬が登場する前から抗悪性腫瘍薬を使用している医師は急性期の強い悪心・嘔吐を経験していることが多く，それらの印象から過剰に認識していることが考えられる。したがって，今回の我々の報告が急性期の悪心・嘔吐に対する適切な判断を行う参考になればと考える。

現状では急性期の悪心・嘔吐において制御率が高いことは明らかであるが，注目すべきは遅発期の悪心・嘔吐である。本調査の結果から，CDDP，CBDCAいずれのレジメンにおいても急性期と比較して遅発期の嘔吐発現率，摂食障害発現率が高いことが認められた。患者の「つらさ」に対する印象は男女共に抗悪性腫瘍薬投与後4日目に最も「つらく」感じており，医師と患者間の認識に乖離があることから，遅発期まで含めた患者の状態の認識と対応が必要と考えられる。また，十分量のステロイド投与群においても急性期と比較して遅発期の嘔吐発現率が高かったことから，従来の第一世代5-HT_3受容体拮抗薬とステロイドだけでは遅発期の悪心・嘔吐のコントロールは不十分であると考えられる。

特に中等度催吐性リスクであるCBDCAレジメンでの悪心・嘔吐は軽視されがちである。MASCCのガイドラインにおいても中等度催吐性リスクの抗悪性腫瘍薬に関して，5-HT_3受容体拮抗薬の中で唯一遅発期に効果があるパロノセトロンとデキサメタゾンの2剤併用療法が推奨されており，パロノセトロンを用いてしっかりと悪心・嘔吐を予防することも重要だと考えられた[4]。

本調査では，新規制吐薬であるパロノセトロンやアプレピタントを使用していない。しかし，新規制吐薬が急速に普及することを考慮すると，従来の制吐薬を用いた肺がん化学療法における悪心・嘔吐の現状を調査した本結果は今後，新薬を含んだ治療法を検討する

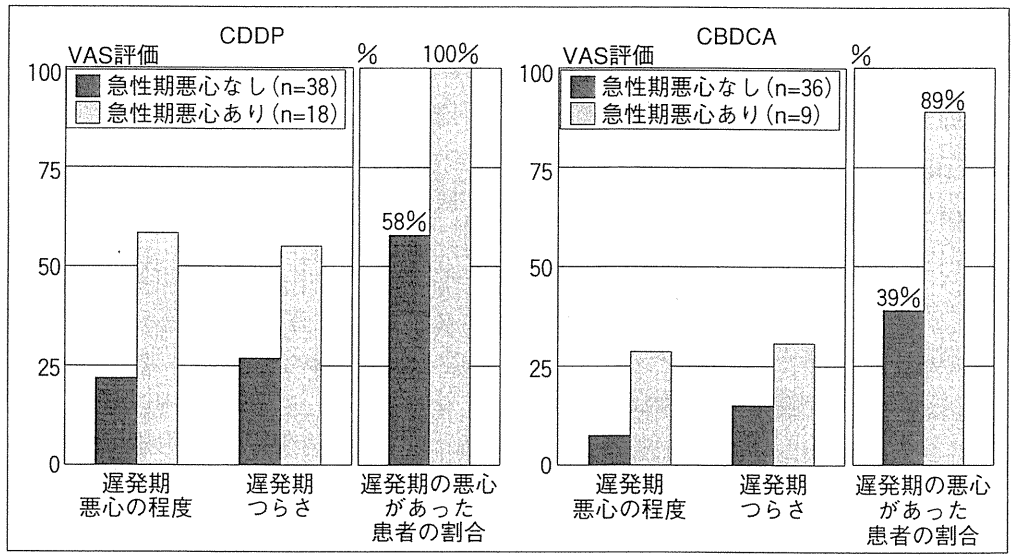

図5　急性期および遅発期の悪心発現の相関

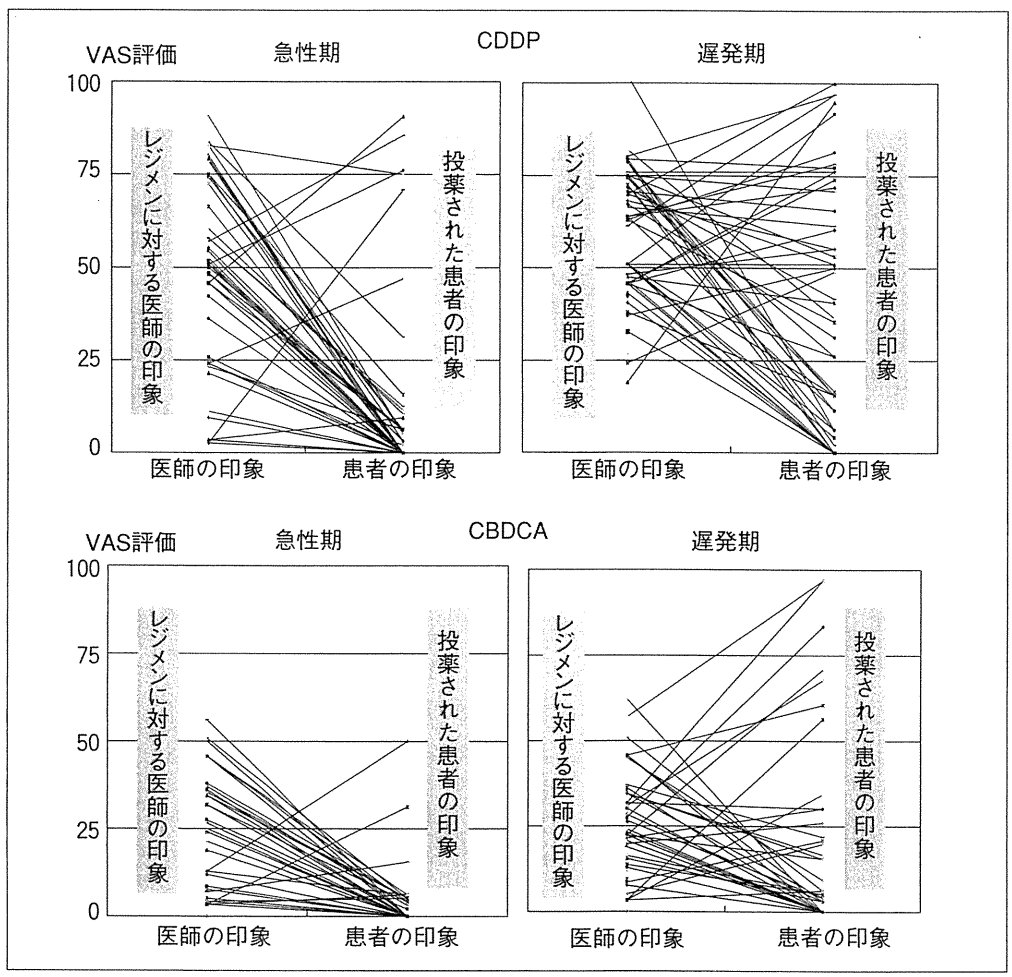

図6　シスプラチン/カルボプラチンレジメン時の医師と患者の認識の相違

上で参考となる。

現在，新潟肺癌治療研究会において本調査をヒストリカルコントロールとした高度催吐性抗悪性腫瘍薬投与に起因する急性及び遅発性の消化器症状に対するパロノセトロン，アプレピタント，デキサメタゾンの三剤併用療法の有効性と安全性を検討する臨床第Ⅱ相試験を実施中である。

また，中等度催吐性抗悪性腫瘍薬投与に起因する急性及び遅発性の消化器症状に対して，パロノセトロン，アプレピタント，デキサメタゾンの三剤併用療法と，従来の二剤併用療法とを比較した臨床第Ⅱ相試験を実施中である。

結　語

制吐薬適正使用ガイドラインでも示されている通り，化学療法に起因する悪心・嘔吐は予防が何よりも重要である。今回調査をした結果，化学療法による悪心・嘔吐に悩まされている患者の実態を把握することが極めて重要であることを改めて認識することができた。また，化学療法による悪心・嘔吐の制御には，さらに改善の余地があることを確認することができた。我々は，上記のような新規制吐薬の評価を通じて，患者がより安心して化学療法を受けられる環境を今後さらに整えていくことが大切であると考える。

深　謝：

本調査の実施にご協力賜りました新潟肺癌治療研究会の先生方に深く感謝いたします。（順不同，敬称略）

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ORIGINAL ARTICLE

Vaccination with CD133$^+$ melanoma induces specific Th17 and Th1 cell–mediated antitumor reactivity against parental tumor

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Abstract Accumulating evidence suggests that cancer cells possess a small subpopulation that survives during potentially lethal stresses, including chemotherapy, radiation treatment, and molecular-targeting therapy. CD133 is a putative marker that distinguishes a minor subpopulation from normal differentiated tumor cells in many cancers. Although it is necessary to eradicate all cancer cells to obtain a cure, effective treatment to eliminate the CD133$^+$ treatment–tolerant cells has not been elucidated. In this study, we demonstrated that a CD133$^+$ subpopulation in murine melanoma is immunogenic and that effector T cells specific for the CD133$^+$ melanoma cells mediated potent antitumor reactivity, curing the mice of the parental melanoma. CD133$^+$ melanoma antigens preferentially induced type 17 T helper (Th17) cells and Th1 cells but not Th2 cells. CD133$^+$ melanoma cell–specific CD4$^+$ T-cell treatment eradicated not only CD133$^+$ tumor cells but also CD133$^-$ tumor cells while inducing long-lasting accumulation of lymphocytes and dendritic cells with upregulated MHC class II in tumor tissues. Further, the treatment prevented regulatory T-cell induction. These results indicate that T-cell immunotherapy is a promising treatment option to eradicate CD133$^+$ drug-tolerant cells to obtain a cure for cancer.

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Keywords Cancer stem cells · Melanoma · CD133 · Th17 · Antitumor immunotherapy

Introduction

The cancer stem cells (CSCs) theory states that a minor subpopulation can initiate differentiated cancer cells and tumor tissues via self-renewal and asymmetrical cell division and that this plays a critical role in metastasis and recurrence [1–7]. It is controversial whether the classical CSC theory is applicable for all solid tumors. However, accumulating evidence suggests that a small subpopulation with unique features plays an important role in cancer recurrence after classical anticancer treatment and molecular-targeting therapy [8–12]. An excess of multidrug efflux transporters, antiapoptotic factors, DNA repair gene products, stem cell–specific growth signaling, and relative dormancy contribute to the ability of these cells to resist treatment. CD133 is a stem cell marker and putative CSC marker [13, 14]. It was demonstrated that all of examined cancer cells surviving after potentially lethal drug treatments uniformly express CD133 [15]. These drug-tolerant cancer cell populations use an altered chromatin state to induce a reversible drug-tolerant state and give rise to a permanent drug-tolerant cell population with genetic mutations. Unless these CD133$^+$ cancer cells are eradicated, it is impossible to achieve a lasting cure.

T-cell-mediated immunotherapy can mediate antitumor reactivity. We previously reported that effector T cells primed in tumor-draining lymph nodes (LNs) possessed antitumor therapeutic efficacy in brain, pulmonary, and skin metastasis models [16–18]. In this study, we found that LN T cells primed with the CD133$^+$ tumor vaccine

mediated potent antitumor therapeutic efficacy by eradicating $CD133^+$ tumor cells in tumors, thereby curing parental melanomas that comprised <1% $CD133^+$ tumor cells. Interestingly, $CD133^+$ melanoma antigens tended to prime type 17 helper T (Th17) cells and Th1 cells but not Th2 cells. These results indicate that T-cell immunotherapy may be a promising strategy to eradicate treatment-tolerant $CD133^+$ cancer cells.

Materials and methods

Mice

Female C57BL/6J (B6) mice were purchased from the CLEA Laboratory (Tokyo, Japan). They were maintained in a specific pathogen-free environment and used for experiments at the age of 8–10 weeks. All animal experiments were conducted with the permission of the Niigata University ethics committee for animal experiments.

Tumor cells

B16F10 melanomas, which originate from B6 mice, were maintained in vitro. Parental tumor cells were labeled with phycoerythrin (PE)-conjugated anti-CD133 monoclonal antibody (mAb; 13A4) and anti-PE microbeads (Miltenyi Biotec, Auburn, CA). $CD133^+$ and $CD133^-$ tumor cells were isolated with autoMACS™ (Miltenyi Biotec) according to the manufacturer's instructions. Cell purity was >90%.

mAbs and flow cytometry

Hybridomas producing mAbs against murine CD4 (GK1.5, L3T4), CD8 (2.43, Lyt-2), CD3 (2C11), and CD62L (MEL14) were obtained from the American Type Culture Collection (Rocksville, MD). Anti-CD4, anti-CD8, and anti-CD62L mAbs were obtained from ascitic fluid of sublethally irradiated (500 cGy) DBA/2 mice. PE-conjugated anti-CD80 (16-10A), anti-CD86 (GL1), anti-CD62L (MEL14), anti-CD8 (2.43), and anti-CD25 (PC61) mAbs; fluorescein isothiocyanate (FITC)-conjugated anti-Thy1.2 (30-H12); and anti-CD4 (GK1.5) mAbs were purchased from BD PharMingen (San Diego, CA). Analyses of cell surface phenotypes were carried out by direct immunofluorescence staining of $0.5–1 \times 10^6$ cells with conjugated mAbs. In each sample, 10,000 cells were analyzed using a FACScan™ flow microfluorometer (Becton–Dickinson, Sunnyvale, CA). PE-conjugated subclass-matched antibodies used as isotype controls were also purchased from BD PharMingen. Samples were analyzed using the CellQuest™ software (BD PharMingen).

Fractionation of T cells

T cells in the LN cell suspension were concentrated by passing through nylon wool columns (Wako Pure Chemical Industries, Osaka, Japan). To yield highly purified (>90%) cells with downregulated CD62L expression ($CD62L^{low}$), LN T cells were further isolated by a panning technique using T-25 flasks pre-coated with goat anti-rat immunoglobulin antibody (Ig Ab) (Jackson ImmunoResearch Laboratories, West Grove, PA)/anti-CD62L mAb (MEL14) and sheep anti-rat-Ig Ab/anti-CD62L mAb-coated Dynabeads M-450 (Dynal, Oslo, Norway). In some experiments, cells were further separated into $CD4^+$ and $CD8^+$ cells by depletion using magnetic beads, as described previously [18]. For in vitro experiments, highly purified $CD4^+$ cells were obtained using anti-CD4 mAb-coated Dynabeads and Detachabeads (Invitrogen) according to the manufacturer's instructions.

Bone marrow–derived dendritic cells

Dendritic cells (DCs) were generated from bone marrow cells (BMs), as described previously. In brief, BMs obtained from femurs and tibias of treatment-naïve mice were placed in T-75 flasks for 2 h at 37°C in complete medium (CM) containing 10 ng/ml of recombinant murine granulocyte–macrophage colony-stimulating factor (rmGM-CSF; a gift from KIRIN, Tokyo, Japan). Non-adherent cells were collected by aspirating the medium and transferred into fresh flasks. On day 6, non-adherent cells were harvested by gentle pipetting. CM consisted of RPMI 1640 medium supplemented with 10% heat-inactivated lipopolysaccharide (LPS)-qualified fetal calf serum (FCS), 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 100 U/ml of penicillin, 100 μg/ml of streptomycin sulfate (all from Life Technologies Inc.), and 5×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO).

DC/tumor-draining LN cells

BMs and DCs were co-cultured with the same number of irradiated tumor cells (5,000 cGy) in CM overnight. B6 mice were inoculated subcutaneously (s.c.) with 10×10^6 BM–DC and tumor cells in both flanks. Inguinal LNs draining BM–DC and tumor cells were harvested. Single-cell suspensions were prepared mechanically as described previously [19].

Adoptive immunotherapy

B6 mice were injected s.c. with parental B16-F10 tumor cells in 100 μl of Hank's balanced salt solution (HBSS) to establish subcutaneous tumors. Two or three days after the

inoculation, mice were sublethally irradiated (500 cGy) and then infused intravenously (i.v.) with T cells isolated from tumor-draining LNs. LN cells were stimulated with anti-CD3 mAb (2C11) and cultured in CM containing 40 U/ml of interleukin (IL)-2 for 3 days to obtain a sufficient number of T cells for in vivo experiments, as described previously [17]. The perpendicular diameter of subcutaneous tumors was measured with calipers.

Cytokine ELISAs

T cells were stimulated with immobilized anti-CD3 mAb or tumor antigen–pulsed BM–DCs in CM. Supernatants were harvested and assayed for IFN-γ, IL-4, and IL-17 content by a quantitative "sandwich" enzyme immunoassay using a murine IFN-γ, IL-4, and IL-17 ELISA kit (Genzyme, Cambridge, MA) according to the manufacturer's instructions.

In vitro proliferation assay

Melanoma cells were labeled with 5 μM 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE; Molecular Probes Inc., Eugene, OR) in HBSS at 37°C for 15 min and washed twice before CD3 stimulation. The ratio of CFSE-labeled tumor cells to unlabeled tumor cells was 1:10. Tumor cells were cultured in CM at 1×10^5/ml. Tumor cells were counted every day and were analyzed using a microfluorometer to determine the number of CFSE-labeled tumor cells. Three wells were analyzed for each condition.

Statistical analysis

Comparison between groups was made by Student's *t*-test. The dynamic tumor growth data was analyzed by multivariate general linear model. Differences were considered significant for $P < 0.05$. Statistical analysis was performed with SPSS statistical software (SPSS, Chicago, IL) or GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA).

Results

$CD133^+$ melanoma cells possessed distinct characteristics

For this study, we obtained purified $CD133^+$ tumor cells from murine B16-F10 melanomas (Fig. 1a) and then tested the properties of these cells. Skin tissues were not produced by 1×10^5 subcutaneously inoculated parental B16 melanoma cells, but 5×10^3 $CD133^+$ melanoma cells were sufficient to establish tumor tissues in vivo (Fig. 1b). In vitro proliferation assays showed that $CD133^-$ tumor cells proliferated more aggressively than $CD133^+$ tumor cells before they became confluent, but their proliferation was impeded by cell–cell contact inhibition. In contrast, the proliferation of $CD133^+$ tumor cells did not stop by contact inhibition, and cells piled together, developing into floating aggregates (Fig. 1c). We also tested whether $CD133^+$ melanoma cells could grow in an anchorage-independent manner. Although $CD133^-$ cells eventually died without anchorage, all $CD133^+$ tumor cells proliferated to become tumor spheres (Fig. 1d, e). $CD133^+$, but not $CD133^-$, tumor cells exhibited colony formation on soft agar (Fig. 1f).

Vaccination with $CD133^+$ tumor cells induced protective immunity against the parental melanoma

To examine whether the immune system can recognize $CD133^+$ melanoma cells, we immunized mice by subcutaneously inoculating them with 5,000 cGy-irradiated 1×10^7 parental, $CD133^-$, or $CD133^+$ melanoma cells mixed with 1×10^7 DCs. Fourteen days after immunization, 3×10^5 parental melanoma cells were subcutaneously injected. The tumor growth curves of mice that were immunized with parental tumor cells or $CD133^-$ tumor cells were identical to those of mice that had not received immunization (Fig. 2a). In contrast, tumor growth was significantly retarded in mice immunized with $CD133^+$ tumor cells.

$CD133^+$ tumor antigen–specific T cells mediated potent therapeutic efficacy

We examined the antitumor efficacy of LN T cells draining irradiated parental, $CD133^-$, or $CD133^+$ melanoma cell vaccinations with DCs. $CD62L^{low}$ (cells with downregulated CD62L expression) T cells that were isolated as antigen-primed T cells from LNs were cultured by the anti-CD3/IL-2 method, as described previously [20]. LN T cells were intravenously infused into mice bearing a 2-day-established parental melanoma skin tumor after sublethal whole-body irradiation (500 cGy). The tumors of mice treated with LN T cells primed with parental or $CD133^-$ tumor cells grew in a pattern similar to those of the untreated mice (Fig. 2b). In contrast, the tumors of mice treated by LN T cells primed with $CD133^+$ tumor cells did not grow, even though the mice had palpable tumors. Interestingly, the antitumor reactivity mediated by the LN T cells primed with $CD133^+$ tumor cells persisted for more than 60 days and no mice died of tumor. In this system, regulatory T (Treg) cells were eliminated by whole-body irradiation before antitumor T-cell infusion; however, generally, host lymphocytes recover approximately 20 days after irradiation, and Treg cells that recover as host

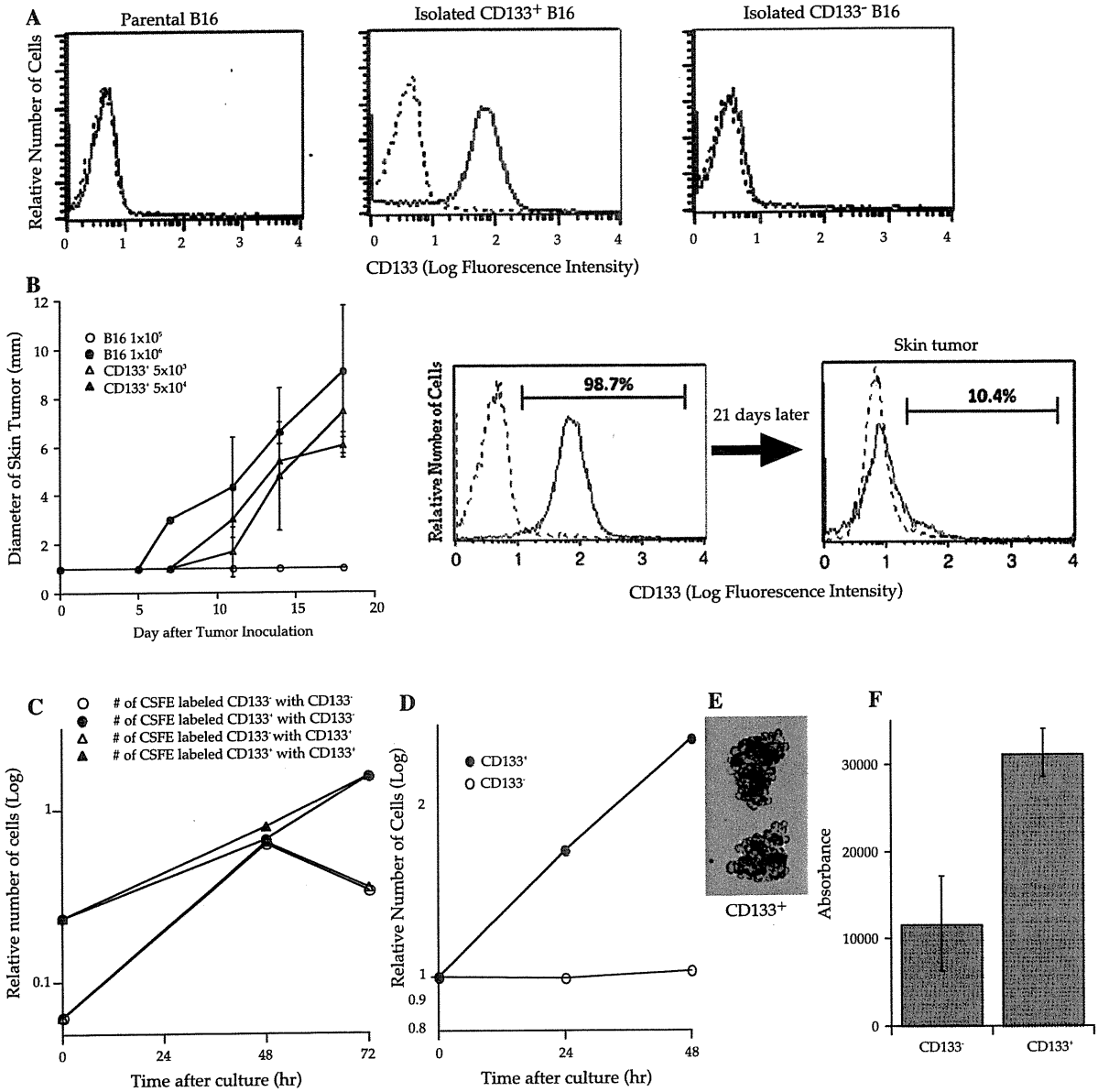


Fig. 1 $CD133^+$ B16 melanoma cells demonstrated high tumorigenicity in vivo (each group contained 5 mice) and proliferated in an anchorage- and cell–cell contact inhibition-independent manner in vitro. **a** One million B16 melanoma cells were stained with phycoerythrin (PE)-conjugated anti-CD133 or PE-conjugated isotype control monoclonal antibodies (mAbs). *Dotted lines* indicate the fluorescence intensity of tumor cells stained with PE-conjugated subclass-matched isotype control mAbs. Each frame consists of 10,000 cells. **b** B6 mice were subcutaneously inoculated along the midline of the abdomen with 5×10^3 or 5×10^4 $CD133^+$ cells, or 1×10^5 or 1×10^6 parental B16 cells. The diameter of the skin tumors was measured twice weekly with calipers, and the size was recorded as the average of 2 perpendicular diameters. Each group contained 6 mice. *Bars* indicate standard deviation. **c** $CD133^+$ or $CD133^-$ tumor cells (0.3×10^6) labeled with carboxyfluorescein diacetate succinimidyl diester (CFSE) were mixed with non-labeled tumor cells (3×10^6) and cultured in complete medium (CM) at 1×10^5 cells/ml. Tumor cells were counted every day and analyzed using a microfluorometer to determine the number of CFSE-labeled tumor cells. Three wells were analyzed for each condition. **d**, **e** One million $CD133^+$ or $CD133^-$ tumor cells were cultured in 10 ml of CM in 50 ml conical tubes that were rotated to avoid cell attachment. Cell counts were performed every 24 h. After 72 h in culture, $CD133^+$ cells proliferated to build spheroid-like cell aggregates. **f** The soft agar colony assay was performed using CytoSelect™ 96 Well Transformation Assay (Cell Biolabs Inc.) according to the manufacturer's instructions; 5×10^3 $CD133^+$ or $CD133^-$ B16 tumor cells were cultured in soft agar for 7 days, and colony formation was examined using a 96-well fluorometer

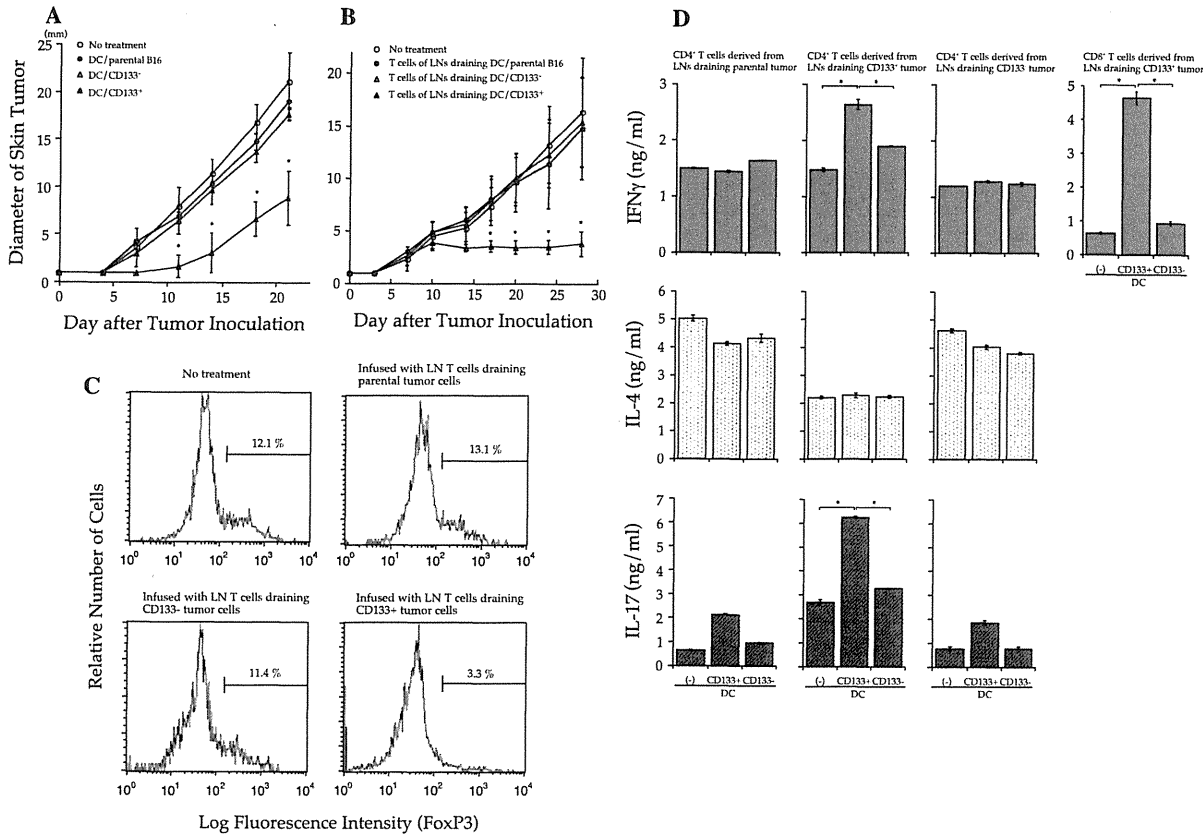


Fig. 2 Irradiated CD133$^+$ tumor cell/dendritic cell (DC) vaccine-primed CD133$^+$ tumor-specific CD8$^+$, and Th1 and Th17 CD4$^+$ T cells mediated therapeutic efficacy against parental B16 melanomas. **a** Bone marrow–derived DCs were co-cultured 1:1 overnight with irradiated (5,000 cGy) parental, CD133$^-$, or CD133$^+$ B16 cells in CM. Non-adherent cells were collected, and 1 × 10^6 cells were subcutaneously injected into mice. Two weeks later, mice were subcutaneously inoculated along the abdominal midline with 1 × 10^6 parental B16 cells. Data are from a representative experiment of 3, with 5 mice/group. *Asterisks* indicate $P < 0.01$. **b** Bone marrow–derived DCs co-cultured with irradiated parental, CD133$^-$, or CD133$^+$ tumor cells were subcutaneously injected into both flanks of mice. Inguinal lymph nodes (LN) were collected 8 days later. CD62L^{low} T cells were isolated as antigen-primed LN T cells and cultured by the anti-CD3/interleukin (IL-2) method. B6 mice were subcutaneously inoculated along the abdominal midline with 1 × 10^6 parental B16 cells in order to establish tumors. Two days later, mice were sublethally irradiated (500 cGy) and intravenously infused with 15 × 10^6 LN T cells. The diameter of skin tumors was measured twice weekly with calipers; size was recorded as the average of 2 perpendicular diameters. The data are from a representative experiment of 2, with 5 mice/group. *Asterisks* indicate $P < 0.01$. **c** Tumors were obtained 30 days after tumor inoculation and were digested with collagenase, hyaluronidase, and DNase. Cells in tumor tissues were stained using FITC-conjugated anti-CD4 mAb and PE-conjugated anti-Foxp3 mAb (e-Bioscience) with the staining kit according to the manufacturer's instructions. Cells in the lymphocyte region were gated for analyses. **d** IFN-γ, IL-4, and IL-17A secretion. In a 96-well plate, 1 × 10^5 CD62L^{low} CD4$^+$ T cells isolated from LNs draining irradiated tumor cells/DCs were stimulated with 2 × 10^4 DCs pulsed with tumor antigens in 200 μl CM for 48 h. DCs for stimulation were purified with CD11c microbeads after overnight co-culture with irradiated tumor cells. *Asterisks* indicate $P < 0.01$

immunity abrogate antitumor reactivity [18]. To elucidate whether T cells primed with CD133$^+$ tumor cells affected Treg induction, we examined Foxp3$^+$ regulatory T cells in tumor tissues 30 days after treatment. As shown in Fig. 2c, very few Foxp3$^+$ CD4$^+$ T cells were detected in tumor tissues of mice treated with LN T cells draining CD133$^+$ tumors. In contrast, there was almost the same number of Foxp3$^+$ CD4$^+$ T cells in tumor tissues of mice that were infused with LN T cells draining parental or CD133$^-$ tumors as in the untreated mice.

T cells primed in LNs draining CD133$^+$ melanoma cells/DCs were specific to CD133$^+$ tumor antigens and exhibited specific IFN-γ and IL-17 production

To examine cytokine release by T cells primed in LNs draining irradiated tumor cell/DC vaccine, CD62L^{low} CD4$^+$ LN T cells or CD62L^{low} CD8$^+$ LN T cells were stimulated with irradiated tumor cells in the presence of DCs for 48 h. Th1 cells have been considered the most important CD4$^+$ T cells for antitumor immunity.

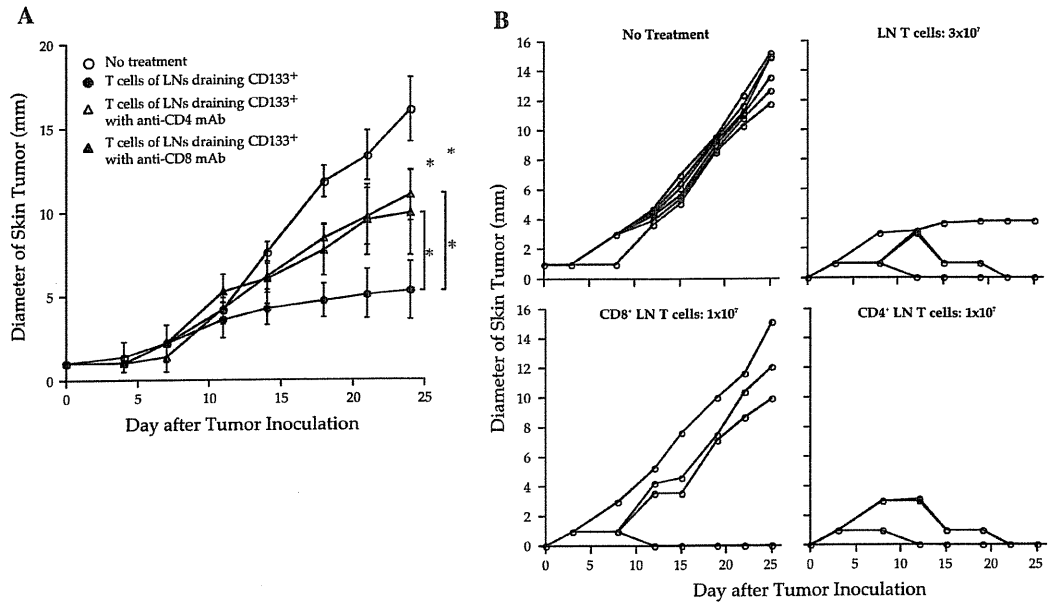


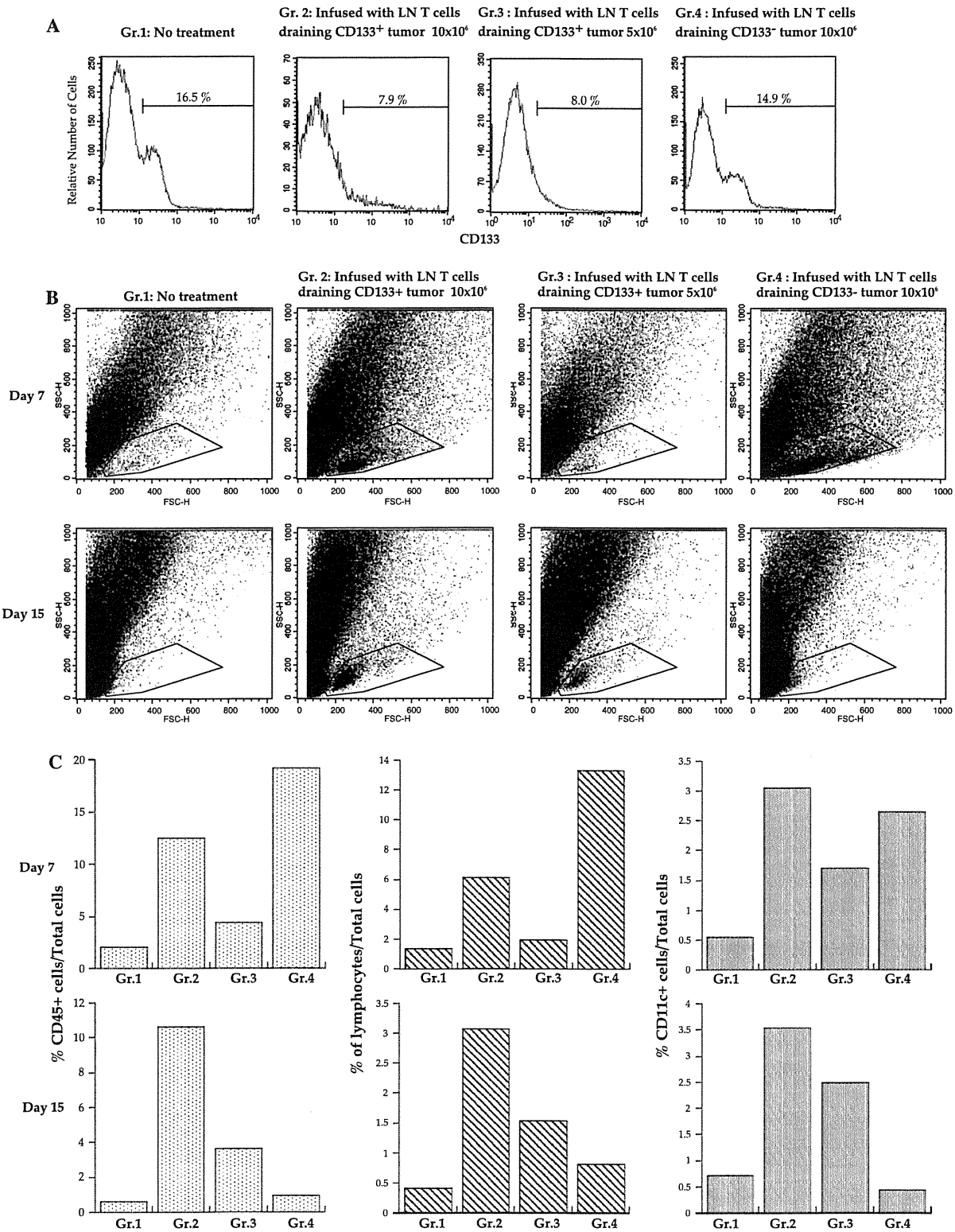
Fig. 3 $CD4^+$ T cells primed with irradiated $CD133^+$ tumor cell/dendritic cell (DC) vaccine mediated potent and long-lasting antitumor reactivity. Both $CD4^+$ and $CD8^+$ T cells were required for optimal antitumor efficacy. **a** Approximately 15×10^6 $CD62L^{low}$ T cells isolated from lymph nodes (LNs) draining irradiated $CD133^+$ melanoma cells/DCs for 8 days were cultured by the anti-CD3/interleukin (IL)-2 method and were infused intravenously into mice bearing 2-day-established skin tumors of parental melanoma. Mice were intraperitoneally injected with either anti-CD4 or anti-CD8 monoclonal antibody (mAb). The diameter of the skin tumors was measured twice weekly with calipers; size was recorded as the average of 2 perpendicular diameters. Each group contained 5 mice. *Asterisks* indicate $P < 0.01$. **b** $CD4^+$ and $CD8^+$ T cells were purified from $CD62L^{low}$ LN T cells draining irradiated $CD133^+$ melanoma cells/DCs with immuno-magnetic beads. Approximately 3×10^7 $CD62L^{low}$ T cells, 1×10^7 $CD62L^{low}$ $CD8^+$ T cells, or 1×10^7 $CD62L^{low}$ $CD4^+$ T cells were isolated from 8-day B16 $CD133^+$ tumor cell/DC-draining LN cells. $CD62L^{low}$ T cells were activated by the anti-CD3/IL-2 method and separated into $CD4^+$ and $CD8^+$ cells with magnetic beads. The diameters of the skin tumors were measured twice weekly with calipers; size was recorded as the average of 2 perpendicular diameters. Each group contained 5 mice. *Asterisks* indicate $P < 0.01$

Recently, it was reported that Th17 $CD4^+$ T cells that preferentially produce IL-17 and IL-6 play a critical role in antitumor immune responses [21–25]. The supernatants were analyzed for interferon (IFN)-γ, IL-4, and IL-17A using cytokine-specific enzyme-linked immunosorbent assays (ELISAs). $CD62L^{low}$ $CD4^+$ T cells derived from LNs draining $CD133^+$ melanoma cells exhibited specific and significantly greater IL-17A, but not IL-4, production upon $CD133^+$ tumor antigen stimulation (Fig. 2d). Conversely, T cells primed in LNs draining parental or $CD133^-$ tumor antigens did not show antigen-specific cytokine release. $CD62L^{low}$ $CD8^+$ T cells derived from $CD133^+$ tumor cell–draining LNs also produced IFN-γ upon stimulation with $CD133^+$ tumor antigens. These cytokine assays indicate that both $CD133^+$ tumor-specific $CD4^+$ and $CD8^+$ T cells were primed in LNs draining $CD133^+$ tumor antigens and that $CD133^+$ tumor antigens preferentially induced Th1 and Th17, but not Th2, cells.

$CD133^+$ melanoma-specific $CD4^+$ T cells mediated superior antitumor reactivity

To determine whether $CD4^+$ or $CD8^+$ T cells contributed to the antitumor efficacy mediated by $CD133^+$ tumor-specific LN T-cell treatment, $CD4^+$ and $CD8^+$ T-cell

Fig. 4 $CD133^+$ tumor-specific LN T cell treatment eradicated ▶ $CD133^+$ tumor cells and induced accumulation of leukocytes in tumor tissues. One million $CD133^+$ tumor cells mixed with 0.5×10^6 $CD133^-$ tumor cells were subcutaneously inoculated at both flanks. Zero (Gr. 1), 10×10^6 (Gr. 2), 5×10^6 $CD133^+$ tumor-specific LN T cells (Gr. 3), or 10×10^6 LN T cells draining $CD133^-$ tumor antigens (Gr. 4) were intravenously infused after sublethal whole-body irradiation. Each group contained 4 mice. Four tumors were collected and digested with collagenase, DNase, and hyaluronidase 7 and 15 days after T-cell infusion. The representative data of 3 independent experiments are presented. **a** CD133 expression of cells in the tumor region. **b** *Dot plots* of forward and side scatter. **c** The percentages of $CD45^+$ leukocytes based on the total number of cells (*left graphs*), of cells in the lymphocyte region (*middle graphs*), and of $CD11c^+$ cells (*right graphs*) in each group


A
Gr.1: No treatment
Gr. 2: Infused with LN T cells draining CD133+ tumor 10x10⁶
Gr.3 : Infused with LN T cells draining CD133+ tumor 5x10⁶
Gr.4 : Infused with LN T cells draining CD133- tumor 10x10⁶
Relative Number of Cells
16.5 %
7.9 %
8.0 %
14.9 %
CD133
B
Gr.1: No treatment
Gr. 2: Infused with LN T cells draining CD133+ tumor 10x10⁶
Gr.3 : Infused with LN T cells draining CD133+ tumor 5x10⁶
Gr.4 : Infused with LN T cells draining CD133- tumor 10x10⁶
Day 7
Day 15
SSC-H
FSC-H
C
Day 7
Day 15
% CD45+ cells/Total cells
% of lymphocytes/Total cells
% CD11c+ cells/Total cells
Gr.1
Gr.2
Gr.3
Gr.4

depletion studies were performed. In vivo depletion studies showed that both $CD4^+$ and $CD8^+$ T-cell depletion significantly diminished antitumor reactivity (Fig. 3a). However, these studies could not assess whether $CD4^+$ or $CD8^+$ LN T cells mediated superior antitumor reactivity, because host $CD4^+$ and $CD8^+$ T cells were also depleted. Therefore, we infused mice with 1×10^7 $CD4^+$ or $CD8^+$ cells purified from $CD133^+$ tumor-specific LN T cells. Two of 5 mice infused with $CD8^+$ LN T cells were cured (Fig. 3b). Interestingly, all mice infused with $CD4^+$ LN T cells were cured. Moreover, the duration of the antitumor effect of $CD133^+$ tumor-specific $CD4^+$ T cells was longer than that of $CD8^+$ T cells. In other words, skin tumors that were not cured within 10 days after tumor inoculation eventually grew in mice infused with 1×10^7 purified $CD8^+$ LN T cells. In contrast, mice infused with 1×10^7 purified $CD4^+$ LN T cells or 3×10^7 total LN T cells that contained approximately 6×10^6 $CD4^+$ LN T cells exhibited long-lasting antitumor reactivity and resulted in the complete remission of the parental melanoma.

$CD133^+$ tumor-specific T-cell treatment resulted in long-lasting accumulation of $CD4^+$ T cells and activated DCs in tumors

To understand the mechanism by which $CD133^+$ tumor-specific T cells mediated antitumor reactivity, we analyzed the cellular composition of skin tumors 7 and 15 days after treatment. One million $CD133^+$ tumor cells mixed with 0.5×10^6 $CD133^-$ tumor cells were inoculated in both flanks of mice. Although the percentage of $CD133^+$ cells did not differ among groups on the seventh day after tumor inoculation (data not shown), tumors of $CD133^+$ tumor-specific LN T-cell recipients lost their $CD133^+$ subpopulation. In contrast, tumors in mice that were infused with $CD133^-$ tumor-draining LN T cells contained approximately the same number of $CD133^+$ tumor cells as the control mice on the fifteenth day after treatment (Fig. 4a). Thus, it is likely that infused $CD133^+$ tumor-specific T cells indeed eradicated $CD133^+$ tumor cells prior to tumor regression.

On the seventh day, the number of $CD45^+$ leukocytes and lymphocytes in tumor tissues depended on the number of infused T cells, as more leukocytes were observed in tumor tissues of mice that were infused with 1×10^7 LN T cells draining $CD133^+$ or $CD133^-$ tumor cells. However, the tumor-infiltrating lymphocytes in mice that were infused with 1×10^7 LN T cells draining $CD133^-$ tumor antigen disappeared, leaving these mice with lymphocyte levels comparable to those in untreated mice by the fifteenth day (Fig. 4b). Conversely, mice treated with 10×10^6 $CD133^+$ tumor-specific LN T cells had 10 times more $CD45^+$ cells and 6 times more lymphocytes than the control animals did (Fig. 4b, c). $CD4^+$ T cells preferentially increased in the tumor tissues of $CD133^+$ tumor-specific LN T-cell recipients. Moreover, $CD133^+$ tumor-specific LN T-cell infusion resulted in a long-lasting increase in $CD11c^+$ DCs that had augmented the expression of MHC class II antigen in tumor tissues (Fig. 5a, b).

To determine whether $CD4^+$ or $CD8^+$ $CD133^+$ tumor-specific LN T cells induce the accumulation of lymphocytes and DCs, we analyzed tumors in mice infused with purified $CD4^+$ or $CD8^+$ $CD133^+$ tumor-specific LN T cells. Leukocyte accumulation was observed only when the mice were infused with $CD4^+$ $CD133^+$ tumor-specific T cells (Fig. 6a). Furthermore, 90 days after T-cell treatment, we examined splenocytes of mice cured with the $CD133^+$ tumor-specific T-cell infusion. Surprisingly, the mice cured with $CD4^+$ $CD133^+$ tumor-specific LN T cells had not only the $CD4^+$ T cells that produced IFN-γ and IL-17 upon $CD133^+$ tumor antigen stimulation but also the $CD4^+$ T cells that recognized and responded to $CD133^-$ tumor antigens, although the infused $CD4^+$ T cells were highly specific for $CD133^+$ tumor antigens (Figs. 2c, 6b).

Discussion

Our study demonstrates that $CD133^+$ melanoma-specific T cells are capable of mediating antitumor reactivity that results in the regression of established parental melanoma in mice. These results are surprising, as $CD133^+$ tumor cells comprised less than 1% of the parental melanoma. $CD133^+$ tumor cells may be so essential for the development of tumor tissues that eradication of $CD133^+$ tumor cells makes it difficult for melanoma cells to establish tumors in vivo, as $CD133^+$ tumor cells possess high tumorigenicity. However, this does not explain how $CD133^+$ tumor-specific T-cell therapy cured the mice with established skin tumors. Notably, $CD4^+$ T cells mediated superior long-lasting antitumor reactivity by inducing the accumulation of activated DCs and lymphocytes, but not Treg, in tumor tissues. Further, although $CD4^+$ T cells that were highly specific for $CD133^+$ melanoma antigens were infused, we detected T cells that secreted IFN-γ and IL-17 upon $CD133^-$ tumor antigen stimulation in cured mice. This observation is consistent with the previous report that Th17 cells expressing TCR for 1 tyrosinase-related protein-1 (TRP-1) epitope induced tumor antigen–specific T cells that were not specific for TRP-1 [21]. Thus, it is likely that the interaction between $CD4^+$ $CD133^+$ tumor-specific T cells and DCs that acquired $CD133^+$ tumor cells resulted in the induction of antimelanoma effector T cells with wide specificity, because $CD133^+$ and $CD133^-$ melanoma cells shared most antigens according to 2-D electrophoresis analyses (*data not shown*). Cumulatively, these results